DOCTORAL DISSERTATION

KACHLEK MARIAM LILIA

UNIVERSITY OF KAPOSVÁR

FACULTY OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES

2017

DOI: 10.17166/KE2018.003

KAPOSVÁR UNIVERSITY

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 B_1, DEOXYNIVALENOL AND ZEARALENONE

Written by:

KACHLEK MARIAM LILIA

KAPOSVÁR

2017

DOI: 10.17166/KE2018.003

LIST OF ABBREVIATIONS

ALB	Albumin
ALKP	Alkaline phosphatase
ALT	Alanine aminotransferase
APCI	Atmospheric-pressure chemical ionization
AST	Aspartate aminotransferase
BEA	Beauvericin
BIL	Bilirubin
BW	Body weight
CCK8	Cell counting kit-8
CD	Conjugated dienes
CFU-GM	Colony forming unit-granulocytes and monocytes
CREA	Creatinine
CT	Conjugated trienes
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
ENB	Enniatin B
ESI	Electrospray ionization
FA	Fumonisin A
FB	Fumonisin B
FC	Fumonisin C
FP	Fumonisin P
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FB_4	Fumonisin B_4
FB_5	Fumonisin B_5
FB_6	Fumonisin B_6

FrA	Fructosamine
FUMs	Fumonisins
GGT	Gamma-glutamyl transferase
GLOB	Globulin
GLU	Glucose
Gn-RH	Gonadotropin-releasing hormone
GPx	Glutathione peroxidase
GSH	Reduced glutathione
IL-	Interleukin
LDH	Lactate dehydrogenase
LMP	Low melting point
MCP-1	Monocyte chemoattractant protein-1
MDA	Malondialdehyde
MN	Micronuclei
mRNA	Messenger ribonucleic acid
MTT	3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NIV	Nivalenol
NMP	Normal melting point
qPCR	Quantitative (Real time) PCR
PA	Phagocytic activity
PBS	Phosphate buffered saline
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PTP	Permeability transition pore
RBCH	Red blood cell hemolysate
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase PCR

tCHOL	Total cholesterol
Sa/So	Sphinganine/Sphingosine
SCFA	Short chain fatty acids
TBARS	Thiobarbituric acid reactive substances
TDI	Tolerable daily intake
TG	Triglyceride
TNFα	Tumour necrosis factor α
TP	Total protein
WST	Water soluble tetrazolium salt
ZEN	Zearalenone

1. INTRODUCTION

Secondary metabolites of fungi are natural products which can be harmful/undesirable or beneficial to humans (Demain and Fang, 2000). They are termed secondary because they do not play a role in the primary metabolism; they may be necessary for sporulation (Calvo et al., 2002). Mycotoxins belong to the harmful/undesirable secondary fungal metabolites.

Ergotism is the oldest mycotoxicosis known and it is referred as St Anthony's fire as well. This mycotoxicosis has as a causative agent the fungus genus *Claviceps*. *Claviceps* fungi invade the plant by replacing the ovary with a mass of fungal tissue (in the female part of the host plant) which is called sclerotium (CAST, 2003). Although ergotism was described during the Middle Ages the foundation of modern toxicology did not take place until the 1960's when the Turkey X disease occurred. Thousands of turkey poults died in England; later it was discovered that the cause was the exposure to aflatoxins, mycotoxins produced by fungi of the *Aspergillus* genus, although later it was disputed involving another mycotoxin cyclopiazonic acid in the etiology of the disease (Cole, 1986).

Since then approximately 400 fungal secondary metabolites have been characterized (Bennet and Klich, 2003). The most important toxin producing genera are *Fusarium, Aspergillus, Penicillium, Claviceps* and *Alternaria* (CAST, 2003). The most important and most thoroughly studied mycotoxins and mycotoxin classes are fumonisins, trichothecenes, aflatoxins, ochratoxins and zearalenone. For the clarification of the erroneously and unequivocally used terminology in the year 2014, a new, systematic definition criterion has been worked out, in which mycotoxins are differentiated in free and unmodified, matrix-associated and chemically modified (Rychlik et al., 2014). The occurrence of mycotoxins worldwide is a very important issue in the aspect of both food and feed safety. The Commission of European Union has set guidance levels (76/2006/5EC) for several mycotoxins in products intended for animal feeding (European Commission, 2006). In Europe, and in particular in the EU, regulatory and scientific interest in mycotoxins has undergone a development in the last decade from autonomous national activities towards more EU-driven activities with a structural and network

character. Harmonized EU limits now exist for 40 mycotoxin-food combinations (van Egmond et al., 2007).

Fusarium mycotoxins are the most frequently occurring mycotoxins worldwide and more specifically fumonisin B1 (FB_1), deoxynivalenol (DON) and zearalenone (ZEN) are the most likely to co-occur (Griessler et al., 2010; Rodrigues and Naehrer, 2012; BIOMIN 2015; 2016; Smith et al., 2016). Thus these mycotoxins were chosen in order to investigate their single and combined effects. To the best of our knowledge, there are not many studies on their binary and especially ternary mixtures both *in vivo* and *in vitro*. Investigation of the interactive effects is very crucial for risk assessment since it has been confirmed - as aforementioned - that mycotoxin co-occurrence is a fact. Hence the single and combined effects of FB1, DON and ZEN were investigated both *in vivo* and *in vitro* in monogastric animals.

2. LITERATURE REVIEW

2.1 *Fusarium* mycotoxins

2.1.1 Fumonisin B1

The 28 fumonisin analogues that have been characterized since 1988 (Gelderblom et al., 1988) can be separated into four main groups identified as the fumonisin A, B, C, and P series (Rheeder, 2002). Among the four primary series of fumonisins (FA, FB, FC and FP), the toxicologically most important compounds are the FB analogues. Fumonisin B group comprises of six compounds; fumonisin B_1, B_2, B_3, B_4, B_5 and B_6 (FB_1, FB_2, FB_3, FB_4, FB_5 and FB_6 respectively), FB_1 is the major metabolite (Kubena et al., 1997; Månsson et al., 2010, Bartók et al., 2013). FB_1 is generally found in the highest concentrations in maize and maize-based products. The main producers of FB_1 are *Fusarium verticillioides* (previously *F. moniliforme*) and *F. proliferatum*, which mainly occur in corn. FB_1 is chemically a diester of propane-1, 2, 3- tricarboxylic acid (tricarballylic acid, TCA) and 2-amino-12, 16- dimethyl- 3, 5, 10, 14, 15-pentahydroxyeicosane, in which the C_{14} and C_{15} hydroxyl groups are esterified with the terminal carboxyl group of TCA (Marasas, 2001; Heidtmann-Bemvenuti et al., 2011). The primary amino group of FB_1 is essential for its toxicity. This is proven by the fact that acetylation of FA_1 to FB_1 inhibits its cytotoxic capability (Stockmann-Juvala and Savolainen, 2008).

The main mechanism of action of FB_1 is a result of its similarity (long-chain hydrocarbon unit) to sphingosine and sphinganine, thus inhibiting the ceramide synthase enzyme. As a consequence FB_1 disturbs the metabolism of sphingolipids resulting in accumulation of free sphingoid bases, altered sphinganine (Sa) to sphingosine (So) ratio (Sa/So), and depletion of complex sphingolipids. This was hypothesized by Wang et al. (1991) and was further confirmed by several research groups (Yoo et al., 1992; Wang et al., 1999; Enongene et al., 2000). These effects essentially lead to impairment of cell membrane function (Riley et al., 2001). Apart from inhibiting ceramide synthase, FB_1 is suggested to stimulate apoptosis in cells but the mechanism is not clear. Some possible explanations could be the induction of lipid peroxidation and the decreased concentrations of antioxidants such as

glutathione (GSH) (Surai and Dvorsrka, 2005). Gelderblom et al. (1997) found altered lipid constituent proportions in exposed rats; in particular, phosphatidylethanolamines were significantly increased in the mitochondrial and even in the plasma membrane fractions. The main targets of FB_1 are the liver and kidney (Stockmann-Juvala and Savolainen, 2008; Voss et al., 2007).

FB_1 is confirmed causative factor of several diseases in livestock. In *Equidae*, neurotoxic and hepatotoxic effects have been reproduced experimentally. Moreover, FB_1 has been shown to induce cardiovascular problems to horses (Smith et al., 2002). The most important disease in horses, caused by FB_1 is equine leukoencephalomalacia (ELEM) (Marasas et al., 1988; Kellerman et al., 1990; Ross et al., 1993). In swine, FB_1 induces pulmonary oedema (porcine pulmonary oedema, PPE) syndrome (Colvin and Harisson, 1992). FB_1 can also induce hepatotoxicity to swine (Gumprecht et al., 1998). Elevated serum cholesterol is another characteristic of FB_1 toxicity (Rotter et al., 1996b; Gumprecht et al., 1998). Poultry and cattle are less sensitive to FB_1 (Bolger et al., 2001). In lambs, acute hepatic and renal toxicity was reported by Edrington et al. (1995).

Carcinogenicity due to FB_1 has been reported in laboratory animals as well. Gelderblom et al. (1994) reported FB_1-induced hepatocellular carcinoma in rats. FB_1 is possibly carcinogenic to humans; it has been classified as a possible carcinogen (group 2B) by IARC (2002). FB_1 has been associated with cancer of the oesophagus in rural regions of Southern Africa and China (Sydenham et al., 1990; Yoshizawa et al., 1994). FB_1 can be teratogenic as well. It can cause neural tube defects (NTD) to embryos (Marasas et al., 2004).

Fumonisins (FUMs) are regulated in the European Union and the levels vary according to the species' sensitivity. In the case of fumonisins (and the rest fusariotoxins), there are guidance (not maximum limits like for aflatoxins) values in both feed and food (European Commission, 2006).

2.1.2 Deoxynivalenol

DON is a member of the trichothecene family, which is consisted of approximately 200 compounds (Grove 2000; Pestka 2007, 2010). Trichothecenes

are structurally related sesquiterpenes which include four basic types, from which the most important representatives belong to types A, B and D (Pestka, 2007). The chemical name of DON is 12, 13-epoxy-3α, 7α, 15-trihydroxythichothec-9-en-8on ($C_5H_{20}O_6$). The epoxide at the positions $C_{12,13}$ makes trichothecenes toxic (Heidtmann-Bemvenuti et al., 2011). The ketone group which is in C_8 is a characteristic of type B compounds (Ueno, 1984). The high melting point of DON gives its resistance to heat (cooking, cereal processing) (Bonnet et al., 2012). DON is produced by *Fusarium graminearum* and *F. culmorum* and despite being the least toxic trichothecene; its ubiquitous occurrence in grains increases its importance for food and feed safety.

Like the rest trichothecenes, DON inhibits the protein synthesis (the initiation and/or elongation of the polypeptide chain is inhibited) because of its ability to bind to eukaryotic ribosomes (Ueno et al., 1968; Ueno, 1984).

In pigs, DON is absorbed rapidly and almost completely through the stomach and proximal intestine (Dänicke et al., 2004). After DON is metabolized a chemical compound is formed; de-epoxy-DON.

The main symptoms of DON are diarrhoea, nausea, reduced feed intake and weight gain (Bonnet et al., 2012). The pig is the most sensitive while ruminants are the least sensitive animal species to DON. Monogastric animals are extremely prone to growth and body weight gain suppression upon DON exposure. In swine, DON is causing feed refusal already at 1 mg/kg, and emesis (vomiting; the minimum oral dose is 100μg/kg of body weight), thus given the trivial name vomitoxin (Vesonder et al., 1973; Ueno, 1984; Forsell et al., 1986). It has been demonstrated that DON can cause histopathological alterations to the gastrointestinal tract to pigs (Zielonka et al., 2009). The effect of DON on the intestine of pigs was observed by Lessard et al. (2015). They reported down-regulation of the genes important for integrity and barrier function. In chickens, DON can cause alterations to the small intestine for both weight and morphology (Awad et al., 2005). Changes in liver morphology (possible hepatic impairment) can be also induced by DON in rats (Bracarense et al., 2017).

DON, as well as other trichothecenes, has immunomodulatory abilities (Pestka and Smolinski, 2005). It was suggested by Rotter et al. (1996a) that the

immunosuppression induced by trichothecenes is due to the inhibition of translation (Bamburg, 1983), whereas the mechanism of immunostimulation is not so clear. Even low DON doses can downregulate the gene expression of cytokines like interleukin 1β (IL-1β) or tumor necrosis factor α (TNFα) in the small intestine (Becker et al., 2011). A recent study in rats demonstrated the immunosuppressive properties of DON by means of histopathological examination of lymphoid organs (Bracarense et al., 2017). Poultry is quite resistant to the negative effects of DON. Despite this, prolonged exposure to low doses of DON can cause problems related to productivity and immunity (Awad et al., 2013).

Reproductive system can be affected by DON as well. In rats, DON caused significant alterations to hormones like luteinizing (LH) and follicle stimulating (FSH) and testosterone in rats. Furthermore, DON affected the weights of the prostate and of the epididymal and seminal vesicle. Abnormal tail incidents were also increased along with a decrease in the numbers of spermatid and cauda epididymal sperm (Sprando et al., 2005).

As mentioned before, there are guidance values for all fusariotoxins including DON (European Commission, 2006). DON has not been classified as carcinogenic yet (Group 3 according to IARC, 1993).

2.1.3 Zearalenone

Chemically ZEN is a resocyclic acid lactone 6-(10-hydroxy-6-oxo-trans-1-undecenyl])-β-resocyclic acid lactone (Urry et al., 1966). ZEN was isolated by Christensen et al. (1965) who named it F-2 toxin. The next year, Urry et al. (1966) characterised its chemical structure and gave its present name zearalenone. ZEN is produced by various species of *Fusarium* genus, namely *Fusarium graminearum* (*Giberella zea), F. culmorum, F. equiseti, F. sambucinum* and *F. crokwellense* (Placinta et al., 1999; CAST, 2003). ZEN very often is co-occurring with DON since two of the producers of both toxins are the same i.e. *F. graminearum* and *F. culmorum*.

ZEN is absorbed rapidly after oral administration (Zinedine et al., 2007). Olsen et al. (1981) suggested two pathways for biotransformation of ZEN, hydroxylation and conjugation. After hydroxylation two metabolites are formed, α-

and β-ZOL with the latter sometimes having a higher potency (Frizzell et al., 2011). These two metabolites are isomers and can be produced by fungi as well but in much lower concentrations (Zinedine et al., 2007). The main site of ZEN's metabolization is the liver (Kiessling and Pettersson, 1978).

ZEN is not considered as a real toxin but rather a mycoestrogen due to its similar chemical structure with oestrogens. This similarity leads to a competitive binding to oestrogenic receptors which affect the reproductive system of both male and female animals (Abdelhamid et al., 1992; Nakaido et al., 2004). In the case of ZEN swine is the most sensitive species as well (Tiemann and Dänicke, 2007).

In female animals, ZEN can induce an early onset of puberty and can alter uterus weight and the morphology of the genital tract (Christensen et al., 1965; Abdelhamid et al., 1992). As reviewed in Kanora and Maes (2009), ZEN can induce swelling of the vagina and the vulva and cause anoestrous or prolonged cycle in pigs. Furthermore, ZEN in high concentration (>25 ppm) can cause stillbirth or neonatal mortality. ZEN can affect male pigs as well; testicular weight decreases, epididymis and vesicular glands decrease in size, libido drops and spermatozoa do not bind as effectively to the zona pellucida (Christensen et al., 1972; Ruhr et al., 1983; Tsakmakidis et al., 2007). The effects of ZEN in male animals have been studied mainly in rats, mice and rabbits. It has been demonstrated that ZEN affects sperm cells, spermatogenesis, testosterone concentration and germ cells. As regards to spermatozoa, ZEN can affect the number, morphology and motility of the spermatozoa (Zatecka et al., 2014; Boeira et al., 2015). Furthermore, spermatogenesis can be adversely affected and one group has investigated the expression of genes essential for spermatogenesis (Cho et al., 2011; Zatecka et al., 2014). ZEN-induced germ cell degeneration has also been reported (Kim et al., 2003).

ZEN despite its pronounced ability to affect the reproductive system may cause effects in non-reproductive systems of the organisms (*in vitro* and *in vivo*). ZEN has been shown to exert cytotoxic and genotoxic effects (Ouanes et al., 2003; Abid-Essefi et al., 2004; Vlata et al., 2006; Gao et al., 2013). ZEN has been reported to be immunotoxic to rats (Hueza et al., 2014) and to exert immunomodulating effects to piglets (Marin et al., 2010). ZEN can be hepatotoxic as well, which has

been reported in gilts, rats and rabbits (Marin et al., 2013b). Oxidative stress is one of the mechanisms associated with genotoxicity. In the following chapter (2.1.4.2), studies regarding ZEN-induced oxidative stress will be discussed.

ZEN has not yet been classified as a carcinogen (Group 3) for humans by IARC (1993). The presence of ZEN, like FB_1 and DON in animal feed is regulated in Europe (European Commission, 2006).

2.1.4 Cyto- and genotoxic effects of FB_1, DON and ZEN

The most popular endpoints used for the investigation of mycotoxin effects *in vitro* are cytotoxicity and genotoxicity.

As regards cytotoxicity, several parameters have been employed; the most frequently used are cell proliferation, metabolism and viability, cell membrane integrity as well as macromolecule synthesis (protein and DNA). Less frequently, cell cycle is investigated along with cell morphology. The most commonly used cell lines are intestinal cell lines of human (Caco-2, HT-29, HCT116) and animal origin (IPECJ-2), peripheral blood lymphocytes isolated usually from either humans or pigs and kidney (porcine, monkey, bovine, canine) cell lines. Genotoxicity has been studied as well but in a lesser extent, especially *in vitro*. Genotoxicity can be assessed studying different parameters like DNA damage, oxidative stress/status, micronuclei (MN) and DNA adducts formation, and sister chromatid exchange.

2.1.4.1 Studies on cytotoxic effects

The effects of mycotoxins on immunity are very important since low immunity can predispose humans and animals to infectious diseases. FB_1 has been shown to be cytotoxic to both human and porcine peripheral blood mononuclear cells (PBMC) (Meky et al., 2001; Marin et al., 2007; Stoev et al., 2009; Mulunda and Dutton, 2014). Similarly to FB_1, DON – known for its immunomodulation properties - has exerted cytotoxic effects to human and porcine PBMC (Lautraite et al., 1997; Thuvander et al., 1999; Meky et al., 2001; Goyarts et al., 2006a, Marin et al., 2006a; Baltriukiene et al., 2007; Taranu et al., 2010). Minervini et al. (2004) studied the effects of FB_1 and DON in human erythroleukemia cell line (K562).

ZEN's cytotoxic effects have been demonstrated only in one study using human PBMC by Vlata et al. (2006).

Cytotoxicity due to FB_1 has been exerted to human (Caco-2 and HT-29 cells) and porcine (IPEC-J2 cells) intestinal cells as well (Clarke et al., 2014; Kouadio et al., 2005; Wan et al., 2013a; Minervini et al., 2014). DON's cytotoxic effects has been assessed in both human (Caco-2 and HCT116) and porcine (IPEC-J2) intestinal cells (Kouadio et al., 2005; Vandenbroucke et al., 2011; Wan et al., 2013a; Bensassi et al., 2014; Manda et al., 2015; Springler et al., 2016). On the other hand, the cytotoxic effects of ZEN have been studied only in human epithelial cells (Abid-Essefi et al., 2003; Abid-Essefi et al., 2004; Kouadio et al., 2005; Wan et al., 2013a; Bensassi et al., 2014).

Another important tissue in mycotoxin research is kidney, thus several studies have been performed in kidney cell lines of different origin. More specifically, FB_1 exerted cytotoxic effects on monkey, porcine and bovine kidney lines (Abado-Becognee et al., 1998; Šegvić Klarić et al., 2008; Clarke et al., 2014). In a study from the late 80's the cytotoxic effects of DON on monkey, porcine and canine kidney cell lines were investigated (Reubel et al., 1989). On the other hand, ZEN's cytotoxic effects on kidney *in vitro* have been assessed by the same group in three different studies (Abid-Essefi et al., 2003; Abid-Essefi et al., 2012; Bouaziz et al., 2013).

The liver is the main site for the metabolism of any substances entering the body of animals or humans. Despite this, only a few studies have been performed on the *in vitro* effects of FB_1, DON and ZEN in the liver. The cytotoxic effects of FB_1 have been studied in liver cells as well (primary hepatocytes; Riedel et al., 2016). The only study on the cytotoxic *in vitro* effects of DON in the kidney is from Ueno et al. (1973). The effects of ZEN in human liver cells (HepG2) have been studied by Hassen et al. (2007).

Cytotoxicity has been scarcely studied *in vivo*. One of the few studies in cows was performed by Dänicke et al. (2011). Lymphocytes were isolated from the DON-treated cows for the assessment of the cytotoxicity (*ex vivo)* and non-treated cows for exposure to DON (*in vitro*) using 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide (MTT) test. In the *ex vivo* experiment, viability was

decreased by 18%. On the other hand for the *in vitro* cell viability was seriously affected (50-80% decrease) when DON concentration was over 0.27 μM (0.08μg/ml). In the study of Marin et al. (2013a) piglets were exposed to ZEN for 18 days. At the end of the trial, lymphocytes were isolated from blood, spleen and lymph nodes and their proliferation was measured by the [methyl-^{3}H]-thymidine proliferation assay. After 72h of incubation, viability decreased by 66.15% in lymphocytes isolated from blood as compared to control cells. On the other hand, no effect was observed in lymphocytes derived from spleen or lymph nodes.

2.1.4.2 Studies on genotoxic effects

One of the most popular methods for the assessment of genotoxic effects is comet assay, which is assessing the DNA damage. Other methods are micronuclei (MN) formation assay, DNA fragmentation assessment and sister chromatid exchange (SCE) assay. Oxidative stress has been investigated in many studies since it has been proposed as a possible mechanism of genotoxicity. In order to assess the oxidative stress, various endpoints are used such as the production of reactive oxygen species (ROS) and the level of thiobarbituric acid-reactive substances (TBARS).

FB_1 has been shown to induce MN formation a dose-dependent manner to porcine kidney epithelial (PK15) cells (Šegvić Klarić et al., 2008). In the study of Galvano et al. (2002), FB_1 induced DNA damage to human fibroblasts. Ehrilch et al. (2002) studied the genotoxic effects of FB_1 in HepG2 cells and found a dose- and time-dependent effect on the induction of DNA damage and MN formation. DON's genotoxic effects have been studied in human epithelial cells. Bony et al. (2006) observed the genotoxic effects of DON on Caco-2 cells with comet assay. HepG2 cells were used by Zhang et al. (2009) for the assessment of genotoxicity caused by DON. Oxidative stress was correlated with the induced DNA damage by measuring the production of ROS and the level of TBARS. ZEN's genotoxic effects have been studied by the same group in monkey (Vero) kidney and human (Caco-2 and Hep-G2) cell lines (Abid-Essefi et al., 2003; Ouanes et al., 2003; Hassen et al., 2007; Abid-Essefi et al., 2012; Bouaziz et al., 2013). In most of the studies, DNA damage

was assessed by comet assay and was correlated with the production of ROS. The formation of MN in Vero cells has been also assessed in one of the studies (Ouanes et al., 2003). In their early trial, DNA fragmentation in Caco-2 was studied (Abid-Essefi et al., 2003). Apart from this group's studies, there is another study on DNA damage and ROS production due to ZEN exposure in the human embryonic cell line (Gao et al., 2013).

In several *in vivo* trials, genotoxic effects are studied in parallel with oxidative stress. Aranda et al. (2000) assessed the genotoxic effect of FB_1 in mice. They studied the formation of MN in the bone marrow of the treated mice. Another study on the genotoxicity of FB_1 was performed by Theumer et al. (2010). DNA damage was assessed by comet assay and MN formation test in spleen mononuclear cells of male Wistar rats. Additionally, malondialdehyde (MDA) was measured in order to assess the effect of oxidative stress with genotoxicity. Kouadio et al. (2013) investigated the effects of low doses of dietary DON and FB_1 on DNA fragmentation of blood lymphocytes in mice. The DON-induced DNA damage was assessed on spleen leukocytes of chickens (Frankic et al., 2006). DON fed to broiler chickens induced DNA damage to blood lymphocytes of broiler chickens (Awad et al., 2014). Apart from the DNA damage, the level of oxidative stress (TBARS) was measured in plasma, heart, kidney and segments of the large intestine (duodenum and jejunum). In the study of Payros et al. (2017) DON exacerbated the genotoxicity of the rats colonized with *Escherichia coli*. Regarding ZEN's genotoxic effects there is only one study in bone marrow and spleen lymphocytes of mice (Ben Salah-Abbès et al., 2009). Two studies on ZEN assessed oxidative stress in chickens and mice (Zourgui et al., 2008; Grešáková et al., 2012).

One of the most important factors determining the toxicity of a mycotoxin in the body of an animal is the metabolism (biotransformation pathways, processes of elimination, microbial transformation) which can explain the different sensitivity among farm animals (D'Mello et al., 1999; Minervini and Dell' Aquila, 2008). The dose levels as well as the duration of the exposure are also affecting differently the final effects of mycotoxins (Forsell et al., 1986). A very good example regarding differences in doses and exposure time are two trichothecene mycotoxins. Although the acute toxicity of T-2 mycotoxin is much higher than that of DON, in practice T-2

occurs in much lower levels hence the chronic exposure of animals to the ubiquitous occurring DON can lead to more adverse effects in the long run. All the aforementioned differences are highlighted by the fact that the same mycotoxin - e.g. FB1 - results in different syndromes in different animal species as mentioned in previous sections of the literature review. At a cellular level different mechanisms of actions, to name a few, compromising of the cell membrane integrity, production of reactive oxygen species (ROS), binding to the DNA or to receptors, inhibiting macromolecular synthesis; cause the manifestation of different effects among cell lines. The issue becomes more complicated when the animals/cells are exposed to combined mycotoxins because all the aforementioned factors are interacting.

2.2 Mycotoxin co-occurrence

As aforementioned the various mycotoxins are regulated worldwide with maximum or recommended limits. However, single mycotoxin exposure is the exception than the rule, and this is a result of several factors. Firstly, many fungal species can produce more than one mycotoxin simultaneously. In addition to that, crops can be infected by different genera of fungi at the same time and last but not least, complete feed is prepared by various commodities. This mycotoxin co-occurrence is reported in several reviews and surveys (Speijers and Speijers, 2004; Griessler et al., 2010; Monbaliu et al., 2010; Rodrigues and Naehrer, 2012, Streit et al., 2013; BIOMIN, 2015, 2016; Smith et al., 2016). In a survey conducted over a period of 4.5 years in countries of Southern Europe (Portugal, Spain, Italy, Greece and Cyprus), the *Fusarium* mycotoxins were found to be the major contaminants (fumonisins, type B trichothecenes and ZEN) of feedstuffs and compound feed samples (Griessler et al., 2010). In the survey of Monbaliu et al. (2010) 75% of the samples were contaminated with at least one mycotoxin. The most frequently occurring mycotoxins were type B trichothecenes and fumonisins. The highest incidence of co-occurrence was in maize (41 samples). In the survey of Streit et al. (2013) that lasted from 2004 to 2012, all (83) the samples were found to be co-contaminated with 7 to 69 metabolites with 28 being the most frequent number of metabolites per sample. Similarly to the other surveys, *Fusarium* mycotoxins were

detected most often. In a most recent survey conducted by BIOMIN (Figure 1) 94% of the samples tested worldwide were contaminated with more than ten mycotoxins, with DON, ZEN and FUMs being present in over 50% (BIOMIN, 2016). Samples were collected from 81 countries and 99066 analyses were performed in 16511 samples.

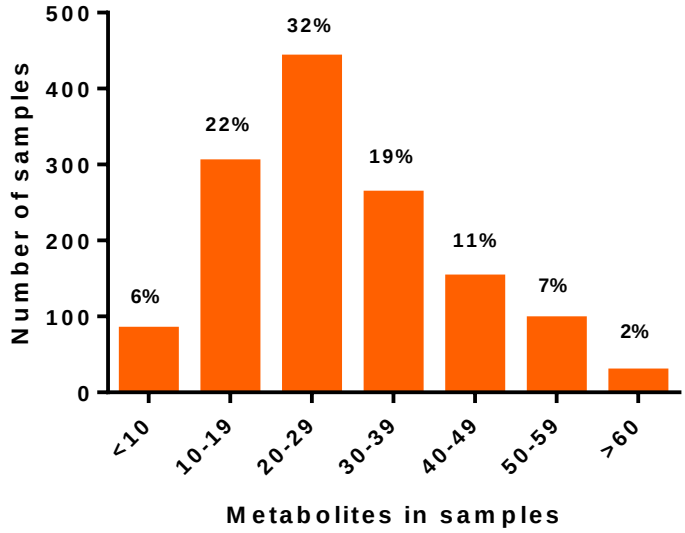


Figure 1: Number of metabolites in samples of cereals, corn and finished feed from around the globe (adapted from BIOMIN mycotoxin survey 2016).

Furthermore, in Europe the most frequently occurring mycotoxins were DON, ZEN and FB_1 with an average of 70%, 48% and 48% contaminated samples (finished feed, corn and cereals), respectively (BIOMIN, 2016). The level of contamination, median and average concentations of the positive samples as well as maximum concentrations of FB1, DON and ZEN in Europe are shown in Table 1.

Table 1: *Fusarium* mycotoxins contamination levels in main commodities in Europe (adapted from BIOMIN mycotoxin survey 2016).

Commodities	**Mycotoxins**		
Finished feed	**FB_1**	**DON**	**ZEN**
Average of positives (µg/kg)	792	420	85
Median of positives (µg/kg)	164	138	20
Maximum (µg/kg)	33970	21300	7400
Corn	**FB_1**	**DON**	**ZEN**
Average of positives (µg/kg)	2057	790	155
Median of positives (µg/kg)	845	422	44
Maximum (µg/kg)	42000	9816	2652
Cereals	**FB_1**	**DON**	**ZEN**
Average of positives (µg/kg)	560	1023	78
Median of positives (µg/kg)	36	400	26
Maximum (µg/kg)	13360	37640	3757

Guidance values for FB_1, DON and ZEN have been recommended by European Commision (Table 2; 2006/576/EC). In the following table only levels for pigs are given because they are the most sensitive species.

Table 2: Guidance values of FB_1, DON and ZEN in European Union (adapted from the Commission Recommendation 2006/576/EC).

	Commodity
Mycotoxins	**Complementary and complete feedings stuffs for pigs (12% moisture content, mg/kg of feed)**
FB_1	5
DON	0.9
ZEN	0.25

In their review, Smith et al. (2016) compiled data from 107 articles and concluded that the most frequent mixtures of fusariotoxins worldwide were DON+ZEN, FUM+ZEN, DON+ nivalenol (NIV), DON+T-2.

According to the aforementioned surveys, the mycotoxin concentrations are usually low. Acute toxicity is rarely occurring thus chronic exposure to low concentrations can be of high importance for human and animal health (Streit et al., 2013).

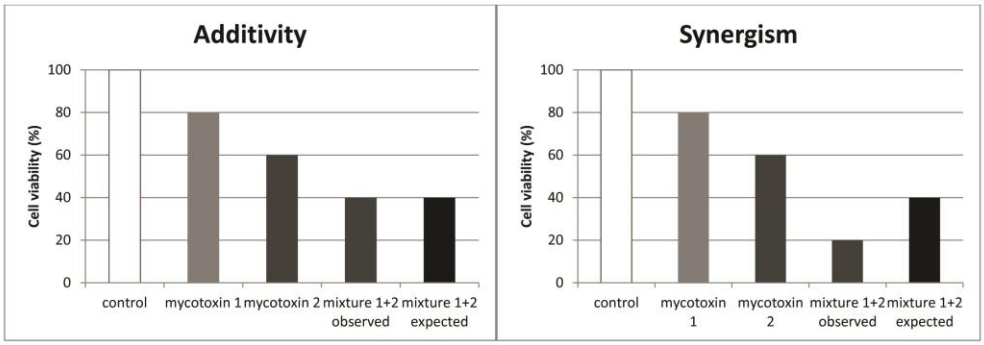
As it can be concluded from the aforementioned, *Fusarium* toxins (i.e. trichothecenes, fumonisins and zearalenone) are of particular interest, because their occurrence is ubiquitous in cereal grains, such wheat and corn, and additionally they co-occur very frequently.

The legislation is enacted based on risk assessment studies, which depend on the toxicity and exposure data of single mycotoxins. However, the effect of co-occurring mycotoxins cannot be predicted based on the effects of the single toxins. When mycotoxins interact with each other, they may interact. Thus the determination of the interactive effects of mycotoxins and especially in low concentrations is essential for the establishment or revision of tolerable daily intake (TDI) (Speijers and Speijers, 2004) and/or maximum/guidance levels (Verstraete, 2006). Recently, the European Food Safety Authority (EFSA) funded a new project for the risk assessment of mycotoxin mixtures in food and feed (EFSA, 2017).

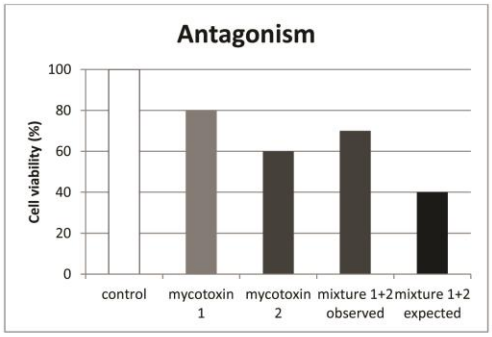
2.3 Combined effects of mycotoxins

There are several studies about interactions between mycotoxins (Grenier and Oswald, 2011; Alassane-Kpembi et al., 2017; Smith et al., 2016), but fewer report the combined effects of three specific *Fusarium* mycotoxins, which are more likely to co-occur; i.e. FB_1, DON and ZEN (Tables 3, 4). Most of the studies about the interactions of FB_1, DON and ZEN regard binary mixtures, although there are some ternary mixture studies (Forsell et al., 1986; Pestka et al., 1987; Harvey et al., 1996; Kubena et al., 1997; Grenier et al., 2011; Bracarense et al., 2012; Ficheux et al., 2012; Bensassi et al., 2014; Cortinovis et al., 2014; Kouadio et al., 2007; Wan et al., 2013a, b; Szabó-Fodor et al., 2015; Albonico et al., 2016; Tables 3, 4).

Interactive effects can fall in one of the three categories; addition, synergism and antagonism. *In vitro*, the definition is more clearly evident since we assess a specific endpoint and after calculating the expected value we compare it with the observed one and make our conclusions (Figure 2).



(a) (b)



(c)

Figure 2: Graphical representation of mycotoxin interactions on cell viability *in vitro* (adapted from Smith et al., 2016).

Table 3: *In vivo* studies on combined effects of fumonisin B_1 (FB_1), deoxynivalenol (DON) and zearalenone (ZEN)

Animal	Mycotoxin	Interaction	Exposure period	Parameters examined	References
B6C3F1 mice	DON+ZEN	Additive	56 days	Productive traits and blood parameters, organ weights, histopathology	Forsell et al., 1986
B6C3F1 mice	DON+ZEN	Synergism	14-21 or 56 days	Immune response	Pestka et al., 1987
Growing barrows	FB_1+DON	Additive and greater-than additive	28 days	Productive traits and blood parameters, immune response, histopathology	Harvey et al., 1996
Broiler chickens	FB_1+DON/ T-2	Additive and less than additive	19 or 21 days	Productive traits and blood parameters	Kubena et al., 1997
Piglets	DON+FB_1	Synergism	35 days	Productive traits and blood parameters, histopathology, immune response	Grenier et al., 2011
Piglets	FB_1+DON	Antagonistic to synergistic	35 days	Morphology, histology, cytokines' expression (intestine)	Bracarense et al., 2012
Swiss mice	FB_1+DON	Additive or more than additive, synergistic	7 days	Serum and urine chemistry, renal DNA methylation	Kouadio et al., 2013
Rabbit bucks	FB_1, DON+ZEN	Antagonistic to synergistic	65 days	Reproductive parameters	Szabó-Fodor et al., 2015
Kunming mice	DON+ZEN	Less than additive	4 days	Serum chemistry, antioxidant status of kidney, cell apoptosis	Liang et al., 2015
Kunming mice	DON+ZEN	Additive and synergistic	4 days	Antioxidant status of spleen, interferon levels, T-cell subsets	Ren et al., 2016

Table 4: *In vitro* studies on combined effects of fumonisin B_1 (FB_1), deoxynivalenol (DON) and zearalenone (ZEN); cell line, mycotoxins, effects, exposure period, endpoint, references)

Cell line	Mycotoxin	Interaction	Exposure period	Parameters examined	References
Mouse fibroblasts (L929)	FB_1, DON, ZEN, NIV[1], T2	Additive and in few exceptions synergistic	Not specified	DNA synthesis	Groten et al., 1998
Mouse fibroblasts (L929)	FB_1, DON, ZEN, NIV, T2	Less than additive to synergistic	24h	DNA synthesis	Tajima et al., 2002
Caco-2	FB_1, DON, ZEN	Antagonistic, less than additive, synergistic	24/72h	Cell viability, protein synthesis, MDA[2] levels, DNA synthesis, methylation and fragmentation	Kouadio et al., 2007
Mononuclear cells (from cord blood from placenta)	FB_1, DON, ZEN, BEA, ENB[3], T2	Antagonistic and additive	14 days	Myelotoxicity	Ficheux et al., 2012
Intestinal porcine enterocytes (IPEC-J2)	FB_1, DON, ZEN, NIV	Additive and synergistic	48h	Cell viability, cytokine expression	Wan et al., 2013a, b
Porcine granulosa cells	FB_1+DON/ZEN	Additive and synergistic	24 and 48h	Cell proliferation, steroid production, gene expression	Cortinovis et al., 2014
Human colorectal carcinoma (HCT116)	DON+ZEN	Less than additive	24h/48h	Cell cycle and viability, mitochondrial transmembrane potential, PTP[4] opening	Bensassi et al., 2014
Bovine granulosa cells	FB_1, DON, α-ZEL[5], β- ZEL	Additive	48h	Cell proliferation, steroid production	Albonico et al., 2016

[1]nivalenol; [2] malondialdehyde; [3]enniatin-B; [4] permeability transition pore; [5α]-Zearalenol

2.3.1 *In vivo* studies

Animal experiments with combined *Fusarium* toxins have been performed on swine, poultry, mice, and rabbits. In the following paragraphs, an overview of these studies will be provided.

Mice were used in the early studies (Forsell et al., 1986; Pestka et al., 1987; Table 1). In the study of Forsell et al. (1986) weanling female mice (B6C3F1) were exposed to dietary DON and ZEN (5 and 10mg/kg of feed respectively). No interaction was observed for any of the physiological traits (production and blood parameters, organ weights, histopathology and immune parameters).

Pestka et al. (1987) studied the interactions of DON and ZEN on the immune function of B6C3F1 mice that were infected with the bacteria *Listeria monocytogenes*. The mycotoxin combination resulted in a decreased resistance to the bacteria suggesting synergism. Kouadio et al. (2013) performed a study on Swiss mice, which superseded their *in vitro* study about FB_1, DON and ZEN on Caco-2 cell line (Kouadio et al., 2007). Differences among male and female mice were also investigated. The combination of DON [45 μg/kg of body weight (BW)] and FB_1 (110 μg/kg of BW) for 7 days, exerted an additive or more than additive effect on the kidney of female mice regarding DNA methylation. Moreover, for both male and female mice the renal creatinine clearance was higher when the two toxins were combined showing a synergistic effect. The most recent study on mice regarding mycotoxins' interactions was performed by Ren et al. (2016). Female Kunming mice were used for the investigation of the combined effects of DON and ZEN (intraperitoneal injection in both studies) by Liang et al. (2015) and Ren et al. (2016). In the study of Liang et al. (2015) the target organ was the kidney. The endpoints used were serum chemistry, the antioxidant capacity of kidneys and cell apoptosis. The combination of DON (1.5 mg/kg of BW) and ZEN (20 mg/kg of BW) resulted in sub-additive nephrotoxic effect. Ren et al. (2016) used the spleen as a target organ. DON was administered at doses of 1.5 and 2.5 mg/kg of BW and ZEN was administered at doses of 20 and 30 mg/kg of BW. The endpoints used were the antioxidant status of the spleen, interferon levels and T-cell subsets. The combined effects were additive and synergistic.

Swine is an animal species quite suitable for mycotoxin research, because of its confirmed sensitivity to *Fusarium* mycotoxins (FB_1, DON and ZEN) (Colvin and Harisson, 1992; Pestka, 2007; Marin et al., 2010; Cortinovis et al., 2013). The first study was conducted by Harvey et al. (1996) on growing barrows (Table 1). The barrows were fed FB_1 (56 mg/kg of feed) and DON (3.6 mg/kg of feed) for 28 days. The interactions were additive and more than additive for most of the investigated endpoints. Ingestion of subclinical doses of DON (3.1 mg/kg of feed or 130 μg/kg of BW) and FBs (6.5 mg/kg of feed- 4.5 mg/kg FB_1 and 2.0 mg/kg of FB2 or 260 μg/kg of BW) respectively for 5 weeks by pigs induced greater histological damages and higher immunosuppression when these two toxins were consumed simultaneously, as compared to the single toxin effect (Grenier et al., 2011). From the same research group, a similar study (using the same toxin concentrations) revealed interactions which varied from synergistic to antagonistic, depending on the parameters examined (Bracarense et al., 2012). For example in jejunum, the villi height was significantly lower for the combined mycotoxins than FB_1 alone (synergistic), whereas in the ileum there was no significant difference among the groups (less than additive). The lymphocytic infiltration was decreased in the ileum of the animals consuming DON+FB_1 (antagonistic). The authors' conclusion was that chronic ingestion of mycotoxins in low doses could predispose farm animals to infections caused by enteric pathogens due to alterations in the intestine.

Poultry is also commonly used in mycotoxins research because it is frequently exposed to mycotoxins due to their cereal based feeds. Although they are not as sensitive as swine, stress and potential high concentrations of mycotoxins due to special weather conditions could lead to hazardous health effects (Kubena et al., 1997). There are several studies concerning combined effects of mycotoxins on poultry (Grenier and Oswald, 2011), but regarding FB_1, DON and ZEN there is only one (Kubena et al., 1997). In this trial, the effects of the combination of FB_1 and DON (300 mg/kg and 15 mg/kg of feed respectively) were studied in a dietary exposure which lasted from hatching till 21 days of age. The exposure interactions observed were synergistic (serum chemistry), less than additive (body weight gain) and antagonistic (relative heart weight).

Rabbits are widely used as model animals in toxicological studies due to their high reproduction rate and the facilitation of measurement of various physiological parameters (Kachlek et al., 2016). Thus they have also been used in mycotoxin research, although mycotoxicoses occur less frequently than in other animal species.

In some of the studies other *fusariotoxins* like trichothecenes (T-2 and NIV), metabolites of ZEN (α-ZEN) and emerging mycotoxins [Beauvericin (BEA), enniatin B (ENB)] were used. The toxins are only referred in the tables (Tables 3, 4) for the convenience of the readers, but they will not be discussed since they are not in the scope of my studies.

2.3.2 *In vitro* studies

In vivo studies are important to determine the effect on complex systems as living organisms. Still unraveling the mechanisms of actions in *in vitro* level is equally important. In addition, spearing animal experiments is in agreement with the Three R guides (replacement, reduction and refinement; Fenwick et al., 2009).

Bensassi et al. (2014) studied the interaction of DON and ZEN in the human colon carcinoma (HCT116) cell line. The endpoints used were cell viability, cycle analysis, mitochondrial apoptosis (mitochondrial membrane potential and permeability transition pore (PTP) opening). The combination of the toxins increased the cell proliferation as compared to the individual toxins thus showing an antagonistic effect on cytotoxicity, whereas a subadditive effect was observed for the mitochondrial apoptosis.

Kouadio et al. (2007) studied the combined effects of FB_1, DON and ZEN in binary and ternary mixtures using the human intestinal cell line Caco-2. In this study, a lot of endpoints were investigated: cytotoxicity, protein synthesis, MDA levels and DNA synthesis, methylation and fragmentation. The least cytotoxic mixture was the FB_1+ZEN (far less than additive) and the most cytotoxic was FB_1+DON+ZEN (synergism). The binary mixtures of DON with ZEN or FB_1 increased lipid peroxidation in a synergistic manner. The binary mixtures acted synergistically since they induced greater DNA damage than that of the individual

toxins. On the other hand, the percentage of inhibition of DNA synthesis of the ternary mixture was lower (25%) than any of the three mycotoxins individually (45, 70 and 43% for ZEN, DON and FB_1 respectively), indicating antagonism.

Two studies by the same research group have been performed on swine jejunal epithelial cells concerning cytotoxicity and the expression of pro-inflammatory cytokines, respectively (Wan et al., 2013a,b). The study about cytotoxicity revealed that even at non-cytotoxic individual mycotoxin concentrations the different combinations of FB_1, DON, and ZEN were already cytotoxic. Particularly, the greatest loss of viability was induced by the quaternary mixture of DON, NIV, ZEN and FB_1 which exerted a synergistic effect (Wan et al., 2013a). The second study investigated the mRNA expression of pro-inflammatory cytokine [(interleukins α, β, 6 and 8- IL1α, IL1β, IL6, IL8), tumour necrosis factor α (TNFα) and monocyte chemoattractant protein-1 (MCP-1)] genes. Non-cytotoxic and cytotoxic concentrations of the toxins were used. Surprisingly, the mixtures in non-cytotoxic concentrations acted in a synergistic manner causing a significant upregulation of pro-inflammatory cytokine mRNA, which can lead to immunostimulation (Wan et al., 2013b).

A recent study on porcine granulosa cells exposed to FB_1 alone and in combination with DON or α-ZEN was performed by Cortinovis et al. (2014). DON inhibited cell proliferation, but when it was combined with FB_1, no significant difference was detected (additive effect). The same was observed regarding progesterone and oestradiol synthesis. For the first time, the interaction of two *Fusarium* mycotoxins was studied on bovine granulosa cells. The combination of FB_1 (30 ng/ml and 100 ng/ml) and DON (100 ng/ml) was additive for either cell proliferation or steroid (progesterone and oestradiol) production (Albonico et al., 2016).

Despite the classic cell lines, there are studies using less common cell lines to assess the adverse effects of mycotoxins. In the study of Ficheux et al. (2012), human hematopoietic progenitors were used for the assessment of *in vitro* myelotoxicity [assessed by colony forming unit granulocyte and macrophage (CFU-GM) assay] after co-exposure to six *Fusarium* mycotoxins (BEA, ENB, T-2, FB_1,

DON and ZEN) in binary mixtures. DON+ZEN and DON+ FB_1 showed additive and antagonistic myelotoxic effects respectively.

The aforementioned studies focused either on binary or ternary mixtures. In the studies of the same research group i.e. Groten et al. (1998) and Tajima et al. (2002), the interactions of five *Fusarium* toxins (FB_1, DON, NIV, T-2 and ZEN) were investigated using DNA synthesis as the only endpoint on mouse fibroblast (L929) cells. The observed effects were mostly addition and in a lesser extent synergism (Groten et al., 1998). In the other study, five components produced less than additive effect and other four toxins revealed significant synergistic interactions (Tajima et al., 2002). These studies were mainly performed to prove the importance of the use of a robust mathematical design (further details in the following chapter) so details of the interactions are not mentioned here.

2.4 Mathematical and statistical analysis of the interactions

A quite crucial issue regarding toxicological interactions is the experimental design in order to be able to characterize the nature of the interaction(s) (Chou, 2006; Šegvić Klarić, 2012). The selection of the right experimental design is essential for accurate mathematical - statistical analysis.

The simplest and most commonly used mathematical model is the arithmetic definition of additivity. This model is mostly used for binary and ternary mixtures. The expected combined effect is the sum of the effects of the individual toxins [cytotoxic effect (mycotoxin 1+ mycotoxin 2) = cytotoxic effect (mycotoxin 1) + cytotoxic effect (mycotoxin 2)]. The arithmetic definition of additivity is a widely used model (Kouadio et al., 2007; Ficheux et al., 2012, Bensassi et al., 2014; Cortinovis et al., 2014).

Factorial (fractional, full, central composite) designs are used when many concentrations and/or combinations of mycotoxins have to be assessed. It is thus dramatically decreasing time and costs since the combinations tested are far less than the originally calculated ones (Groten et al., 1998; Tajima et al., 2002). The interactions occuring in factorial desings are determined by univariate analyses of variance; significant differences indicate synergistic or antagonistic effects (Wan et al., 2013a, b).

2.5 Alleviation of mycotoxin adverse effects by medicinal plants

In order to counteract the effects of mycotoxins, various strategies have been implemented. Several agricultural practices are used in order to prevent fungal infestation of the plants and subsequently mycotoxin production (Jouany, 2007). When prevention of mycotoxin production is not successful, several dietary strategies are applied, such as binding agents (e.g. clay like minerals), mycotoxin degrading enzymes or additives, like antioxidant compounds (vitamin E, selenium) or plant extracts (Galvano et al., 2001; Jouany, 2007).

In traditional medicine, plants have been widely used for thousands of years in many countries such as China, India and several African countries (Fabricant and Farnsworth, 2001). In ancient literature, approximately 500 medicinal plants are mentioned (Verma and Singh, 2008). Nowadays, medicinal plants are often used to improve the health status of animals, especially after the ban of antibiotics in the European Union (European Union Commission, 2005).

Oxidative stress is one of the main pathways for cell damage (cytotoxicity, genotoxicity and immunotoxicity) thus many plants that possess antioxidant properties have been used in studies investigating their possible protective effect against mycotoxins (Abdel-Fattah et al., 2010; Abid-Essefi et al., 2012). *Carduus marianus* (syn. *Silybum marianum*) is a member of Asteraceae (Compositae) family which contains several species used in herbal medicine e.g. *Echinacea* (Gao et al., 2010). One of the most popular colloquial names of *C. marianus* is milk thistle (Dunnick and Nyska, 2012). The main extract of *C. marianus* is silymarin, a mixture of polyphenolic compounds, from which the main subclass consists of seven flavonolignans, i.e. silychristins A and B, silydianin, silybins A and B and isosylibins A and B (Shibano et al., 2007). *C. marianus* has been widely used in traditional medicine in Africa and it is known for its hepatoprotective (Flora et al., 1998) as well as for its antioxidant effect (Henning et al., 2014). Some studies suggest a potential positive effect in inhibition of chemically induced diabetes (Shakeel and Yar, 2014) or anti-atherosclerotic effect (Białecka, 1997). The antioxidant effect of silybins was observed in the study of Trappoliere et al. (2009) on human hepatic stellate cells. Its antioxidant effects could be a key factor in

protecting against mycotoxins which are known to induce oxidative stress as a mechanism of action.

C. marianus has been used as a protective agent against dietary administered mycotoxins (AFB_1 and FB_1) in several trials with pigeons, mice, rats and broiler chickens. The plant was included in the diet in two different forms, either the pure extract (silymarin alone or in conjunction with phospholipids) or the seeds in powder form (He et al., 2004; Tedesco et al., 2004; Grizzle et al., 2009; Chand et al., 2011; El-Adawi et al., 2011; Muhammad et al., 2012; Amiridumari et al., 2013, 2014; Malekinejad et al., 2015).

Although one of the main protective effects of *C. marianus* derives from its antioxidant properties, in only one from the above-mentioned studies (El-Adawi et al., 2011) this parameter was investigated. Most of the studies investigated the possible protective effects of the plant on post mortem lesions and serum biochemistry (hepatoprotective effect) of the treated animals. In most of the trials, *C. marianus* exerted protective effects against AFB_1 and FB_1 on productive performance parameters, lesional scores of tissues and liver enzymes' concentration. Detailed information on the experimental design and the effects of *C. marianus* in each study can be found in Chapter 4.1.2.

In conclusion it is evident that FB_1, DON and ZEN mycotoxins are of worldwide importance and their co-occurrence is increasing every year. Despite this, their mixtures and especially the ternary one have not been studied thoroughly (*in vitro*). Furthermore, it is very interesting to investigate how their combinations in low concentrations (*in vivo*). Therefore the following aims were determined.

The aims of the present work were the following:

1.) Investigation of the single and combined effect of low doses of FB_1, 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 effect of the medicinal plant *Carduus marianus* against DON.

3.) Determination of the IC_{50} value (half maximal inhibitory concentration**)** for FB_1, DON and ZEN using porcine lymphocytes. Additionally, the *in vitro* investigation of their combinations in binary and ternary mixtures on cytotoxicity and genotoxicity.

3. MATERIALS AND METHODS

In order to reach the aims mentioned above two *in vivo* and a series of *in vitro* experiments were conducted.

Regarding *in vivo* experiments both research protocols were reviewed by Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office, under permission number SOI/31/1679-11/2014.

All of the data have been published.

3.1 *In vivo* experiments

3.1.1 Effects of dietary exposure to three *Fusarium* mycotoxins

3.1.1.1 Experimental animals, housing and diets

The experimental animals were Pannon White rabbit breeding bucks (24 weeks of age, 4.0 ± 0.5 kg mean body weight, n=60). The bucks were individually housed in wire mesh cages (42 × 50 cm) in a closed building and the light cycle was 16h light /day. Average temperature ranged from 16°C to 18°C and the farm had overpressure ventilation.

The animals received a commercial diet containing 10.3 MJ digestible energy/kg, 15.5% crude protein, 4.0% crude fat and 14.7% crude fibre for a total of 65 feeding days. The feedstuffs provided were available *ad libitum*, and the rabbits also had free access to drinking water.

Bucks were divided into 4 experimental groups. One group of the experimental animals served as control. The feed of the other three groups contained fungal culture in a predefined concentration (Table 5).

Table 5: Experimental groups

Group	Mycotoxin concentration (mg/kg) of the feed	n
C (control)	0	15
F (FB_1)	5	15
DZ (DON+ZEN)	1+0.25	15
FDZ (FB_1+DON+ZEN)	5+1+0.25	15

The daily feed intake was registered by measuring back the left-over feed amount (in the first 3 weeks daily, and weekly thereafter), while body weight was recorded once every week. The health status of the animals was observed throughout the experiment, morbidity and mortality were logged daily.

3.1.1.2 Mycotoxin production

Fusarium verticillioides (for FB_1) and *Fusarium graminearum* (for DON and ZEN) (NRRL 20960 [MRC 826] and NRRL 5883, respectively) fungal culture (7 days old) was grown on 0.5 strength potato dextrose agar (PDA; Chemika-Biochemica, Basle, Switzerland). Agar discs (5 mm) were prepared with a cork borer (Boekel Scientific, Pennsylvania, USA), which were then stored at 10°C in darkness in test tubes containing sterile distilled water (10 discs/10 ml).

For toxin production, maize (40 g) was soaked in distilled water (40 ml) at room temperature for 1 hour in Erlenmeyer flasks (500 ml), closed with cotton wool plugs. This was followed by the addition of the inoculated agar discs (10 agar discs per flask) to the twice autoclaved (20 min.) matrix. The cultures were then stored and incubated at 24°C (FB_1), 28°C (DON) and 18°C (ZEN) for 3 weeks, respectively. The flasks were shaken twice every day during the first week of incubation. When the incubation time was complete the fungus-infected cereal was dried at room temperature and ground.

The homogenized fungal cultures contained FB, DON and ZEN at concentrations of 3300, 2010 and 1298 mg/kg, respectively. The European Commission has made recommendations (2006/576/EC) regarding the maximum level of several mycotoxins in complete diets (European Commission, 2006) and introduced regulations (2003/100/EC) regarding aflatoxins (European Commission, 2003). However, these only apply to certain cases, particularly with regard to rabbit feed. More specifically, guidance values on finished feed for rabbits exist only for FB1, based on which FB1 dose was pre-determined. On the other hand, the dose of DON was pre-determined according to the EU limits (based on the European Commission Recommendation 2006/576/EC) in finished feed for young pig as the most sensitive animal towards these Fusarium mycotoxins among livestock. Fungal

cultures were mixed into the feed of experimental animals, based on the presented dose in Table 5.

3.1.1.3 Samplings

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). Blood was sampled from the marginal ear vein, while sperm was collected after a training period into artificial vaginas and seminal plasma was separated by centrifugation. Samples for clinical chemistry were taken into native tubes. Heparinized blood was used for the determination of antioxidant parameters and testosterone concentration. Plasma was obtained by centrifugation (Janetzky T23, VEB, Leipzig, Germany) at 900 g for 15 minutes. Parameters of the antioxidant status were determined also from red blood cell haemolysate (RBCH) at day 60, which was prepared by adding 900 µl sterile distilled water to 100 µl RBC.

At the end of the study (on day 65), animals were exsanguinated after stunning. The weight of liver, kidneys, testicles and spleen was measured and macroscopic changes were analysed and recorded. After dissection (n=15/group) samples were taken for histopathological analysis; the testis, liver and kidney were fixed in 10% neutrally buffered formalin. For the analysis of antioxidant status, 2 g samples were taken from the liver.

3.1.1.4 LC-MS analysis of mycotoxin concentration in feed

LC-MS analysis was 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) comprised a vacuum degasser, a binary pump (20AD), a column oven (CTO 20A) autosampler (SIL 20ACHT), and mass analyser (MS 2020) with both atmospheric-pressure chemical ionization (APCI) and electrospray ionization (ESI) systems. Optimized mass spectra were obtained with an interface voltage of 4.5 kV, a detector voltage of 1.05 kV in negative mode, 1.25 kV in positive mode. Heat block temperature was 200°C and a desolvation gas temperature was 200°C. For nebulizing and drying gas, nitrogen was used (1.5 L/min and 15 L/min flow rate, respectively).

Chromatographic separation was performed at 50°C and achieved on an RP-18 (2.1 × 100 mm, 2.6 μm, KinetexTM, Phenomenex USA) stationary phase applying gradient elution 0.3 mL/min eluent total flow rate for mycotoxins and 0.4 mL/min for silymarin flavonoids, with A: 0.1% AcOH and B: 0.1% AcOH in methanol as eluent. With optimum method performance characteristics, analytes were quantified using external calibration.

Rabbit-feed samples were milled and extracted using 1% AcOH containing 75:25=MeOH: H2O (v/v) for F-2 CH3CN: H2O 1:1 (v/v) for FB_1 and water for DON and ZEN as the extraction solvent. The extracts were shaken at room temperature for an hour then decanted and the supernatant was collected. 1 mL of clean water extract was applied to the immunoaffinity column (IAC; Vicam, DON test) which contains specific antibodies for DON for purification. The IAC was washed with 5 mL water, and DON was slowly eluted in 2 mL methanol. ZEN and FB_1 were measured by the dilute and shot method. Romer Mix 4 (containing trichothecenes+ zearalenone at 10 mg/L) and Romer MIX 3 (containing FB_1-2 at 50 mg/L) primary stock solution were used as reference. 1 μL of each sample were analysed with a gradient: (0 min) 5% B, (3 min) 60% B, (8 min) 100% B, followed by a holding time of 3 min at 100% eluent B and 3 min column re-equilibration at eluent A pumped at a flow rate of 0.3 mL/min. DON is detected as [M+AcO]– at m/z=355, ZEN at m/z=317 as [M-H]–, FB_1 at m/z=722[M+H]+. The limit of detection (LOD) for FB, ZEN and DON was 3.0, 5.0 and 5.0 μg/kg, while the limit of quantification was (LOQ) 10, 1.0 and 2.0 μg/kg, respectively.

3.1.1.5 Clinical chemical parameters

The plasma total protein (TP), albumin (ALB), globulin (GLOB), total cholesterol (tCHOL), triglyceride (TG), glucose (GLU), fructosamine (FrA), creatinine (CREA) and bilirubin (BIL) concentrations, and the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), lactate dehydrogenase (LDH) and alkaline phosphatase (ALKP) were determined in a veterinary laboratory (Vet-Med Laboratory, Budapest,

Hungary), using Roche Hitachi 912 Chemistry Analyser (Hitachi, Tokyo, Japan) with commercial diagnostic kits (Diagnosticum LTD., Budapest, Hungary).

3.1.1.6 Antioxidant status

For the determination of lipid peroxidation, the samples of blood plasma, RBCH (1:9) and liver were stored at -70°C until analysis. Lipid peroxidation was determined by the quantification of malondialdehyde (MDA) levels with 2-thiobarbituric acid method in blood plasma and RBCH (Placer et al., 1966) and liver homogenate (Botsoglou et al., 1994), and determination of conjugated dienes (CD) and trienes (CT) according to the AOAC (1984) method in the liver. Among the components of the antioxidant system, some parameters of the glutathione redox system were determined in blood plasma, RBCH and liver. The amount of reduced GSH measured by the method of Sedlak and Lindsay (Sedlak and Lindsay, 1968) and the activity of glutathione peroxidase (GPx) according to Lawrence and Burk (1978). Analyses were performed in the laboratory of SZIU Faculty of Agricultural and Environmental Sciences (Gödöllő, Hungary).

3.1.1.7 GnRH test and determination of testosterone concentration

Experimental and control bucks were treated intramuscularly with 0.2 ml GnRH analogue (Receptal; Intervet, Boxmeer, The Netherlands) for the analysis of the toxin's effect on the Leydig cell function. The levels of testosterone hormone were determined from blood samples taken just prior to GnRH analogue injection (0 min) and thereafter in the subsequent 2 hours in every 25 minutes (a total of 6 blood samplings).

The testosterone concentration was determined with a direct 3H-radioimmunoassay method (Csernus, 1982) adapted and validated for rodents' (chinchilla rabbit and Angora rabbit) plasma as described previously (Kovács et al., 2011).

3.1.1.8 Spermatology

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, Beernem, Belgium) of the spermatozoa. Motility was evaluated with a computer assisted sperm analyser (MedealabTM CASA System, Erlangen, Germany). Moreover, vital test, hyposmotic and peroxidase tests were carried out. A minimum number of 200 spermatozoa were examined for morphology and 500 for motility evaluation (WHO, 1999).

Testosterone concentration and spermatological parameters were determined in the laboratory of University of Veterinary Medicine (Budapest, Hungary).

3.1.1.9 Comet assay

For comet assay sperm was sampled on day 60 of the experiment (n=15/group). The method was adapted from human spermium examination protocols (Gopalan et al., 2011; Simon and Carell, 2013), with the following modifications. The semen was washed three times in phosphate buffered saline (PBS) and resuspended in PBS to a final of 4×10^6 cell/ml number. Onto the microscope slides pre-coated with 1% normal melting point (NMP) agarose 10 µl cell suspensions and 75 µl 1% low melting point (LMP) agarose were loaded. The decondensation was performed in two steps, first the slides were soaked for 1 hour at 4°C in a lysis buffer with dithiothreitol, and second the slides were soaked for 1 hour at 37°C in a lysis buffer with proteinase K. After lysis, the slides were washed in sterile redistilled water to eliminate the salt adhered to the gel. Electrophoresis was performed at 300 mA and 25 V for 30 minutes at 4°C. After washing and drying the slides were stained with ethidium bromide. All chemicals used in this study were obtained from Sigma-Aldrich Ltd. (Budapest, Hungary). Specialized chemicals used were: Histopaque-1077 and RPMI-1640 medium (Sigma-Aldrich Ltd., Budapest, Hungary).

The fluorescence images were generated using an Alpha-Optika B-600TiFL fluorescence microscope (Optika Microscopes, Bergamo, Italy). Scoring was carried out according to Singh et al. (1988) and Collins et al. (1997), in which comets are classified into scores of ‘0’, ‘1’, ‘2’, ‘3’ and ‘4’ according to DNA damage and head/tail migration. Each single comet was scored visually and assigned to an

arbitrary unit from 0 to 4, depending on the relative intensity of DNA fluorescence in the tail; 800 cells/ group were counted.

3.1.1.10 Histopathological analysis

After registering the macroscopic pathological signs on the internal and external organs, testicles, liver, kidneys and spleen were stored in 10% neutrally buffered formalin and were embedded in paraffin. For light microscopic analysis microtome slides of 5 μm were prepared and stained with hematoxylin-eosin.

The histopathological analysis was performed according to the Act/ 2011 (03.30) of the Hungarian Ministry of Agriculture and Rural Development. The analysis was in accordance with the ethical guidelines of the OECD Good Laboratory Practice for Chemicals (1997) and was carried out by the Autopsy Ltd. (Budapest, Hungary).

3.1.1.11 Determination of type of interaction

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 was greater than expected from the sum of the individual effects of the two (or three) toxins. A synergistic effect was determined also if one (or the mixture) of the toxins didn't display any effect, but the effect of their combination was greater than the effect of the other toxin alone. Additive effect means that the effect of combination can be calculated as the sum of the individual effects of the two (or three) toxins.

3.1.1.12 Statistical analysis

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), correlate and descriptive statistics modules. For the determination of the interactions between groups and time (days 30 and 60), general

linear model (GLM) repeared mesaures was used. In the case of comet assay, crosstabs options were used for chi-square test.

3.1.2 Effects of DON and possible protective effect of *Carduus marianus* in growing rabbits

3.1.2.1 Experimental animals, housing and diets

Seventy-two (12/ group) weaned, 7-week-old Pannon White rabbits were housed in metal wire mesh cages (3 rabbits per cage). The animals were checked daily for mortality and morbidity; weight and feed intake were recorded weekly.

The animals received three different diets during the first experimental period (3 weeks). Control (C) diet was a non-supplemented basal diet formulated in such way to meet the nutritional needs of weaned rabbits, control diet supplemented with the herb (*C. marianus*) in a concentration of 5 g/kg (H1) and control diet supplemented with the herb in a concentration of 10 g/kg (H2; Table 6). During the second experimental period (3 weeks) the rabbits received six different diets after the subdivision of the three initial groups. The diets were formulated in a similar way of the first period but this time supplemented with DON mycotoxin. The groups formed 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 10 mg/kg (H1T) and control supplemented with the herb at 10 g/kg and DON at 10 mg/kg (H2T) (Table 6).

Table 6: Nutrient content of the experimental diets

	C	**H1**	**H2**	**CT**	**H1T**	**H2T**
Nutrient content			**g/100g of DM**			
Dry matter (DM)	91.2%	90.4%	91.0%	90.8%	90.9%	93.9%
Crude protein (CP)	18.5	18.8	18.1	18.6	18.3	18.4
Crude fat (EE)	2.8	2.9	2.8	2.9	2.8	2.7
Crude fibre	16.7	16.4	16.4	16.3	17.2	16.3
Ash (ASH)	8.2	8.6	8.3	8.2	8.3	8.3
Starch	18.8	18.6	19.8	18.5	18.0	18.6
Natural detergent fibre (NDF)	35.5	36.0	34.7	35.1	35.2	34.9
Acid detergent fibre (ADF)	22.3	22.4	21.6	22.1	22.0	21.9
Acid detergent lignin (ADL)	6.1	6.3	5.7	6.1	6.2	6.2
Acid insoluble ash (AIA)	1.2	1.4	1.1	1.0	1.6	1.0
Digestible energy (DE, MJ/kg DM)	11.0	11.0	11.2	11.1	11.1	11.1

The rabbits had free access to water (pacifiers) and feed (*ad libitum*). The temperature was 16-18^{o}C and the photoperiod was set to 16h of light and 8 hours of dark. The weight and feed intake were recorded on a weekly basis, whereas the morbidity and mortality were monitored daily.

3.1.2.2 Mycotoxin production and plant purchase

Toxin was produced as described in chapter 3.1.1.2 with a modification. The *F. graminearum* IFA 77 strain was used, which produces only DON at 28^{o}C in order to avoid co-occurrence of ZEN. The LC-MS analysis of the concentration of DON in the diets was performed as described in chapter 3.1.1.4.

The homogenized fungal cultures contained DON at a concentration of 7140 mg/kg. Fungal cultures were mixed into the feed of experimental animals, based on the presented dose in Table 7.

Table 7: Concentration of the toxin in the experimental feeds in the different groups

Groups	DON (mg/kg)
CT	10.1
H1T	11.5
H2T	10.6

CT= control+toxin; H1T= *Carduus marianus* (0.5%)+ toxin; H2T= *C. marianus* (1%)+ toxin

C. marianus was purchased from Parceval (Pty) Ltd Pharmaceuticals, South Africa in powder form (seeds). The nutrient content, mineral profile, vitamin content and gross energy and silibinin concentration of the plant can be found in Table 8.

Table 8: Nutrient content (g/kg feed), mineral profile (mg/kg), vitamin content (mg/kg), silibinin concentration (mg/kg) and gross energy (MJ/kg) of *Carduus marianus*

Nutrient content	*Carduus marianus*
Dry matter (DM)	939
Crude protein (CP)	159
Crude fat (EE)	276
Crude fibre	276
Ash (ASH)	43.4
Starch	4.70
Natural detergent fibre (NDF)	380
Acid detergent fibre (ADF)	292
Acid detergent lignin (ADL)	99.5
Acid insoluble ash (AIA)	0.70
Ca	6.57
P	4.91
K	4.21
Mg	2.76
Na	0.12
Fe	0.06
Zn	0.04
Vitamin E	117
Vitamin B1	0.86
Vitamin B2	4.0
Gross energy	23.6
Silibinin (H1)	76.7±2.5
Silibinin (H2)	164.6±8.4

3.1.2.3 Samplings

Blood sampling was performed on day 21 from the marginal ear vein into native and heparinized tubes. RBCH (9:1) was prepared by adding 900 µl sterile distilled water to 100 µl washed red blood cells bulk. On the same day, 6 rabbits from all groups were euthanized by cervical dislocation and exsanguinated. The digestive tract was immediately removed and caecum was separated. 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 measurements. Ceacal chyme samples were taken for pH, microbiota and short chain fatty acids (SCFA) analysis.

3.1.2.4 Haematology, clinical chemistry, antioxidant parameters

Haematological and serum clinical chemical parameters were determined in a professional veterinary laboratory (Vet-Med Laboratory, Budapest, Hungary), using a Cell-Dyn 3500 automated haematology analyser (Abbott, Chicago, USA) and a Roche Hitachi 912 Chemistry Analyser (Hitachi, Tokyo, Japan) with commercial diagnostic kits (Diagnosticum Ltd., Budapest, Hungary), respectively (see 3.1.1.5).

GPx activity, GSH and MDA concentration were measured in blood plasma and RBCH (1/9 v/v), as described previously (see 3.1.1.6).

3.1.2.5 Determination of immune parameters

Peripheral white blood cells were isolated by density gradient centrifugation (400 x g for 15 minutes) using Ficoll-Paque (Pharmacia) according to standard protocols. The number of viable blood leukocytes was determined by trypan blue exclusion in a haemocytometer. The cells were diluted in Dulbecco's modified Eagle's medium supplemented with penicillin and streptomycin antibiotics and 10% foetal bovine serum. Cells were plated at $1x10^5$ cells/well density into 6 well plates. The cultures were incubated for 4 days at 37^oC under 5% CO_2 tension. Phagocytic ability (PA) was determined with Congo-red stained yeast cells by incubating overnight at 37^oC. PA was expressed as a percentage of phagocytic cells quantified from 100 cells observed under a microscope.

Cytokines (IL-1, IL-2, IFN-γ) were detected by reverse transcriptase real-time polymerase chain reaction (rt-RT-PCR), detecting mRNA. Whole blood was used for total RNA isolation with the Analytik Jena innuPREP Blood RNA Kit (Biometra, Germany), following the manufacturer's instructions. RNA templates were transcribed into cDNA by the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Fermentas, Lithuania) according to the manufacturer's protocol. Amplification using cytokine-specific primers was performed in an Eppendorf

Realpex^2 Mastercycler. Primers for IL-1 and -2 were designed using the Primer3 Programme (Rozen and Skaletsky, 2000) and the available GenBank sequences. For IFN-γ, primers reported by Godornes et al. (2007) were used. The mixture was the following: 2 µl cDNA solution, 5 µl 10x DreamTaq Buffer, 2 µl of 25 pmol/µl of each primer, 1 µl of 1 mmol dNTP Set (Fermentas), 2.5 µl EVA Green (Biotium, USA) and 0.2 DremTaq^{TM} DNA Polymerase, 5 U/µl DremTaq^{TM} DNA Polymerase (5 U/µl, Fermentas) in a final volume of 20 µl. The PCR reactions were as follows: preheating at 95 °C for 5 min, 40 cycles of 54 °C for 30 s, and 72 °C for 45 s, followed by a final extension step of 72 °C for 7 min.

3.1.2.6 Histology

Samples from the liver, heart, kidney, spleen and the small intestine were placed into 10% neutral buffered formaldehyde solution. Five µm thick sections were embedded in paraffin and stained with haematoxylin-eosin (HE). To assess gut morphology, the general status of the mucosa, the epithelial layer, the structure of the stratum villosum, the quality and quantity of the cells and the cytomorphology of the gut-associated lymphoid tissue (GALT) were examined. The ocular micrometric method was used to measure villus height (VH) and crypt depth (CD) (five villi were measured in each sample, and the mean values were calculated). Analyses were performed by the Autopsy Ltd. (Budapest, Hungary).

3.1.2.7 Comet assay

On day 21 to assess DNA damage in the lymphocytes of rabbits comet assay was performed as described previously (Horvatovich et al., 2013), with the following modifications: after mixing 50 µl of whole blood with 1 ml of 1% LMP, 140 µl of this mixture was placed (in 2 drops) on a pre-coated (1% NMP) slide and then immediately covered with a cover slip to avoid dehydration. Tail intensity (% DNA in the tail) was determined with an Epifluorescent Microscope (B600 TiFL; optimum filter 4 and λ = 302 nm) and Comet IV (version 4.3.1.) software (Perceptive Instruments Ltd, Bury St Edmunds, UK), examining 100 comets per gel.

3.1.2.8 Determination of composition of caecal microbiota and SCFA concentrations

The caecal contents' pH values were determined using a pH meter (OP-110, Radelkis, Hungary).

From one gram of caecal digesta, serial dilutions (1 g caecal sample + 9 ml diluent (0.9% NaCl)) were made immediately after sampling and used for microbiological determination. The obligate anaerobe organisms were cultured on Schaedler's agar (Sharlan Chemie, Barcelona, Spain), the selectivity of which was increased by the addition of esculin (Merck, Darmstadt, Germany), neomycin (Merck, Darmstadt, Germany) and iron ammonium citrate (Sharlan Chemie, Barcelona, Spain). Gamma sterile Petri dishes (Biolab, Budapest) were placed into Anaerocult culture dishes (Merck, Darmstadt, Germany), in which the anaerobic conditions were ensured with the help of an "Anaerocult A" (Merck, Darmstadt, Germany) gas-producing bag. Subsequently, the samples were incubated in an LP 104 type thermostat (LMIM, Esztergom, Hungary) at 37°C for 96 hours. Total aerobic bacteria were cultured on media supplemented with 5% calf blood. The samples were incubated at 37°C for 72 hours. Coliform bacteria were cultured on a Cromocult differentiation medium (Merck, Darmstadt, Germany). The samples were incubated at 37°C, under aerobic conditions, for 24 hours. The amount of lactobacilli colonies was measured on MRS agar (Scharlan Chemie, Barcelona, Spain) after anaerobic incubation at 37°C for 24 hours.

After the incubation time had elapsed, the colonies were counted according to standard (ISO 4833:2003) with Acolyte colony counter (Aqua-Terra Lab, Veszprem). The colony counts were expressed in $\log_{10}$ colony-forming units (CFU) related to 1 g of sample.

Approximately 3 g of caecal digesta was homogenized with 4.5 mL metaphosphoric acid (4.16%), then centrifuged at 10.000 *g* for 10 minutes and filtrated. The concentration of SCFA was measured from the supernatant fluid by gas chromatography (Shimadzu GC 2010, Japan; equipped with Nukol 30 m x 0.25 mm x 0.25 μm capillary column - Supelco, Bellefonte, PA, USA; FID

detector, 1:50 split ratio, 1 µl injected volume, helium 0.84 mL/min. Detector conditions: air 400 mL/min, hydrogen 47 mL/min, temperature: injector 250ºC, detector 250ºC, column 150ºC). For quantification 2-etil-butyrate (FLUKA Chemie GmbH, Buchs, Switzerland) was used as internal standard.

3.1.2.9 Determination of silibinin concentration

Detection and quantification was performed using a single-quad LC–MS system (Model 2020, Shimadzu, Kyoto, Japan) comprised a vacuum degasser, a binary pump (20AD), a column oven (CTO 20A) autosampler (SIL 20ACHT), and mass analyser (MS 2020) with both atmospheric-pressure chemical ionization (APCI) and electrospray ionization (ESI) systems. Optimized mass spectra were obtained with an interface voltage of 4.5 kV, a detector voltage of 1.05 kV in negative mode, 1.25 kV in positive mode. Heat block temperature was 200°C and a desolvation gas temperature was 200°C. For nebulizing and drying gas, nitrogen was used (1.5 L/min and 15 L/min flow rate, respectively). Chromatographic separation was performed at 50°C and achieved on an RP-18 (2.1×100 mm, 2.6 µm, Kinetex™ Phenomenex USA) stationary phase applying gradient elution 0.3 mL/min eluent total flow rate 0.4 mL/min for silymarin flavonoids, with A: 0.1% AcOH and B: 0.1% AcOH in methanol as eluent. With optimum method performance characteristics analytes were quantified using external calibration (Polettini, 2006).

3.1.2.10 Statistical analysis

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

3.2 *In vitro* experiments

3.2.1 Chemicals

FB_1, DON and ZEN were purchased from Sigma Aldrich (Schnelldorf, Germany) and Cayman Chemical (Ann Arbor, Michigan, USA). The toxins were dissolved in pure ethanol (stock solutions). The CCK-8 solution was provided by Dojindo EU GmbH (Munich, Germany). All other chemicals used were supplied by Sigma Aldrich (Schnelldorf, Germany).

3.2.2 Experimental design

3.2.2.1 Single toxin experiments

Preliminary experiments were performed (n=3) to establish the IC_{50} (the concentration that induces a 50% decrease in cell viability) value for each mycotoxin. Five concentrations and three exposure periods (24, 48 and 72h) were used. The concentration ranges were chosen on the basis of data reported in the literature (Minervini et al., 2004; Cetin and Bullerman, 2005; Vlata et al., 2006; Maenetje et al., 2008; Mwanza et al., 2009) as well as the results of preliminary experiments (data not shown). The concentration ranges were 50–150 μg/ml, 0.07–0.84 μg/ml and 1–50 μg/ml for FB_1, DON and ZEN, respectively.

3.2.2.2 Combinations' studies

The mycotoxins were tested in binary and ternary mixtures (n=3) at low concentrations (below the IC_{50}) to assess any interactive effects. The binary and ternary mixtures of the toxins were investigated at 5 μg/ml (FB_1 and ZEN), and 0.07 μg/ml (DON) for cytotoxicity and genotoxicity. Higher concentrations (still below the IC_{50}) were used for the second series of genotoxicity experiments (25, 0.21 and 10 μg/ml for FB_1, DON and ZEN respectively). The combinations tested were: DON+FB_1 (DF), DON+ZEN (DZ), FB_1+ZEN (FZ), DON+FB_1+ ZEN (DFZ).

3.2.3 Lymphocytes' isolation and cell enumeration

Blood was taken from the *v. cava cranialis* of healthy adult pigs. Blood (3 ml) was mixed with an equal volume of culture medium (RPMI-1640) in a 12 ml centrifuge tube. Then the mixture was gently overlaid onto an equal quantity of Histopaque 1077 (6 ml) and centrifuged for 30 min at 3000 rpm. The interface layer (mononuclear cells) was carefully transferred with a sterile Pasteur pipette into a new centrifuge tube, suspended in 12 ml RPMI-1640 and centrifuged at 4000 rpm for 20 min. After removing the supernatant cells were re-suspended in fresh medium (12 ml) and centrifuged at 4000 rpm for 15 min. Then the cells were gently re-suspended in 10 ml of complete cell medium [CCM; RPMI supplemented with penicillin and streptomycin solution (1%) and foetal bovine serum (10%)].

The cell suspension (100 µl) was mixed with an equal volume of 0.4% trypan blue and allowed to stand at room temperature for 2 min. Using a sterile pipette, a small amount (10 µl) was then transferred to both chambers of a Neubauer haemocytometer, covered with a cover slip and counted under light microscope. The cell viability (%) was calculated as total number of viable cells × 100/total number viable plus non-viable cells. The cell number was adjusted to 2×10^6 cells/ml and $4\text{-}5\times10^6$ (minimum 95% cell viability) for cytotoxicity and genotoxicity assays respectively.

3.2.4 Assays

3.2.4.1 Cytotoxicity assay

The conversion of the tetrazolium salt, 3-[4,5, dimethylthiazol-2, -yl]-2,5 diphenyl-tetrazolium bromide (MTT) to MTT formazan in living cells is used for determination of cell viability and proliferation (Mossman, 1983). In our study, a water soluble tetrazolium salt was used [WST-8; cell counting kit-8 (CCK-8)]. The use of a water-soluble tetrazolium salt eliminates the additional step of crystal solubilisation. In addition, the detection sensitivity of CCK-8 is higher and its toxicity is lower than that of MTT.

After isolation, the cell culture was subdivided in a 96-well microtiter plate with each well containing 100 µl of medium ($2x10^5$ cells per well). After 24h

incubation, 20 µl of phytohaemagglutinin (PHA) and 100 µl of toxin or solvent were added to the wells. Control cells were treated with mycotoxin-free vehicle (0.9% NaCl sterile distilled water). Each treatment had 5 replicates and the incubation times were 24, 48 and 72h. After each incubation period, 22 µl of CCK-8 were added to the wells and after 4h incubation, the absorbance was read at 450 and 620 nm under a Microplate Reader (DIALAB GmBH, Wiener Neudorf, Austria).

3.2.4.2 Genotoxicity (comet) assay

The comet assay (single-cell gel electrophoresis) is a simple and sensitive method for measuring deoxyribonucleic acid (DNA) strand breaks in eukaryotic cells. Cells embedded in agarose on a microscope slide are lysed with detergent and high salt to form nucleoids containing supercoiled loops of DNA linked to the nuclear matrix. Electrophoresis at high pH results in structures resembling comets, observed by fluorescence microscopy; the intensity of the comet tail relative to the head reflects the number of DNA breaks. The likely basis for this is that loops containing a break, lose their supercoiling and become free to extend toward the anode.

After isolation, the cell culture was subdivided (100 µl) into microcentrifuge tubes which were incubated overnight in a humidified incubator at 37^{o}C and 5% CO_2. Then toxin or vehicle/carrier solutions were added to the tubes and were incubated for 24 h, 48 h and 72 h. After each incubation period, the procedure described by Horvatovich et al. (2013) was followed, with slight modifications. The slides were washed with phosphate-buffered saline (PBS) instead of neutralising buffer and for the staining of the slides ethidium bromide (EtBr) was used instead of DAPI.

3.2.5 Calculations and statistical analysis

Dose-response curves were fitted to the best linear or nonlinear (dose response) models and the concentration that induced 50% loss of viability (IC_{50}) was calculated using the software OriginPro version 9.0 (OriginLab, Northampton, MA, USA).

The values obtained in the combination experiments 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 a second or a third substance (Šegvić Klarić et al., 2007):

$$\text{Mean \% (expected for myc1 + myc2)} = \text{mean \% (myc1)} + \text{mean \% (myc2)} - 100\% \text{ control}$$

$$\text{Mean \% (expected for myc1 + myc2 + myc3)} = \text{mean \% (myc1)} + \text{mean \% (myc2)} + \text{mean \% (myc3)} - 100\% \text{ control}$$

Calculation of expected SD/SEM:

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

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

The results were interpreted according to Šegvić Klarić et al. (2007), 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.

Analysis and scoring for comet slides were performed with Epifluorescent Microscope (B600 TiFL) and Comet IV (version 4.3.1) software (Perceptive Instruments Ltd, Bury St Edmunds, UK). 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).

The statistical difference between observed and expected values was calculated with unpaired t-test and the significance level was $p<0.05$.

4. RESULTS and DISCUSSION

4.1 *In vivo* experiments

4.1.1 Effect of multi-toxic dietary exposure on rabbit bucks

The aim of this study was the determination of the effect of a 65-day oral *Fusarium* mycotoxin administration in combination, on the reproductive system of 24-week old breeding Pannon White rabbit bucks. The concentrations of the mycotoxins included in the feed were chosen taking into account the 2006/576/EC recommendation. The concentrations of FB_1, DON and ZEN in the feed (5, 1 and 0.25 mg/kg feed, respectively) corresponded to 169-193 μg FB_1/kg BW, 33.7-38.7 μg DON/kg BW, and 8.5-9.7 μg ZEN/kg BW exposure. As previously demonstrated in the case of T-2 toxin (Kovács et al., 2013), the results of this study show possible adverse effects on male reproduction after a prolonged low-dose mycotoxin exposure. The interactions observed were of varying nature depending on the specific parameter examined. All kinds of interactions (addition, synergism and antagonism) could be detected in our study. Only FB_1 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 hypothetic discussion is for the two single toxins.

For some parameters (clinical chemistry, antioxidant parameters and testosterone production) two samplings were performed. The expectation of the time scale changes was that a longer exposure would result in higher alterations (Čonková et al., 2001). However, in some cases adaptation can also occur, so a temporary change is also interesting. In the study of Gumprecht et al. (1995) an increase of ALKP was observed on day 3 while the levels decreased on day 5. On the contrary, ALT levels decreased on day 3 and increased on day 5 (Gumprecht et al., 1995). In the study of Kovács et al. (2013), a temporary decrease in the concentration of albumin, urea and GPx was observed on day 30 compared to day 63 (Kovács et al., 2013).

4.1.1.1 Feed consumption and body weight

No significant differences were observed in the feed intake of the rabbit bucks among the groups. In the study of Hewitt et al. (2012) DON did not induce any feed refusal at 5 mg/kg of feed. This is in agreement with our results since at a 5-fold lower concentration no anorexia was observed. Gbore and Akele (2010) reported a decrease in dry matter intake in female rabbits that were exposed to dietary fumonisin at 5 and 10 mg/kg of feed. However, the decrease in the 5 mg/kg group was just 6.32% whereas in the 10 mg/kg FB_1 treated group the decrease was 50.13%. There were no significant differences in body weight among groups at any of the 12 timepoints (Table 9).

Table 9 : Body weight of rabbits on 12 timepoints

	Group			
BW	**C**	**F**	**DZ**	**FDZ**
Jan 22	3982±238	4078±310	4025±403	4093±335
Jan 27	4054±258	4186±318	4111±449	4178±345
Feb 3	4101±279	4241±340	4117±481	4218±355
Feb 10	4163±295	4275±347	4136±520	4274±367
Feb 17	4195±370	4323±361	4200±520	4300±391
Feb 24	4255±367	4385±356	4219±554	4357±409
Mar 3	4230±293	4419±341	4231±536	4330±373
Mar 10	4325±314	4419±361	4220±518	4329±396
Mar 17	4244±293	4442±365	4252±470	4368±366
Mar 24	4247±311	4424±360	4256±507	4370±404
Mar 31	4252±346	4442±360	4259±497	4382±426

The average body weight in the groups was between 4252 and 4442 g by the end of the experiment. No specific sign of mycotoxicosis was detected.

4.1.1.2 Organ masses

The weight (g) of liver, kidneys, spleen and testicles was measured. A significant difference was observed only in the case of the spleen (Table 10).

Table 10: Organ/gonad weights

	Weight of organs/gonad	C	F	DZ	FDZ
mean	**testis**	10.73	11.45	11.48	10.62
±SD		± 2.27	±1.24	±1.65	±2.24
mean	**kidney**	19.31	18.78	19.35	18.40
±SD		±1.85	±2.97	±2.57	±2.20
mean	**liver**	87.45	94.65	90.29	89.33
±SD		±16.21	±16.78	±18.27	±17.68
mean	**spleen**	1.46^{a}	1.65^{ab}	1.84^{b}	1.65^{ab}
±SD		±0.38	±0.30	±0.49	±0.37

4.1.1.3 Clinico-chemical parameters

Clinical chemistry is an important tool in the process of monitoring the health status of animals during an experiment. Any potential damage to the liver or kidneys (and other organs or tissues) can be determined through the investigation of specific enzymes.

The values of ALB, TC, GLU, FrA, CREA and BIL did not differ ($P \geq 0.05$) significantly among the groups. Similarly, the activities of AST, ALT, GGT, LDH and ALKP showed no significant difference ($P \geq 0.05$). Čonková et al (2001) studied the effects of purified ZEN at low (10 µg/kg BW) and high (100 µg/kg BW) oral doses in rabbits. The low concentration of ZEN induced a significant increase in the activity of ALP, while the high concentration induced significant increases in the activities of AST, ALT, AP, GGT, and LDH activity, which indicates possible liver toxicity due to the chronic effects of the toxin. A significant increase in ALT, AST and ALKP activities and the level of CREA was demonstrated in female rabbits that consumed FB_1 at 5 or 10 mg/kg of feed (Gbore and Akele, 2010). The activity of ALKP and concentration of CREA were significantly higher only when the highest concentration (10 mg/kg of feed) of the mycotoxin was administered.

The significant differences that were observed in the case of TP, GLOB and tCHOL at the two sampling dates (day 30 and 60) are summarized in Table 11. There was no interaction regarding clinical chemical parameters with the exception

of TP and GLOB. In the same table effect of time within the same groups can be found (Table 11).

Table 11: Clinico-chemical parameters of rabbits on day 30 and 60

		Day 30		Day 60	
		mean	**SD.**	**mean**	**SD.**
AST_GOT	**C**	21.0^{A}	9.1	55.7^{B}	21.3
	F	25.0^{A}	11.4	42.0^{B}	15.0
	DZ	18.0^{A}	8.1	39.2^{B}	14.1
	FDZ	23.2^{A}	8.6	46.8^{B}	18.0
ALT_GPT	**C**	28.3	8.9	41.5^{b}	14.7
	F	29.2	5.9	32.8^{ab}	10.1
	DZ	29.5	10.9	29.6^{a}	9.4
	FDZ	26.7	4.8	30.8^{ab}	11.1
ALKP	**C**	119.0	43.5	116.9	25.7
	F	160.7^{A}	25.4	111.8^{B}	27.2
	DZ	118.0	23.3	120.8	33.4
	FDZ	124.3	28.3	119.9	15.6
GGT	**C**	2.0^{A}	1.8	6.5^{B}	4.4
	F	2.5^{A}	1.7	5.4^{B}	2.1
	DZ	2.4^{A}	2.4	5.5^{B}	2.3
	FDZ	3.3	3.3	3.9	2.5
TP	**C**	69.9^{b}	5.9	62.2^{a}	3.0
	F	68.6^{b}	1.7	64.7^{ab}	4.0
	DZ	61.3^{a}	4.3	65.8^{ab}	7.3
	FDZ	69.6^{b}	1.9	67.7^{b}	3.4
ALB	**C**	41.2^{A}	2.7	39.8^{B}	3.7
	F	41.4	1.1	41.8	3.2
	DZ	41.1	2.1	41.7	2.3
	FDZ	41.6	1.7	41.1	3.0
GLOB	**C**	$28.7^{b,A}$	3.5	$22.4^{a,B}$	2.2
	F	$27.1^{b,A}$	1.6	$22.9^{a,B}$	2.1
	DZ	20.2^{a}	3.4	24.1^{ab}	6.0
	FDZ	27.98^{b}	1.6	26.7^{b}	3.1
ALB/GLOB	**C**	$1.5^{a,A}$	0.1	1.8^{B}	0.3
	F	$1.5^{a,A}$	0.1	1.8^{B}	0.2
	DZ	$2.1^{b,A}$	0.3	1.8^{B}	0.4
	FDZ	1.5^{a}	0.1	1.7	0.2
tCHOL	**C**	1.5^{ab}	0.1	1.5^{c}	0.4
	F	$1.6^{b,A}$	0.2	$1.2^{a,B}$	0.1
	DZ	1.3^{a}	0.2	1.3^{ab}	0.1
	FDZ	1.6^{ab}	0.2	1.4^{bc}	0.1

^{a,b} numbers with different superscripts indicate significant differences ($P \leq 0.05$) between groups (C= control, F= FB_1, DZ= DON+ZEN, FDZ= FB_1+DON+ZEN).
^{A,B} numbers with different superscripts indicate significant differences ($P \leq 0.05$) within groups (rows) between different sampling dates
[1]total protein; [2]globulin; [3]total cholesterol

However, these differences were only slight and within the normal range, which were reported to be 54-75 g/L for TP, 15-27 g/L for GLOB and 0.3-3.00 mmol/L for tCHOL (Rotter et al., 1996b). Although TP concentration did not exceed the normal range, GLOB concentration exceeded the upper limit in two groups (C and FDZ), but only on day 30 (29 and 28 g/L, respectively). The amount of TP is a sum of the globulin and the albumin concentrations. No changes were observed in the concentration of albumin thus the alteration found in the TP was as result of the concentration changes of globulin. Kim et al. (2008) observed a decrease in immunoglobulins in mice fed DON but no effect was observed when ZEN was administered. This could provide a possible explanation for the slight temporary decrease in GLOB and TP of DZ animals at day 30. On the other hand, on day 60, the results are difficult to explain, since the difference among groups was rather due to the lowered GLOB level in control animals than the toxin treatments. Despite that, fumonisin treatment decreased GLOB concentration at day 60, compared to day 30. Tessari et al. (2006) demonstrated that FB_1 (in combination with AFB_1) has a primary additive effect on immune response in broiler chickens, in accordance with our results of the FDZ group on day 60. Interestingly, FB_1 in single exposure at a higher concentration (i.e. 8 mg/kg of feed) exerted a sex-related effect on the immune response in swine (Marin et al., 2006b). It is not clear whether the reason for the augmented immune response in the FDZ group was a result of FB_1 solely or the interaction of the mycotoxins. Some irregularity in the function of the immune system was depicted in the histological results (see later) as well. In both DZ and FDZ animals, slight lymphocyte depletion (slight thinning of the T and B dependent zones of the Malpighian tubules) was observed in the spleen, without any change in the cytomorphology of lymphoblasts and lymphocytes. Similar findings were observed in the study of Sprando et al. (2005) in rats orally exposed to DON (5 mg/kg of feed).

The pathophysiological significance of the changes in tCHOL level, which was around 5-20%, is questionable, taking the broad physiological ranges (0.3-3.00 mmol/L) into consideration.

4.1.1.4 Antioxidant status

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 (Table 12).

Table 12: **Antioxidant parameters measured on day 30 in plasma**

Parameter	Groups			
	C	**F**	**DZ**	**FDZ**
GSH[3]	2.2 ± 0.1	2.6 ± 0.5	2.3 ± 0.3	2.2 ± 0.1
GPx[1]	3.0±0.2	2.8±0.6	3.1±0.4	2.8±0.2
MDA[2]	20.1 ±2.8	21.1 ± 4.0	25.6 ± 9.2	19.6 ± 3.8

[1]Units/g protein, [2]nmol/ml, [3]µmol/g protein

The significant differences of the different treatments on the antioxidant status on day 60 are summarised in Table 13.

Table 13: Antioxidant parameters measured on day 60

Parameter	Groups			
	C	F	DZ	FDZ
GSH-RBCH	2.5±0.6	2.4±0.7	2.5±0.5	2.7±0.7
GPx-RBCH[1]	2.7 ± 1.0[a]	3.5 ± 1.2[a]	4.9 ± 1.1[b]	3.1 ± 0.8[a]
MDA-RBCH[2]	30.3 ± 3.0[ab]	28.3 ± 3.2[a]	33.9 ± 7.5[b]	28.8 ± 3.2[a]
GSH-plasma[3]	2.2 ± 0.2[a]	2.2 ± 0.5[a]	2.0 ± 0.2[a]	2.6 ± 0.5[b]
GPx-plasma	2.8±0.4	2.7±0.4	2.7±0.2	3.0±0.4
MDA-plasma[2]	18.9 ±2.8[a]	18.7 ± 3.0[a]	24.5 ± 8.0[b]	16.8 ± 2.9[a]
CD liver[4]	0.51 ± 0.03[ab]	0.51 ± 0.02[ab]	0.53 ± 0.05[b]	0.48 ± 0.05[a]
CT liver[4]	0.19 ± 0.01[a]	0.20 ± 0.01[ab]	0.21 ± 0.04[b]	0.18 ± 0.01[a]

[1]Units/g protein, [2]nmol/ml, [3]µmol/g protein, [4]Abs 232nm
GPx= glutathione peroxidase; RBCH= red blood cell hemolysate; MDA=malondialdehyde; GSH= glutathione; CD= conjugated dienes; CT= conjugated trienes; C= control; F= FB_1; DZ= DON+ZEN; FDZ= FB_1+DON+ZEN

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. DZ exposure induced peroxidation of dienoic and trienoic fatty acids which increased the concentration of conjugated dienes and trienes, as compared to the combined effect of three mycotoxins (FDZ). No interaction between group and time was observed for any of the analysed parameters on both days (GSH, GPx and MDA).

An increased lipid hydroperoxide production could be guessed due to the increased GPx activity in the red blood cells of DZ animals by day 60 because the role of the enzyme is to reduce lipid hydroperoxides to their corresponding alcohols

and in this way to reduce free hydrogen peroxide to water. This is depicted by the higher MDA concentrations in both the red blood cells and the blood plasma, referring to an increased in vivo lipid peroxidation (Mead et al., 1986), and also by the decreased GSH, as co-substrate of GPx, level in plasma. One of the main mechanisms of DON toxicity is the induction of oxidative stress within the cells (Mishra et al., 2014). ZEN is also known to induce the generation of reactive oxygen species (ROS) and thus lipid peroxidation, whereas the oxidative capacity of fumonisin is rather moderate (El Golli-Bennour and Bacha, 2011). Minervini et al. (2014) demonstrated that FB_1 at a level of the EC regulation did not induce oxidative stress in intestinal cells. This is in general agreement with our results. FB_1 has been proven to induce ROS production in broiler chicks, but only at a very high (100 mg/kg feed) dose (Poersch et al., 2014). According to our findings, FB_1 at a low dose may act antagonistically towards Z and D, which is reported for the first time, though there is no literature on the mitigating property of FB_1 on lipid peroxidation. In conclusion, DZ combination was the most harmful in terms of initial (CD and CT) and terminal (MDA) phases of lipid peroxidation after prolonged exposure (60 days). At the same time, FB_1 at the EC Regulation level did not cause significant oxidative stress and it additionally relieved the effects triggered by ZEN and DON.

4.1.1.5 Gn-RH induced testosterone production

No effect of the mycotoxin exposure was observed on day 30 regarding Gn-RH induced testosterone production (Table 14).

Table 14: Gonadotropin-releasing hormone (mean and standard deviation) induced testosterone production at day 30

	Group			
Day 30	**C**	**F**	**DZ**	**FDZ**
Time 0	6.2±3.8	5.9±4.0	8.6±4.8	8.9±5.8
Time 15	16.9±3.5	14.8±1.8	16.3±3.7	13.3±5.5
Time 50	17.8±2.6	20.3±1.2	18.9±4.2	18.0±5.0
Time 75	17.8±3.6	19.0±12.2	22.9±12.2	15.3±3.9
Time 90	17.3±3.4	19.6±2.1	20.6±5.0	15.1±3.1
Time 115	19.2±4.0	17.6±3.6	18.0±2.1	15.7±3.4

Mycotoxin exposure affected the concentration of testosterone in a significant manner only at day 60 at the sampling minutes of 75, 90 and 115 (Figure 3).

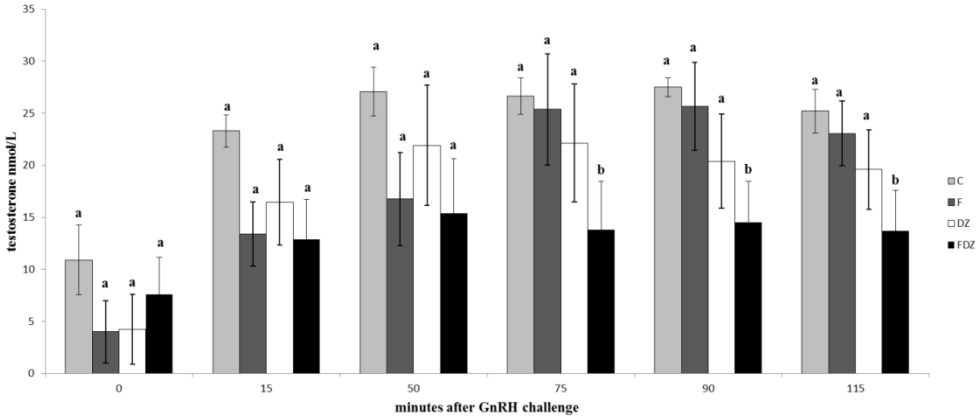


Figure 3: Gonadotropin-releasing hormone (GnRH; mean and standard deviation) induced testosterone production at day 60.
[a,b] numbers with different superscripts indicate significant differences (P ≤0.05) between groups (C=control, F=FB_1, DZ=DON+ZEN and FDZ=FB_1+DON+ZEN)

In figure 3 it is clearly demonstrated that Leydig cells of the animals intoxicated with the mixture of the three mycotoxins (FDZ) for a period of 60 days, synthesized significantly less testosterone as a response to exogenous GnRH. Compared to control, FB_1 induced a slight, not significant decrease in the blood testosterone level, while DON and ZEN in combination (DZ) induced a more intense decrease in the concentration, albeit the differences were not statistically significant.

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.

Sprando et al. (2005) investigated the effects of DON on several reproductive traits of male rats. The animals were exposed to DON via gastric intubation at concentrations of 0.5, 1, 2.5 and 5 mg/kg BW, which resulted in a dose-dependent downregulation of testosterone synthesis after 28 days of exposure. Trichothecene toxins may decrease the secretion of testosterone by the Leydig cells by inhibiting early steps of the steroidogenic pathway, i.e. inhibition of pregnenolone and/or the conversion of pregnenolone to progesterone (Fenske and Fink-Gremmels, 1990), as it was previously observed in one of the studies with T-2 toxin (Kovács et al., 2011). These *Fusarium* toxins may act indirectly as well, on the

pituitary-testicular axis, or affect Sertoli cell inhibin production, as shown in the case of DON (Sprando et al., 2005). No direct effect of the mycotoxin on Leydig cells could be supported by the histopathological findings. The most potent factor is ZEN, as confirmed by Liu et al. (2014) who demonstrated that ZEN inhibits testosterone biosynthesis in Leydig cells in a dose or time-dependent manner via changing the nuclear/oestrogen receptor signaling (Liu et al., 2014), although the exact molecular mechanism is not elucidated yet. Regarding FB_1 direct toxicological studies are lacking, but according to Lu et al. (2003), elevated ceramide levels are directly apoptotic towards Leydig cells, which is inhibited by FB_1, a ceramide-synthase blocking substance. However, no deleterious effects of FB_1 were observed in rat testicular Leydig cell function *in vitro*. In summary, the main inhibitor of testosterone synthesis in our study was primarily attributed to ZEN. Some kind of synergism occurred between FB_1 and DON+ZEN. This could be attributed to the common cellular modes of actions of these toxins, with respect to the DNA synthesis inhibiting effect. FB_1 has previously been shown to decrease the DNA synthesis along with ZEN in a rather synergistic way using a DNA inhibition assay, although this effect was not fully confirmed when it was further analysed in a second stage (Tajima et al., 2002).

4.1.1.6 Comet assay

The integrity of DNA is essential for both the foetal and post-natal development. Thus the DNA damage induced by the toxin treatments in our study was assessed. DNA damage was induced by all the mycotoxin treatments. More than 90% of the treated cells (98.6, 91.6 and 91.8% in F, DZ and FDZ, respectively) could be categorized as having 1 to 3 scores (Figure 4).

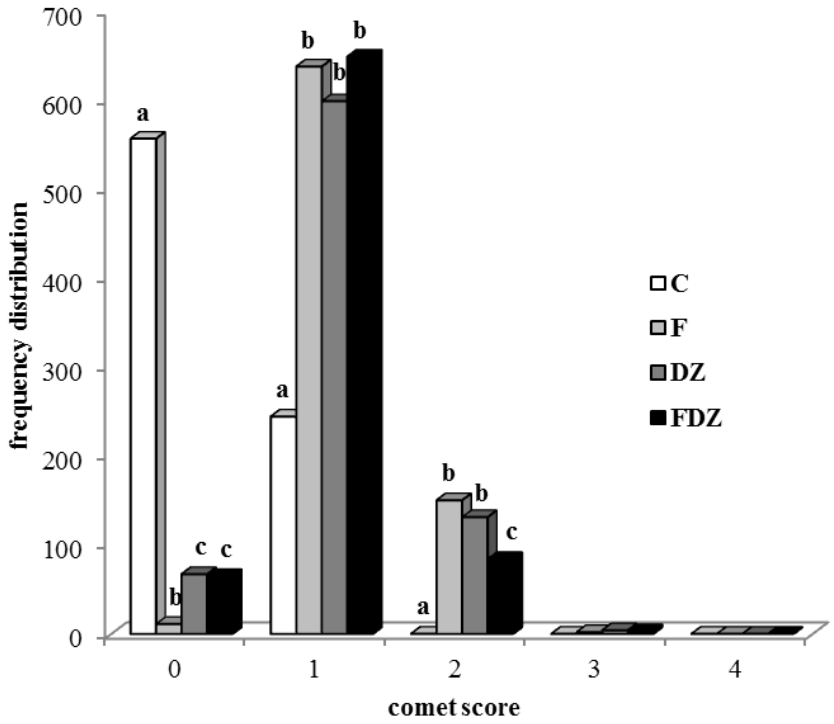
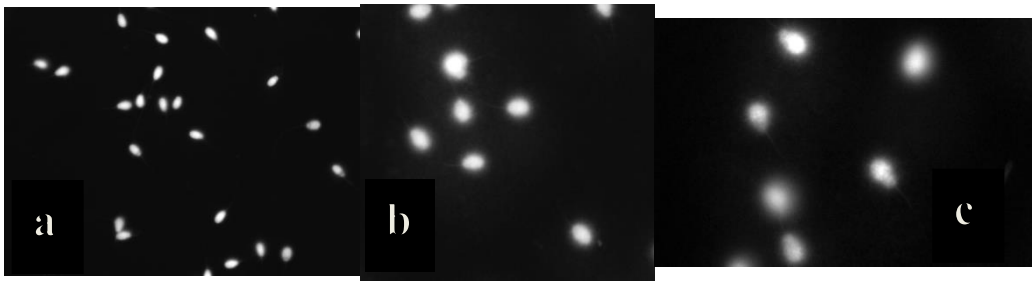


Figure 4: Number of cells with the respective comet values (values of comet scores: 0 to 4).
[a,b] columns with different superscripts indicate significant differences (P<0.0001)
C=control, F=FB_1, DZ= DON+ZEN, FDZ=FB_1+DON+ZEN

The highest prevalence of cells with no damage, i.e. 0 score, was observed in C group. The prevailing score in all treated groups was 1, which corresponds to the slightest damage. Furthermore, comet score 1 occurred in all three treatments in similar proportions (Picture 1a). Cells derived from the FB_1 treated animals had significantly less 0 comets (intact cells) compared to the other treatments. On the other hand, comet value 2 (Picture 1b) showed the highest prevalence in samples of F and DZ animals whereas FDZ treatment resulted in significantly fewer cells of this type suggesting an antagonistic effect of FB_1 against DZ. Similarly, in our *in vitro* study (Kachlek et al., 2017), the interactive effect of the ternary mixture of FB_1, DON and ZEN was antagonistic. This should be further elucidated in future studies. The number of cells with score 3 (Picture 1c) occurred only in 0.6% of the categorized cells. In consideration of the lack of cells with a comet score of 4 in the

experiment, it can be concluded that none of the toxins exerted a strong genotoxic effect.



Picture 1: Comet assay (DNA damage) scoring: images of comets from bucks' spermatozoa stained with EtBr. Classes 0 (a), 1 (b) and 2 (c) were used for visual scoring.

To the best of our knowledge, the genotoxic effects of DON on rabbit (or any animal) spermatozoa have not been assessed by the means of comet assay previously. There is only one study on human spermatozoa in which 12 dietary/environmental compounds - among which DON - were tested (Baumgartner et al., 2012). It was observed that spermatozoa were more sensitive towards DON (regarding DNA damage) in comparison with lymphocytes. A possible explanation for this could be that spermatocytes belong to germ cells whereas lymphocytes to somatic ones. The mammalian sperm cells have a totally different cell composition and structure. In order to protect the genetic integrity through the passage from the genital tract of the male and female, the sperm chromatin is very condensed (Andrabi, 2007). This specialisation of sperm cells could be the reason for their higher sensitivity.

The genotoxic effects of *Fusarium* mycotoxins on sperm cells have been reported previously regarding ZEN (Benzoni et al., 2008; Minervini et al., 2010), using sperm chromatin structure assay (SCSA), while no data on genotoxicity of FB_1 related to sperm cells are available. DON has been demonstrated to decrease cell viability, cause damage to the membrane, the chromosomes and the DNA in human lymphocytes, and potentially induced genotoxicity by the depletion of the antioxidant capacity (Yang et al., 2014). On the contrary according to El Golli-Bennour and Bacha (2011), oxidative stress does not contribute significantly to DON toxicity, and its genotoxicity is the result of a direct effect on DNA

fragmentation and caspase dependent apoptosis. In the case of ZEN, Minervini et al. (2010) found a high degree of individual variability, when chromatin structure stability was checked *in vitro*, using equine spermatozoa. Interestingly no increase in ROS production was observed even in those cases when genotoxicity was detected, so the damage was attributed to mitochondrial dysfunction related to the altered ceramide metabolism. This mode of action might influence motility as well, knowing that spermatozoa are used as biosensors when testing mitochondrial toxicity, because of the strong influence of mitochondrial function on motility (Minervini et al., 2010).

4.1.1.7 Spermatology and histopathology

No significant differences in semen pH (ranging from 6.4 to 8.2 in each group), the quantity of the semen (on average 1 ml in each group) or the concentration of spermatozoa (ranging from 2.4 to 2.6 × 10^7/ml) were observed. Sperm motility did not differ significantly among the groups as well. A ratio of 80% of the spermatozoa showed progressive forward motility at the beginning of the experiment, while it decreased from 80 ± 1.7% to 67 ± 4% in the semen of FDZ animals showing a significant difference when compared to the other three treatment groups.

Just a few studies on the individual effects of FB_1, DON and ZEN on sperm motility are available. Dietary exposure of male rabbits to FB_1 at concentrations of 7.5 and 10.0 mg/kg induced 18% decrease in sperm motility. The authors suggested the inhibition of cyclic 3'5'AMP activity and calcium ion transport as a possible explanation for this decrease. Additionally, the decreased formation of the acidic epididymal glycoprotein synthesis which is required to maintain motility could contribute as well (Ewuola and Egbunike, 2010). FB_1 had a dose-dependent depressive effect on the motility of sperm cells of cocks fed FB_1 (0.2-15.2 mg/kg feed) (Ogulande et al., 2015). An increase in the velocity of sperm cells after oral exposure to DON (1-5 mg/kg of feed) was not consequent, and was considered to be random (Sprando et al., 2005), while no significant effect of DON on motility was found in rabbit spermatozoa *in vitro* (Medvedová et al., 2012). Boeira et al. (2015)

reported that ZEN decreased spermatozoa motility in mice, while a dietary exposure of ZEN at 12 mg/kg of feed for 8 weeks did not alter sperm motility in adult rams (Milano et al., 1991). The lack of effect of ZEN on sperm motility is supported by an *in vitro* study on boar spermatozoa (Benzoni et al., 2008). However, Rajkovic et al. (2007) and Tsakmakidis et al. (2006), described impairment of progressive motility in boar spermatozoa. Intriguingly, ZEN was reported to induce hyper-activation of the motility of stallion sperm (Filannino et al., 2011). Since the cellular mode of action of these *Fusarium* mycotoxins that affect motility have not been fully elucidated, it is rather difficult to explain their combined effect. The decrease of sperm motility observed on day 60, as a result of the three toxins treatment (FDZ), can be associated with the decreased testosterone concentration due to the synergistic effect between the three mycotoxins observed by day 60 as well.

The ratio of spermatozoa with normal morphology was significantly different between the groups C and DZ (Table 15).

Table 15: The ratio of spermatozoa with normal morphology in the semen after 65 days of toxin treatment (%, mean ± S.D.)

Group	Spermatozoa with normal morphology
C	80.2 ± 11.2^{b}
F	76.0 ± 9.0^{ab}
DZ	66.3 ± 23.7^{a}
FDZ	68.9 ± 14.1^{ab}

$^{a, b}$: numbers with different superscripts indicate significant differences ($P \leq 0.05$)
C= control; F= FB_1; DZ= DON+ZEN; FDZ= FB_1+DON+ZEN

The most frequent morphological abnormalities were: abnormality of the tail, retention of the cytoplasmic drop, the absence of the acrosome and altered head. A cell was considered altered if at least one defect was present.

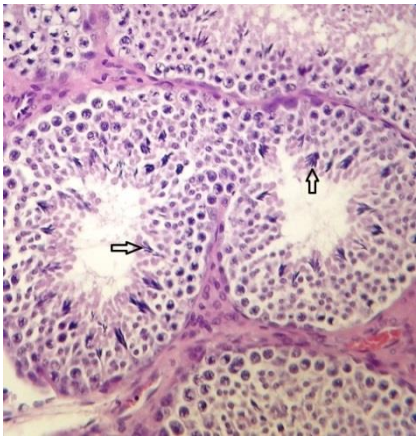
According to the histology of the testes spermatogenesis decreased by 43, 31 and 64% in animals fed with the diets F, DZ and FDZ, respectively (Table 16). The decreased spermatogenesis was reflected by the lack of differentiated spermatozoa, thinning of the germinal epithelium, the appearance of multinuclear giant cells indicative of the disturbance of meiosis and mitosis of the germinal epithelial cells and in some cases the lack of spermatogonia.

Table 16: Decreased intensity of spermatogenesis as revealed by the histopathology of the testicles

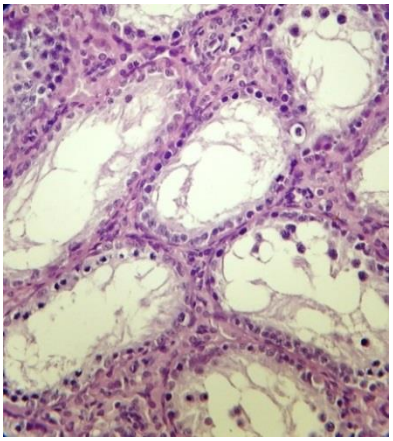
Group	No	Number of animals showing alteration				
	(n=15/group)	grade 1	grade 2	grade 3	All together	%
C	15	0	0	0	0	0
F	8	6	0	0	6	43
DZ	9	4	0	0	4	31
FDZ	5	7	1	1	9	64

No= number of animals without alteration, grade 1= slight / small area / low occurrence frequency, grade 2= intermediate severity / intermediate area / intermediate occurrence frequency, grade 3= marked / extensive / high occurrence frequency
C= control; F= FB_1; DZ= DON+ZEN; FDZ= FB_1+DON+ZEN

These histological findings were observed in different degrees of severity in the seminiferous tubules. In the case of slight alteration (grade 1) disturbance of sperm cell formation was observed in 20-30% of the seminiferous tubules, while in the case of grade 2 and 3, 30-70 or 100 % of the tubules, respectively, showed decreased spermatogenesis. Due to the lack of well differentiated sperm cells, the lower number of primary and secondary spermatocytes and spermatids and the thinning of the seminiferous epithelium, spermatogenesis was decreased (grade 1). In the case of intermediate severity (grade 2), these alterations were observed in a higher ratio of the tubules, the disturbance of meiosis and mitosis was indicated by the appearance of multinuclear cells. When all tubules were altered and even spermatogonia (initial spermiogenetic cells) were absent, the classification was marked damage (grade 3) (Pictures 2 and 3).



Picture 2: Active spermatogenesis in a control animal's seminiferous tubules. The cells at different differentiation levels in the epithelium germinativum (spermatogonia, stage I/II spermatocytes, spermatids and mature spermia (⇑) are as well visible. (Haematoxylin-eosin staining, ×400)



Picture 3: A part of the seminiferous tubule after FDZ treatment. The marked lack of germinal epithelial cells is visible. (Haematoxylin-eosin staining, ×400)

The toxin treatments exerted no significant effect on the morphology and proportion of the Leydig interstitial cells in the testes.

In the livers and kidneys, no detectable alteration attributable to mycotoxins was found. The liver and the kidney are known to be the primary target organs of many mycotoxins (including trichothecenes and FB_1) in all species examined. Čonková et al. (2001), have also suggested that prolonged exposure to dietary ZEN causes liver damage. Nevertheless, in our study, no gross hepatic lesions and disorder were 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 absence histopathological alterations among all groups. Thus we can report that these organs were likewise tolerant towards the exposure of low dose of these mycotoxins, without providing degenerative processes. Our observations are in agreement with Sprando et al. (2005) who reported a similar conclusion, namely few lesions of various tissues in control and treated rats, either incidental or related to experimental techniques (gavage) and were not attributed to the test substance.

The male reproductive organs are strongly androgen-dependent in respect of structure, as well as function. According to de Kretser and Kerr (1994), testosterone

supports spermatogenesis, sperm maturation, seminal plasma production and sexual functions. Thus, decreased testosterone secretion may give an explanation also for the more morphologically abnormal cells, and the decreased intensity of spermatogenesis shown by histology. However, the direct effect of the particular toxins is also presumable. Interestingly, FB_1 affected cell morphology and spermatogenesis only to a small degree. FB_1's main toxic effect - the inhibition of the sphingolipid synthesis - is causing cellular ceramide depletion and elevation of sphinganine, which in turn is cytotoxic, and has growth inhibitory and preapoptotic effects (Müller et al., 2012). These events induce disturbances to cell growth, differentiation and morphology. Dietary FB_1 fed to rabbits led to similar morphological and structural abnormalities in sperm cells and spermatogenesis overall. In the same study, a LOAEL (lowest observed adverse effect level) of 7.5 mg/kg of feed was suggested for FB_1 (Ewuola and Egbunike, 2010). Even at a lower dose (5 mg/kg of feed), that was used in our study, FB_1 exerted adverse effects. Regarding DZ, a similar compromise to F group was observed. For both F and DZ, disturbance of sperm cell formation was observed only in 20-30% of the seminiferous tubules, although the DZ induced abnormal morphology to more cells. An additive of F and DZ was observed since, in one of FDZ animals 30-70% of the seminiferous tubules were compromised, while in another 100% of the seminiferous tubules were concerned, indicating additive effect. ZEN's effect on spermatogenesis has been more thoroughly studied in comparison to FB_1 and DON. Zatecka et al. (2014) observed a ZEN-induced decrease of sperm concentration and an increase of abnormal and apoptotic spermatozoa. In the study of Kim et al. (2003), it was shown that the main effect by which ZEN causes atrophy of the testes and germ cell depletion is apoptosis. Impaired spermatogenesis as a result of germ cell apoptosis was described also by Cho et al. (2011). In the *in vitro* study of Benzoni et al. (2008), ZEN exerted cytotoxic effects on boar sperm cells as proved by the significantly decreased viability. Sprando et al. (2005) showed that DON at concentrations of 2.5 and 5 mg/kg of feed induced germ cell degeneration, decreased sperm cell release and abnormal cell development. According to the authors, DON may inhibit protein, RNA and DNA synthesis, and be cytotoxic to certain cells,

which could cause the harmful effect in the most sensitive preleptotene spermatocytes.

4.1.2 Effects of DON and possible alleviation from *Carduus marianus* on growing rabbits

4.1.2.1 Production parameters

Feed intake, body weight and body weight gain showed no significant treatment effects (Table 17). The toxin exposed rabbits did not show clinical signs, feed refusal effect was not observed, and no animals died as a result of toxin treatment.

Table 17: Production parameters of rabbits exposed to deoxynivalenol (10 mg/kg of feed) and the medicinal herb *Carduus marianus* (5 and 10 g/kg of feed)

Period (days)	Group					
	C	**CT**	**H1**	**H1T**	**H2**	**H2T**
	feed consumption (g)					
0-7	170.2±20.5	157.3±26.7	166.3±19.0	168.4±13.8	172.9±17.4	168.7±14.7
7-14	183.4±18.3	187.8±13.5	183.0±21.9	182.01±11.7	197.5±4.6	180.6±12.8
14-21	188.8±27.0	191.78±27.0	193.5±16.5	193.4±16.5	203.0±7.1	192.7±12.7
	Body weight (g)					
0-7	2385.5±364.4	2277.4±540.5	2317.5±370.3	2317.8±195.7	2287.0±399.8	2246.8±459.8
7-14	2745.5±370.1	2660.9±501.8	2720.0±337.2	2703.4±157.9	2689.4±376.9	2652.2±403.9
14-21	3116.0±382.2	3200.8±333.9	3089.6±346.1	3094.7±146.7	3067.3±383.1	3030.1±467.1
	Body weight gain (g)					
0-7	59.3±13.7	50.7±1937	60.2±18.9	64.6±21.2	59.6±17.9	56.3±5.1
7-14	54.1±9.1	54.8±10.5	55.1±12.0	55.1±7.8	57.5±13.8	57.9±12.3
14-21	50.5±12.0	46.0±12.0	51.8±10.6	51.9±10.8	52.8±12.8	51.5±12.4

C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

Our results contradict with the feed refusal and anorexia that DON normally induces even at such high concentration; in swine, anorexia is induced already at 1 mg/kg of feed. In the study of Hewitt et al. (2012) DON (0.25, 4.2 and 4.9 mg/kg) did not affect feed consumption. The above-cited authors found some slight numerical differences in certain clinical chemical parameters and a change in the number of eosinophilic granulocytes in the lamina propria of the gastrointestinal tract of rabbits fed the DON-containing diet. Based on these findings they concluded that rabbits could be adversely affected by *Fusarium* mycotoxins but appear to be less sensitive than other animal species. This is in contrast with the hypothesis that rabbits could be re-exposed to toxins through caecotrophy (Fekete et al. 1989), and thus it could be presumed that they are more sensitive to dietary toxin exposure than other monogastric animals. No significant differences were observed when the plant was fed to the rabbits either solely or in combination with DON. Due to the resistance of the rabbits to the usual adverse effects of DON on productive performance, the observation of any possible protective effect of the plant was not possible in this study. *C. marianus* has been included in diets of animals (pigeons, rats, mice and most frequently broiler chickens) exposed to either aflatoxin B_1 (AFB_1) of FB_1 (He et al., 2004; Tedesco et al., 2004; Grizzle et al., 2009; Chand et al., 2011; El-Adawi et al., 2011; Muhammad et al., 2012; Amiridumari et al., 2013; Amiridumari et al., 2014; Malekinejad et al., 2015). But only in three of the studies were the interactive effects on productive performance investigated. Silymarin was used as a complex with a phospholipid in order to assess any interaction with AFB_1 on broilers. The silymarin-phospholipid complex was able to alleviate the decrease in body weight gain and feed intake caused by the toxin (Tedesco et al., 2004). Similarly, the use of *C. marianus* seeds at a concentration of 10 g/kg of feed resulted in significantly higher body weight gain and feed intake for the animals consuming the contaminated feed (Chand et al. 2011; Muhammad et al. 2012).

4.1.2.2 Haematology, clinical chemistry, antioxidant parameters

The control group had a higher neutrophil percentage in comparison 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 (Table 18).

Table 18: Haematological indices (mean ± S.D.) of rabbits exposed to deoxynivalenol (10 mg/kg) and the medicinal herb *Carduus marianus* (0.5 and 1%)

Para-meter	Group					
	C	CT	H1	H1T	H2	H2T
WBC[1] G/l	4,0±0,9	3,6±1,0	3,8±1,4	5,0±2,7	3,2±0,5	4,6±1,9
RBC[2] T/l	5,9 ±2,3	5,7±0,5	5,7±0,3	6,0±0,4	5,8±0, 4	5,9±0,5
Neutro-phils (%)	71,8±2,6	64,5±6,7	72,2±1,6	71,6±4,2	67,7±5,3	68,4±5,8
Lympho-cytes (%)	17,5±5,1	19,9±6,3	13,1±6,7	21,4±7,3	18,7±10,7	20,6±6,5
Monocytes (%)	8,7±4,2[a,b]	13,2±2,1[b]	12,9±4,6[b]	3,9±2,6[a]	11,9±6,1[b]	8,1±5,0[a,b]
Eosinophils (%)	0,0±0,0	0,8±0,8	0,0±0,0	0,0±0,0	0,6±0,9	0,4±0,5
Basophils (%)	1,3±1,8	1,6±1,4	1,2±0.95	0.5±0.8	0.7±0.7	0.8±0.9
Haemo-globin g/l	123,3±5,4	122,5±5,5	123,0±2,0	123,5±8,1	124,3±5,2	119,7±8,4
Platelets G/l	254,3±153,8	349,5±161,9	335,5±11,9	334,0±112,8	339,8±35,5	336,7±188,8
MCV[3] fl	76,6±2,2	78,1±4,1	77,6±1,7	75,1±2,3	78,7±2,6	74,8±2,5
MCH[4] pg	20,9±0,6	21,6±1,0	21,7±0,3	20,8±0,6	21,8±0,7	20,5±0,6
MCHC[5] g/dl	27,4±0,2	27,6±0,2	27,6±0,4	27,7±0,3	27,7±0,2	27,5±0,6
HCT[6] g/l	45,2±2,0	44,3±1,8	44,6±1,0	44,7±3,2	45,0±1,8	43,7±2,7
RDW-CV[7]	16,0±1,7	15,1±0,9	16,1±1,5	15,6±2,3	15,2±2,3	15,3±2,0

[a,b] differences of treated animals in comparison with control and among toxin groups ($P<0.05$)
[1]white blood cells; [2]red blood cells; [3]mean corpuscular volume; [4]mean corpuscular haemoglobin; [5]mean corpuscular haemoglobin concentration; [6]haematocrit; [7]red cell distribution width-corpuscular volume
C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

The higher monocyte and eosinophil and the lower neutrophil ratio in the toxin-treated animals were the most pronounced effects of DON in this experiment.

As the absolute monocyte count was not influenced significantly by the treatment, the shift in the ratio of the different leukocytes was due to the decrease of neutrophil and the increase in eosinophil cell counts. As neutrophil granulocytes are the most numerous among the phagocytic cells, impairment of the non-specific immune response might be a consequence. Upon activation, eosinophils also become capable of phagocytosis; they secrete a range of toxic proteins and free radicals that are effective in killing parasites but may also cause tissue damage through an allergic reaction. While the effect of DON on the innate and the adaptive immune system has been thoroughly studied both *in vitro* and *in vivo*, most information concerns macrophages, cytokine production and expression, lymphocytes and natural killer cells, and very few data are available on the effect exerted by DON on neutrophil and eosinophil granulocytes. The haematotoxicity of other trichothecene toxins (T-2 toxin and diacetoxyscirpenol) has been thoroughly investigated but DON has been less studied. DON preferentially inhibits late-aggregate-forming cells, specifically colony-forming cells, in the rat, but is 100 times less toxic than T-2 (Lautraite et al. 1997). According to Chowdhury et al. (2005), 12 mg/kg DON in the feed was not haematotoxic and did not affect the ratio of peripheral blood leukocytes in laying hens. These differences in the immunomodulation that DON exerts among animal species can be attributed to the diffences in toxicokinetics as it has also been reviewed in Pestka (2007).

C. marianus exerted no effect (either protective or negative) on blood indices apart from monocytes. To our surprise, the percentage of monocytes in H1T was the lowest (3.9%) of all groups and was significantly lower than in the groups CT and H2T. *C. marianus* was reported to possess immunomodulatory effects that are dose-dependent as it was reviewed in Darvishi Khezri et al. (2016). More specifically, it has been observed that in low doses the plant can stimulate the immune system but at higher doses to suppress it (Gharagozloo and Amirghofran, 2007; Gharagozloo et al., 2013). Ahmadi et al. (2012) reported an increase in the number of white blood cells in rainbow trout after oral exposure to silymarin extract for 15 or 30 days at a concentration range 0.1-0.8 g/kg of feed. *In vitro*, *C. marianus*

extract has been demonstrated to exert immunostimulatory effects on murine spleen lymphocytes (Wilasrusmee et al., 2002).

Serum total protein, albumin, glucose, triglyceride, cholesterol, urea and creatinine concentrations as well as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma-glutamyl transpeptidase (GGT) activities did not differ significantly according to treatment, and the mean values were within the physiological ranges (Harcourt-Brown, 2002) (Table 19).

Table 19: Clinical chemistry indices (mean ± S.D.) of rabbits exposed to deoxynivalenol (10 mg/kg) and the medicinal herb *Carduus marianus* (0.5 and 1%)

Parameter	Group					
	C	CT	H1	H1T	H2	H2T
AST[1] IU/l	28,8±5,7	28,8±5,0	38,0±14,4	28,8±11,4	27,8±6,8	25,7±6,2
ALT[2] IU/l	38,8±7,3	33,3±9,6	34,3±11,1	34,7±15,2	30,2±4,5	28,7±8,4
ALKP[3] IU/l	480,3±94,6	602,8±133,6	495,3±21,07	568,3±136,7	681,3±143,3	566,5±158,4
Total protein g/l	65,2±2,0	62,3±2,7	65,0±4,6	64,4±4,9	66,9±4,3	64,8±4,0
GGT[4] IU/l	5,2±3,0	5,0±2,8	3,2±1,7	5,7±1,4	3,6±2,4	5,5±1,7
Albumin g/l	43,5±1,3	41,9±2,0	42,3±1,9	42,3±1,4	43,6±2,1	43,4±1,6
Albumin/globulin (%)	2,0±0,2	2,0±0,1	1,9±0,3	2,0±0,3	1,9±0,3	2,1±0,3
Triglycerides	0,8±0,2	1,2±0,7	0,8±0,5	0,7±0,1	0,7±0,4	0,9±0,4
Creatinine µmol/l	113,2±9,4	99,7±10,7	105,3±15,2	102,0±8,4	108,0±10,9	105,0±12,0
Cholesterol mmol/l	1,8±0,2	2,1±0,2	1,7±0,2	1,6±0,1	1,8±0,1	1,7±0,1
Urea mmol/l	5,9±0,6	6,0±0,5	5,5±1,0	6,1±0,6	5,9±0,8	6,4±0,5
CK[5] IU/l	2095±668	2771±1507	3018±1544	2772±1598	3219±1512	2475±860

[1]aspartame transaminase; [2]alanine transaminase; [3]alkaline phosphatase; [4]gamma-glutamyl transferase; [5]creatine kinase

C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

Clinical chemical parameters are indicators of the health status of animals and may indicate dysfunction or damage of the different target organs, e.g. the liver, kidney and heart. The liver is considered to be the primary target organ of many mycotoxins including trichothecenes (such as DON) in most species examined (Bergsjø et al., 1993; Sun et al. 2014). In the present experiment, DON caused slight fibrosis in the liver without degenerative alteration of the hepatocytes. The latter was confirmed by the fact that the activities of liver enzymes (AST, ALT and GGT) remained unchanged and within the physiological ranges. As discussed above the lack of DON-induced liver toxicity prohibited us from observing any protective effect of the plant. *C. marianus* is primarily known for its hepatoprotective effects thus the most studied endpoint is serum biochemistry. In many studies, *C. marianus* alleviated or completely restored the changes on serum enzyme activities (AST, ALT, ALKP) or concentrations of glucose, cholesterol, total protein and creatinine (He et al., 2004; Tedesco et al., 2004; Grizzle et al., 2009; El-Adawi et al., 2011; Muhammad et al., 2012; Amiridumari et al., 2013; Amiridumari et al., 2014; Malekinejad et al., 2015).

The antioxidant parameters measured (GSH, GPx and MDA) are shown in Table 20.

Table 20: Level of the antioxidant parameters (mean ± S.D.) after exposing rabbits (n=6) to feed containing 10mg/kg deoxynivalenol

Antioxidant parameters	Group					
In blood	**C**	**CT**	**H1**	**H1T**	**H2**	**H2T**
GSH (µmol/g)	4.0±1.4	4.8±0.8	4.5±1.2	4.1±0.9	3.9±0.8	3.8±0.9
GPx (E/g)	5.3±1.5	5.1±2.5	5.2±0.4	4.8±1.3	5.1±0.8	5.4±0.5
MDA (nmol/g)	21.6±3.7	22.2±4.6	21.3±2.4	21.5±2.1	21.8±3.3	21.5±1.6
In plasma	**C**	**CT**	**H1**	**H1T**	**H2**	**H2T**
GSH (µmol/g)	4.6±0.5	4.5±0.4	4.8±0.8	4.8±0.4	4.8±0.5	4.7±0.4
GPx (E/g)	5.1±0.5	5.2±0.5	5.4±1.0	5.5±0.2	5.5±1.2	5.3±0.7
MDA (nmol/g)	15.1±2.0	14.2±3.3	15.4±1.1	14.5±2.3	15.0±3.7	13.9±1.4

C=control; CT=control+ 10mg/kg of mycotoxin; H1=plant at 5mg/kg of feed; H1T=plant at 5mg/kg of feed+ 10mg/kg of mycotoxin; H2=plant at 10mg/kg of feed; H2T=plant at 10mg/kg of feed+ mycotoxin;
GSH=reduced glutathione; GPx= glutathione peroxidase; MDA = malondiadelhyde
C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

In the present study, DON at the dose applied had no effect on the antioxidant status of the animals. According to El Golli-Bennour and Bacha (2011), oxidative stress is not a major contributor to DON toxicity because it does not induce over-expression of Hsp70 and has a negligible effect on hydrogen peroxide and MDA production. While there are several *in vitro* studies on oxidative stress induced by DON, *in vivo* studies are lacking as reviewed by Mishra et al. (2014): DON increased lipid peroxidation and altered the antioxidant status (GSH, GPx) in rats, mice and broiler chickens, while it did not significantly alter antioxidant parameters (MDA, TAS, GPx) in a study with broiler chicken and male pigs. In our previous study (Szabó-Fodor et al., 2015) DON in combination with ZEN, after prolonged exposure period (60 days), resulted in significantly higher MDA levels in plasma and significantly higher levels of GPx in RBCH. Although the concentration of DON was lower (0.9 mg/kg of feed) the longer exposure period (60 vs 21 days) could be the reason for the observed oxidative stress. Since no significant difference was observed for the CT group the antioxidant effects of the plant could not be revealed. Surprisingly despite its pronounced antioxidant capacities there is only one study in which antioxidant parameters were investigated (El-Adawi et al., 2011). In FB_1 treated rats, GPx was depleted to 48% while pre-treatment with *C. marianus* elevated GPx by 30%. In a similar manner the high elevation (137%) of MDA (an indicator for lipid peroxidation) was minimized to 38% when *C. marianus* was coadministered.

4.1.2.3 Immune parameters

No significant differences were observed for any of the gut cytokines (IL-1, IL-2 and INF-γ) (Table 21).

Table 21: Level of some gut cytokines (mean ± S.D.) of rabbits exposed to deoxynivalenol (10 mg/kg) and the medicinal herb *Carduus marianus* (5 and 10 g/kg of feed)

Cytokines[1]	Group					
	C	CT	H1	H1T	H2	H2T
IL-1	16.9±0.5	17.6±3.4	17.4±2.1	18.0±1.2	18.3±3.2	17.4±1.2
IL-2	11.2±2.8	12.4±3.8	10.3±1.2	10.7±1.5	9.8±1.1	10.1±0.8
IFN-γ	12.2±2.6	12.1±2.6	11.6±2.2	11.9±2.4	12.8±1.1	12.0±0.9

[1]Expressed in Ct values (cycle threshold, the number of cycles required for the fluorescent signal to cross the threshold)
C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

As mycotoxins enter the animals' body mostly via the consumption of contaminated feed, the gastrointestinal tract is the first organ exposed to them. This was the reason why some of the gut cytokines (IL-1, IL-2 and IFN-γ) were studied since they have not yet been investigated in rabbits. In domestic pigs, a lower expression of IL-1β and IL-8 occurred in the blood and ileal tissue after the feeding of low doses of DON (Becker et al. 2011).

The macrophage count per ml and the phagocytic activity of macrophages did not differ significantly between the groups (Table 22).

Table 22: Macrophages' number and phagocytic activity % (mean ± S.D.) of rabbits exposed to deoxynivalenol (10 mg/kg) and the medicinal herb *Carduus marianus* (5 and 10 g/kg of feed)

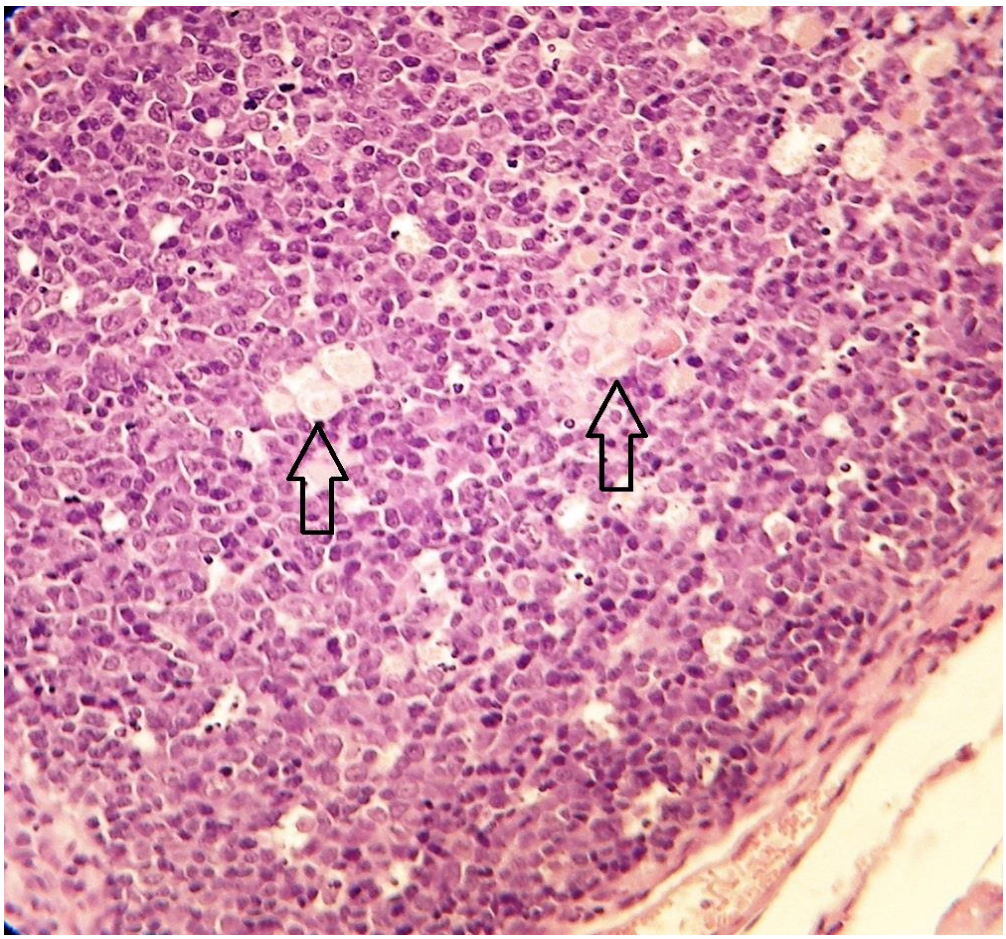
Parameter	Group					
	C	CT	H1	H1T	H2	H2T
Number of macrophages (per ml)	372.5±18.9	378.3±17.2	375.0±8.4	366.7±35	343.3±45.9	353.3±29.4
Phagocytic activity (%)	72.0±5.7	74.7±2.1	74.3±2.3	77.0±5.2	76.3±4.6	72.3±2.7

C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

No data regarding the effects of *C. marianus* on gut cytokines have been recorded so far. The effects of the plant in combination with AFB_1 was assessed on antibody titers of animals vaccinated against Newcastle disease, infectious bronchitis and infectious bursal diseases (Chand et al., 2011; Amiridumari et al., 2014). *C. marianus* acted as an immunostilmulant against the immunosuppressive properties of AFB_1.

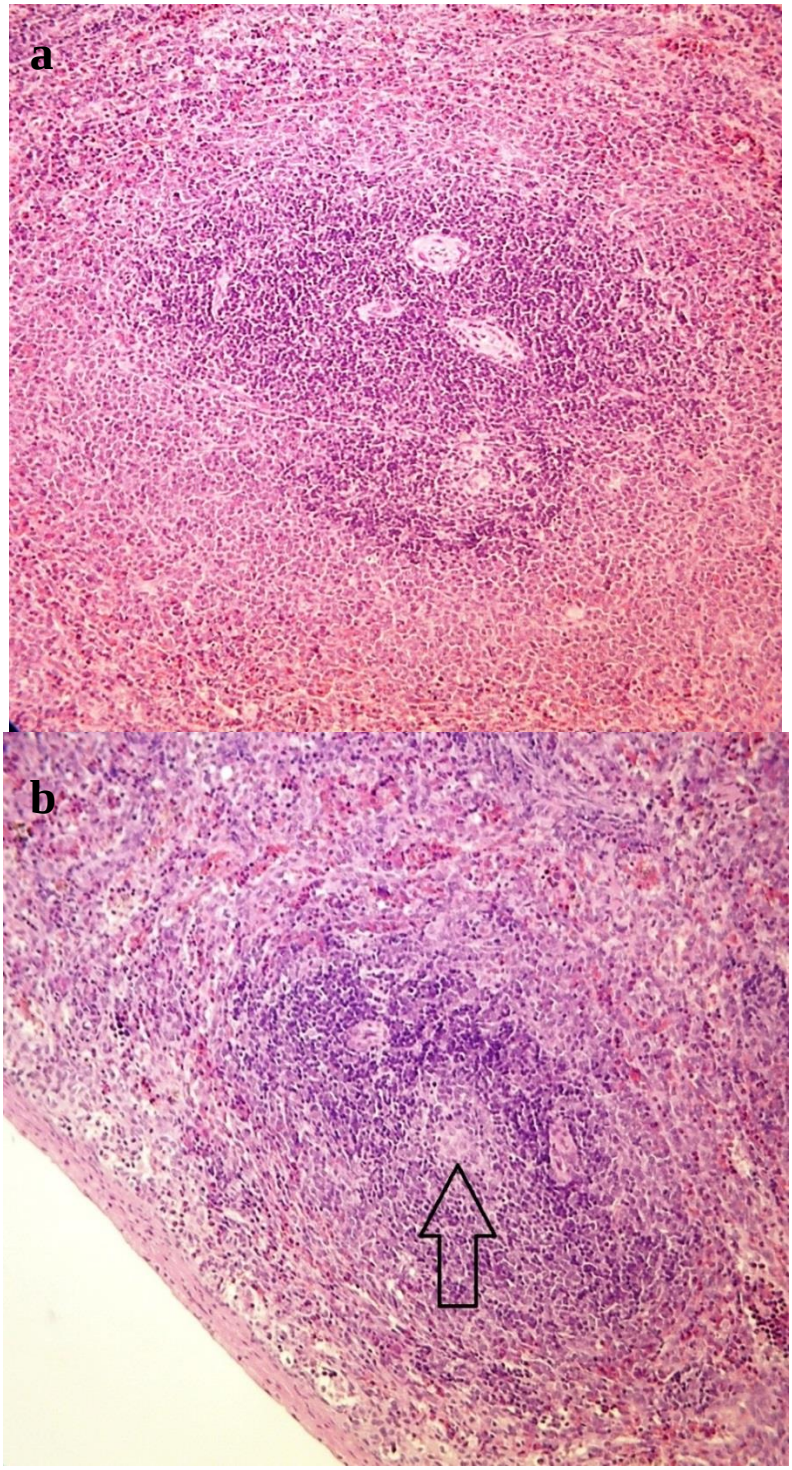
4.1.2.4 Histology

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. In the existing literature, there is no explanation for this observation. 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 lymphoblast proliferation and simultaneous apoptosis shifted towards apoptosis (Picture 4).



Picture 4: Jejunal mucosa of rabbit exposed to deoxynivalenol (DON). Increased apoptotic processes in the lymphatic tissue of Peyer's patches (⇑)
(Haematoxylin-eosin staining, ×400)

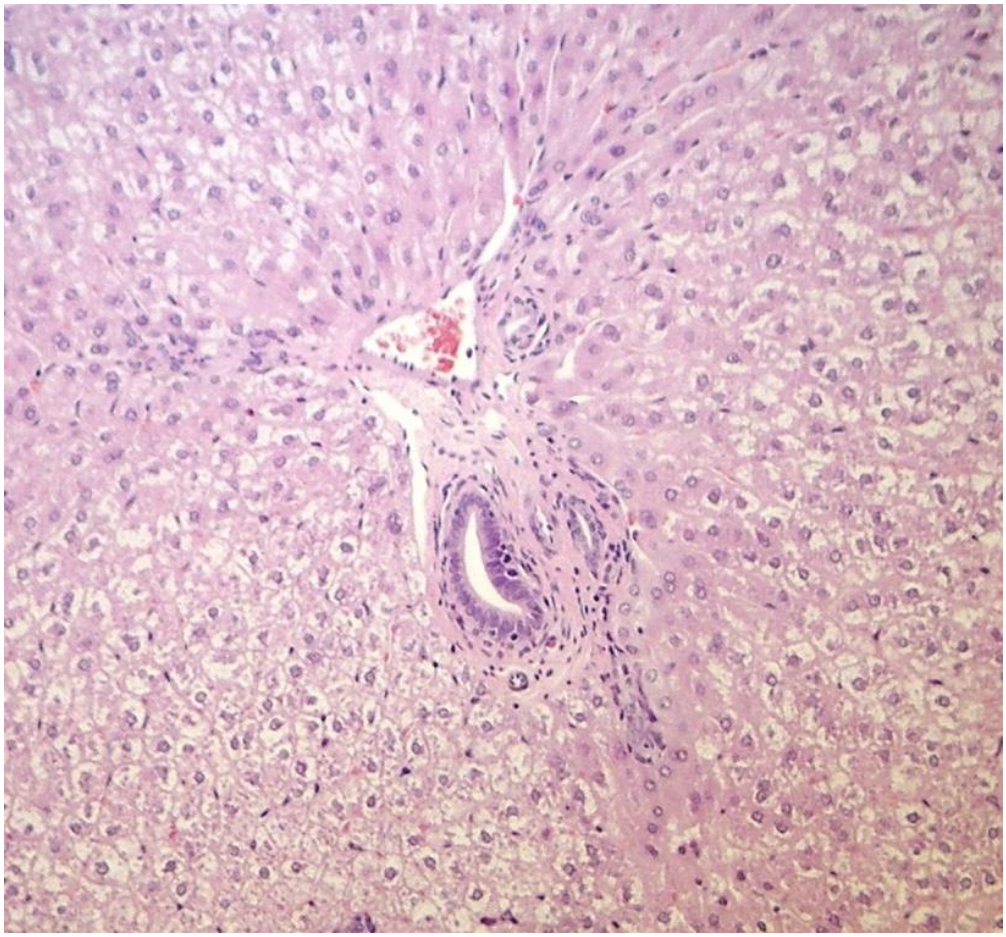
The spleen was also affected by DON treatment in all animals. In the central part of the lymphoid follicles, in the Malpighian bodies, lymphocyte depletion resulting in smaller follicles (follicular atrophy) could be detected (Picture 5b) compared to the control animals (Picture 5a).



Picture 5: (a) In control animals, the lymphatic nodules in the spleen are big and rich in lymphocytes; (b) In rabbits exposed to deoxynivalenol (DON) the Malpighian body is smaller and lymphocyte depletion can be detected
Haematoxylin and eosin staining, ×200

The lymphocyte depletion and follicular atrophy caused by DON was completely inhibited by the plant at both concentrations. Chand et al. (2011) observed reduced spleen weight in the toxin treated animals whereas *C. marianus* alleviated this in the toxin treated animals.

DON caused slight fibrosis in the liver, without degenerative alteration of the hepatocytes (Picture 6).



Picture 6: Interstitial fibrosis (proliferation of fibres) in the liver of deoxynivalenol exposed rabbits (Haematoxylin and eosin staining, ×200)

In almost all of the studies aforementioned, the protective effect of *C. marianus* was tested against the mycotoxin aflatoxin. Aflatoxin is a known carcinogen and the target organ is the liver. In our study, DON caused only slight deleterious effects on the liver thus no protective effect could be observed.

DON did not cause any pathological changes in either the heart or the kidneys. Although no pronounced immunomodulatory effect of DON was observed in this experiment, the histological findings revealed lymphocyte depletion and follicular atrophy in the spleen of DON-treated rabbits. In the GALT, the rate of lymphoblast apoptosis increased in the DON-treated animals. These observations support the results suggesting that DON may alter the functioning of the immune system. The intestinal villi increase the surface area available for brush border digestion and nutrient absorption. In a meta-analysis of experiments on the modulation of intestinal functions following mycotoxin ingestion, Grenier and Applegate (2013) reviewed 11 experiments about DON and nutrient uptake, which are limited to poultry and pigs. Some villus abnormalities (reduced villus height, villus fusion, and atrophy) after DON exposure were described in pigs (Bracarense et al. 2012) and poultry (Awad et al. 2006). In our experiment, thickening and fusion of the villi could be observed in only one of the toxin-treated animals. Thus, the lack of a pronounced effect on the gut may also explain the fact that the toxin-exposed animals had a similar weight gain as the controls.

The histological results are summarised in Table 23.

Table 23: Histological alterations in spleen, liver, kidney, heart, Peyer's plaque and gut of rabbits exposed to deoxynivalenol (10 mg/kg) and the medicinal herb *Carduus marianus* (5 and 10 g/kg of feed)

Alterations	Group					
	C	**CT**	**H1**	**H1T**	**H2**	**H2T**
SPLEEN						
- lymphocyte depletion	0[1]	3	0	0	0	0
- follicular atrophy	0	3	0	0	0	0
LIVER						
- interstitial fibrosis	0	3	0	1	0	0
KIDNEY						
- tubulonephrosis	0	0	0	0	0	0
HEART						
- myocardial degeneration	0	0	0	0	0	0
PEYER PLAQUE						
- apoptosis	0	6	6	6	6	6
GUT						
- villus thickening	0	1	0	0	0	3
- villus fusion	0	1	0	0	0	3

[1] number of animals showing pathological alteration within the group (n=6/group)
C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

4.1.2.5 Comet assay

According to the results obtained, DON did not prove to be genotoxic. The tail intensity and tail moment are presented in Table 24.

Table 24: Tail intensity (mean±SEM) and tail moment (mean±SEM) of lymphocytes isolated from rabbits exposed to deoxynivalenol (10 mg/kg) and the medicinal herb *Carduus marianus* (5 and 10 g/kg of feed)

Parameter	Group					
	C	**CT**	**H1**	**H1T**	**H2**	**H2T**
Tail Intensity (%)	28.6±0.9	21.4±1.0	33.2±0.9	27.8±1.5	22.1±1.1	25.1±1.1
Tail moment*	7.3±0.2	8.5±0.4	10.5±0.3	10.9±5.6	5.4±0.3	6.0±3.7

C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

*Tail moment is defined as the product of the tail length and the fraction of total DNA in the tail. Tail moment incorporates a measure of both the smallest detectable size of migrating DNA (reflected in the comet tail length) and the number of relaxed / broken pieces (represented by the intensity of DNA in the tail).

No significant differences were observed for CT which was compared to groups C, H1T and H2T. To our knowledge, this is the first time that genotoxic effects of DON are investigated on rabbits' lymphocytes. The absence of severe genotoxic effects on the lymphocytes prevented us – similarly with other aforementioned endpoints - to observe any protective effect of *C. marianus*. Reactive oxygen species (ROS) are able to oxidize DNA bases and cause DNA damage. DON fed to broiler chickens at 10 mg/kg of feed for 5 weeks increased the percentage of tail DNA (tail intensity) in the comet assay performed with the isolated lymphocytes (Frankic et al. 2006). Although DON did not increase MDA in the plasma or liver in the above-cited study, the oxidative stress pathway was suggested as a plausible mechanism for the observed DNA damage. Similarly, DNA damage was reported by Frankic et al. (2008) in pigs (4 mg DON/kg of feed for 14 days) and Awad et al. (2012) in chicken (10 mg DON/kg of feed for 35 days). In our previous study (Szabó-Fodor et al., 2015), DON in combination with ZEN resulted in in significantly higher levels of MDA and GPx in plasma and RBCH, respectively. However, it has to be highlighted that the exposure was much longer (30 and 60 days sampling), which can have different effects on oxidative stress. According to the literature cited DON is considered to have negligible oxidative

stress inducing effect and thus, it is supposed that DON is directly genotoxic or acts via different epigenetic mechanisms (e.g. DNA-adduct formation), causing DNA fragmentation. We concluded that 10 mg/kg DON in the feed had no significant effect on the antioxidant status of the rabbits, and no direct or indirect (oxidative stress induced) genotoxicity could be observed.

4.1.2.6 Short chain fatty acids (SCFA), caecal composition and pH

There was no significant difference in the total SCFA (mmol/kg) or the particular SCFA (acetic, propionic and butyric; Table 25).

Table 25: Total short chain fatty acid (SCFA) content, proportion of the fatty acids (% of total fatty acid content) and pH of the caecal chyme (mean±SD)

Parameter	Group					
	C	**CT**	**H1**	**H1T**	**H2**	**H2T**
Total SCFA mmol/kg	46.6 ± 7.7	48.9 ± 7.7	45.8 ± 11.9	39.8 ± 18.8	46.6 ± 16.0	43.4 ± 12.6
Acetic acid (%)	78.6 ± 1.5	78.7 ± 3.7	78.1 ± 2.9	77.2 ± 2.6	79.8 ±2.4	78.6 ± 2.8
Propionic acid (%)	7.2 ± 0.8	7.0 ± 1.2	6.2 ± 0.6	8.0 ± 1.1	7.1 ± 1.8	7.0 ± 1.1
Butyric acid (%)	14.3 ± 1.5	14.3 ± 3.7	14.3 ± 1.1	14.7 ± 2.1	13.2 ± 1.2	14.4 ± 2.1
pH	6.04 ± 0.15	6.05 ± 0.39	6.06 ± 0.14	6.08 ± 0.22	5.88 ± 0.15	6.01 ± 0.23

C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

The composition of caecal microbiota also did not differ regarding anaerobic bacteria and Bacteroides (Table 26).

Table 26: Composition of the caecal microbiota (CFU log10/g, mean±SD)

Microbiota	Group					
	C	**CT**	**H1**	**H1T**	**H2**	**H2T**
Aerobic bacteria	4.9 ± 0.3^{a}	5.8 ± 0.6^{b}	$5.3 \pm 0.6^{a.b}$	5.7 ± 0.6^{b}	$5.2 \pm 0.2^{a.b}$	5.7 ± 0.6^{b}
Anaerobic bacteria	8.9 ± 0.4	9.1 ± 0.4	8.8 ± 0.3	9.0 ± 0.6	8.7 ± 0.5	9.0 ± 0.5
Bacteroides	8.3 ± 0.5	8.5 ± 0.2	8.5 ± 0.3	8.4 ± 0.5	8.3 ± 0.5	8.6 ± 0.4

^{a,b} indices indicate significant differences (in the same row) among the groups; $P<0.05$
C= control; CT= control+toxin; H1= *Carduus marianus* (0.5%); H1T= *C. marianus* (0.5%)+ toxin; H2= *C. marianus* (1%); H2T= *C. marianus* (1%)+ toxin

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 groups (i.e. CT, H1T and H2T; Table 3), a fact that suggests an effect of DON on aerobic bacteria regardless the supplementation with the herb. This is confirmed by the t-test performed using as a factor the consumption of mycotoxin solely (C, H1, H2 and CT, H1T, H2T respectively). The large intestine is an anaerobic environment, thus any increase in the aerobic bacteria could affect host's health negatively. Our results are in agreement with the the study of Waché et al. (2009), who observed an increase of the aerobic and decrease of anaerobic bacteria numbers in pigs' faeces after dietary exposure to DON. In a rather old study, other trichothecenes, i.e. T-2 and diacetoxyscirpenol (DAS) caused an increase of aerobic bacterial count in piglets and Wistar rats (Tenk et al., 1982).

The interaction of DON and caecal microbiota should be further investigated since it can adversely affect intestinal health in the long run, even when no clinical symptoms are present. Several studies have demonstrated that DON increases the susceptibility of different animals to pathogenic bacteria. DON was shown to render the intestinal epithelium of pigs more susceptible to *Salmonella typhimurium* and corroborated the inflammatory response (Vandenbroucke et al., 2011). Payros et al. (2017) demonstrated that DON exacerbated the genotoxicity in rat intestinal epithelial cells (IEC-6), which was induced by a colibactin producing strain of *E.coli* (which is a facultative anaerobe microorganism).

To our knowledge, this is the first time the combined effect of *C. marianus* and a mycotoxin on caecal microbiota and fermentation were investigated.

4.2 *In vitro* experiments

4.2.1 Cytotoxicity assay

Cytotoxicity was assessed by the means of CCK-8 assay using porcine lymphocytes obtained from healthy animals, as target cells. CCK-8 is a water-soluble version of the 3-(4, 5-Dimethylthiazol-2-Yl)-2, 5-Diphenyltetrazolium Bromide (MTT) test. Viable cells can convert the tetrazolium salt to crystals and consequently the optical density can be read under a Microplate reader. The viability of each concentration is a percentage of the control cells' viability multiplied by 100.

A time- and dose-dependent decrease in cell viability was observed for all three toxins (Figure 5).

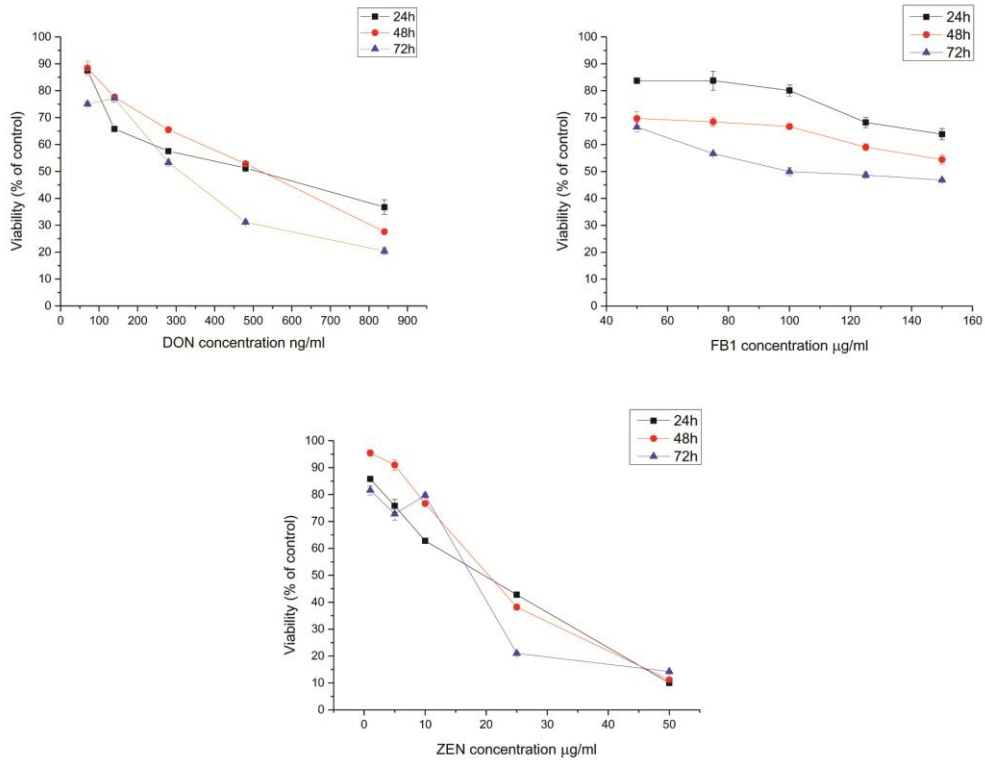


Figure 5: Decrease of cell viability of porcine lymphocytes after 24, 48 and 72h of incubation with FB_1 (µg/ml), DON (ng/ml) or ZEN (µg/ml) determined by CCK8 test (n=5 / treatment)

For DON and ZEN, IC_{50} values could be calculated for all incubation times (Table 27). In contrast, FB_1 decreased cell viability by 50% only after 72h.

Table 27: IC_{50} (mean±SD) values calculated on porcine lymphocytes following 24h, 48h and 72h exposure to FB_1 (50-150 µg/ml), DON (0.07-0.84 µg/ml) or ZEN (1-50 µg/ml)

	IC_{50} (µg/ml)		
	24h	**48h**	**72h**
FB_1	NA*	NA	101.15±7.80
DON	0.43±0.02	0.41±0.02	0.31±0.01
ZEN	19.55±1.20	20.60±1.07	16.60±2.05

*NA: not applicable

In the present study, DON was the most potent among the three mycotoxins that were investigated, with a potency order FB_1<ZEN<DON, which is in agreement with the results of Wan et al. (2013a) on swine jejunal epithelial cells. Kouadio et al. (2007) previously reported that the potency of FB_1, DON and ZEN on Caco-2 cells was ZEN>DON> FB_1 in decreasing order, confirming that FB_1 has low toxicity towards several cell lines.

In our experiments, the calculated IC_{50} value was 101.15 µg/ml for FB_1 after 72 h exposure. On the other hand, Kouadio et al. (2007) could not calculate an IC_{50} value for FB_1 (the highest concentration used was 150 µM which is approximately 108 µg/ml) even after 72h. In the study of McKean et al. (2006) only concentrations higher than 100 µM (72 µg/ml) exerted a cytotoxic effect (24 h exposure) to HepG2 and BEAS-2B cells and the IC_{50} values were 399.2 µM (288 µg/ml) and 355.1 µM (256 µg/ml), respectively. These IC_{50} values are 2.5-fold higher than those found in our study, which could be attributed to the higher sensitivity of porcine lymphocytes to FB_1. The lowest IC_{50} value for DON was 0.31 µg/ml after 72 h in our experiments, which is in total agreement with the findings of Goyarts *et al.* (2006a, b) who investigated the effect of DON on the proliferation of porcine blood lymphocytes using MTT assay. In the study of Meky et al. (2001) the IC_{50} value of DON on human lymphocytes was 0.216 µg/ml, which is significantly lower than the IC_{50} calculated in our study. This difference could be attributed to the longer exposure period (5 days). In our study, the IC_{50} value of ZEN was 19.55 µg/ml after 24h and decreased to 16.6 µg/ml after 72h of exposure. According to the literature, it appears that lymphocytes are less sensitive to ZEN than other cell lines, such as

Vero kidney or Caco-2 cells. In the study of Abid-Essefi *et al.* (2004) the cytotoxic potential of ZEN was determined by the MTS assay (a modified version of MTT) using Vero kidney and Caco-2 cells and the determined IC_{50} values were 7 and 15μM (2.2 μg/ml and 4.8 μg/ml), respectively. Surprisingly, Chinese hamster ovarian cells (CHO-K1) were less sensitive to ZEN with IC_{50} values of > 100 μM (31.8 μg/ml), 60.3 μM (19.2 μg/ml), 68 μM (21.6 μg/ml) for 24 h, 48 h and 72 h exposure, respectively (Tatay *et al.* 2014).

4.2.1.1 Interactive effects of binary and ternary mixtures

Although the number of studies with mycotoxin combinations tends to increase, there are still only a few studies with the mixtures (and especially the ternary combination) of FB_1, DON and ZEN.

In the present study, the mixtures of FB_1, DON and ZEN resulted in antagonism which was more evident after 72h of exposure (Figure 6).

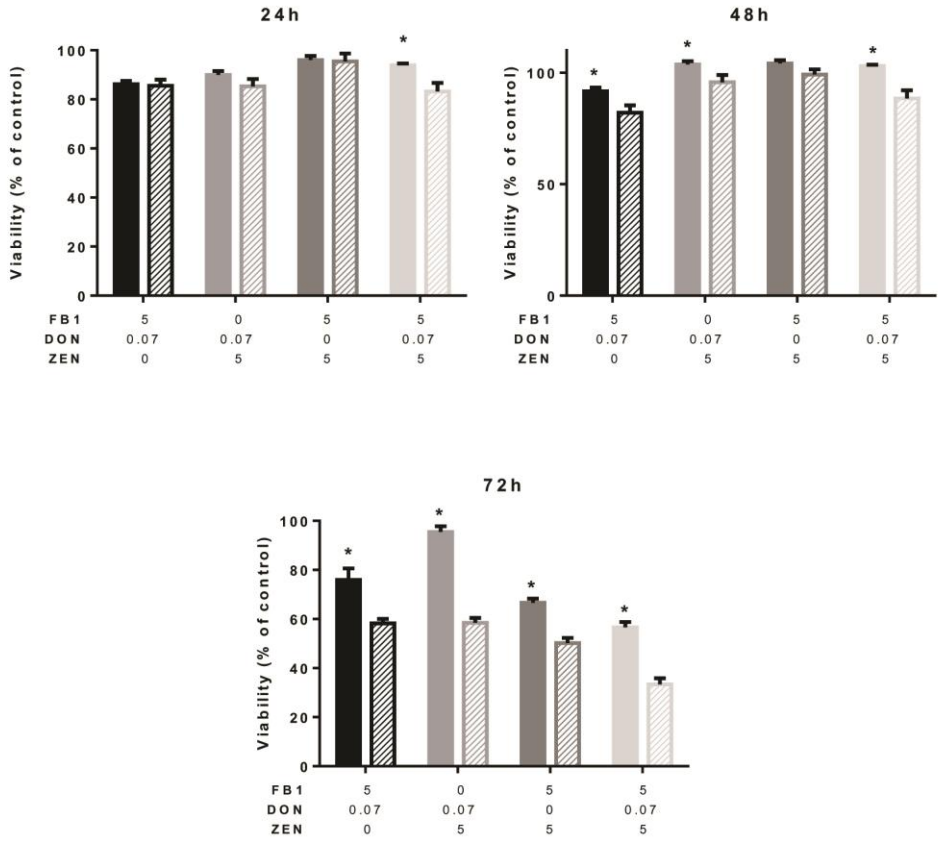


Figure 6: Cytotoxic effects of binary and ternary mixtures of FB_1, DON and ZEN (µg/ml) on porcine lymphocytes after 24h, 48h and 72h of incubation by CCK8 test (sketched bars represent the calculated expected values,*: indicates the statistically significant difference of calculated and expected values, P<0.05 **)**

After 24h of incubation, the binary mixtures did not show interactions, whereas the ternary (DFZ) mixture exhibited a significant antagonism which was expressed at all incubation times. After 48h an antagonistic effect was revealed for the binary mixtures of DON (DF and DZ), which was more expressed after 72 h. After 72h an antagonistic effect was evident for the FZ mixture as well. Ficheux et al. (2012) used myelotoxicity as an endpoint and observed an antagonistic effect of the mixture of DON and FB_1 on haematopoietic progenitor cells. Another study investigating the effects of binary and ternary mixtures of DON, FB_1 and ZEN on porcine intestinal (jejunal) epithelial cells demonstrated antagonistic effects of the mixture when the lowest dose was used (Wan et al., 2013a). DON and ZEN were tested in combination on human colon carcinoma cells and their effects were sub-

additive for all the endpoints used (cell viability and cycle, mitochondrial inner membrane potential and permeability transition pore opening) (Bensassi et al., 2014). On the contrary, Dąbrowski et al. (2016) reported a synergistic effect of DON and ZEN in porcine subpopulations of lymphocytes. Lymphocytes were isolated from pigs that were exposed to dietary DON and ZEN at no observed effect level (NOAEL) doses. The immunomodulatory effect that the single mycotoxins exerted was stronger when they were combined. Kouadio et al. (2007) observed additive effects for the mixtures of DF and DFZ but an antagonistic effect for FZ. Kouadio et al. (2013) reported an antagonistic effect for FB_1 and ZEN for lysosomal and mitochondria; damage but an additive effect for necrosis.

FZ was the most cytotoxic among the binary mixtures, and DFZ was significantly antagonistic at all incubation time points. The potency of the mixtures was consistent after 24 and 48 h of incubation DF<DZ<DFZ<FZ whereas after 72 h the order was reversed, DFZ<FZ<DF<DZ. This phenomenon could be due to the delayed cytotoxic effect of FB_1 in a similar manner as with the single exposure (IC_{50} calculated for 72 h only). Differences found in the interactions between different time points indicate the difficulty to foresee the effects of chronic multi-mycotoxin exposure. Kouadio et al. (2007) reported that the reduction of cell viability as assessed by the neutral red (NR) test was FZ<DF<DZ<DFZ in increasing order as assessed by NR test. These results are partly consistent with the findings of the present study regarding DFZ and DF combinations, whereas DZ and FZ showed opposite results. In the study of Wan et al. (2013a) the binary and ternary mixtures of FB_1, DON and ZEN were cytotoxic although non-cytotoxic concentrations of the individual mycotoxins were used. The least toxic mixture was FZ after 48 h of incubation, like in our study. From the data of the present study, it can be concluded that in the case of porcine lymphocytes ZEN does not determine the interactions of the binary mixtures as strongly as does FB_1 and/or DON.

4.2.2 Comet assay

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 (Table 28).

Table 28: Tail intensity (mean±SEM) measured in porcine lymphocytes following 24, 48 and 72h exposure to single and combined mycotoxins

Cell treatment	**Tail Intensity**		
	24h	48h	72h
Concentrations	**5, 0.07, 5 µg/ml (FB_1, DON, ZEN)**		
Control	6.21±0.73	5.11±0.53	5.39±0.65
FB_1	7.98±0.93	4.52±0.66	7.91±1.00
DON	7.05±0.85	5.21±0.60	6.81±1.17
ZEN	8.76±1.08	11.89±1.37	5.50±0.72
FB_1+DON	6.41±0.83	11.20±1.42	4.53±0.58
DON+ZEN	6.89±1.01	10.31±1.36	5.12±0.80
FB_1+ZEN	8.34±1.03	11.36±1.37	6.70±0.86
FB_1+DON+ZEN	6.97±0.91	11.27±1.33	11.45±1.50
Concentrations	**25, 0.21, 10 µg/ml (FB_1, DON, ZEN)**		
Control	11.17±0.68	7.36±0.99	8.20±0.82
FB_1	12.81±0.65	10.12±0.74	9.65±0.68
DON	10.60±0.85	6.94±0.57	6.79±0.77
ZEA	6.05±0.55	7.13±0.55	8.42±0.79
FB_1+DON	7.47±0.62	9.34±0.76	6.54±0.57
DON+ZEN	6.59±0.88	9.33±0.84	11.50±0.88
FB_1+ZEN	7.74±0.90	8.32±0.89	13.40±0.89
FB_1+DON+ZEN	7.39±0.94	8.44±0.68	12.26±0.76

When the lower set of concentrations (5, 0.07 and 5 µg/ml for FB_1, DON and ZEN, respectively) was used, antagonism was the main interaction observed at all exposure periods (figure 7).

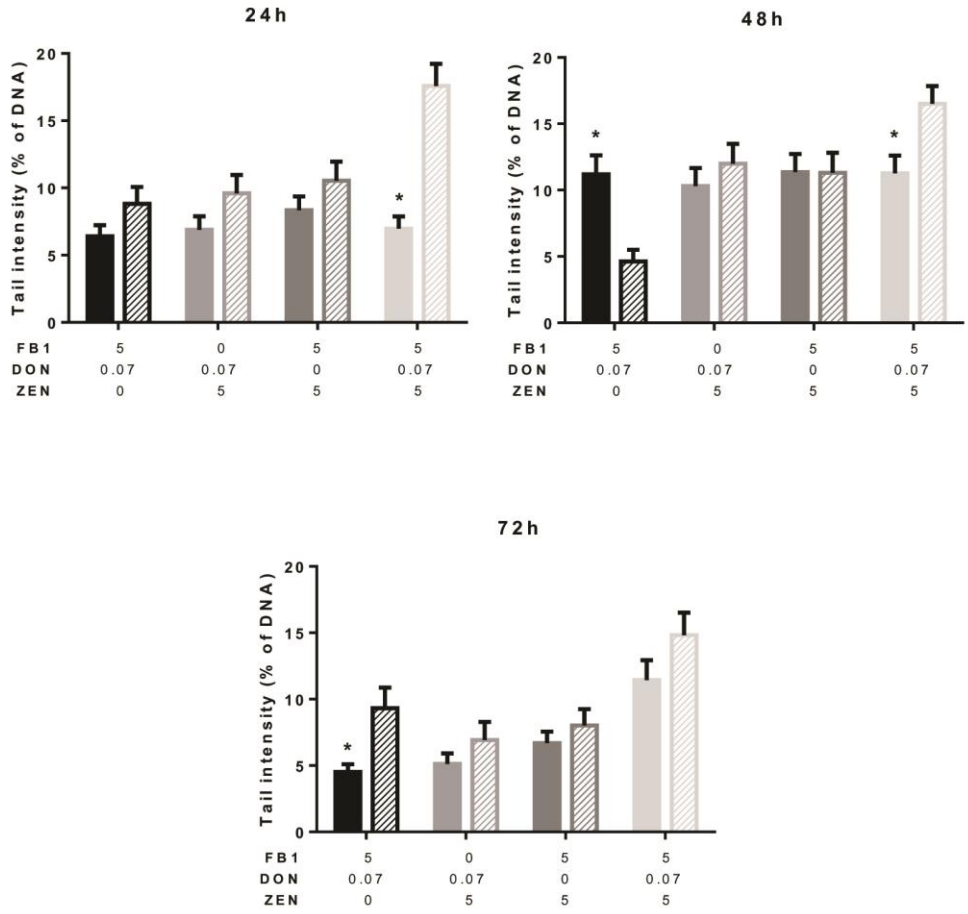


Figure 7: Genotoxic effects of binary and ternary mixtures in lower) concentrations (µg/ml) of DON, FB_1 and ZEN on porcine lymphocytes after 24h, 48h and 72h of incubation (sketched bars represent the calculated expected values, *: indicates the statistically significant difference of observed and expected values, P<0.05).

After 24h antagonism was observed for all mixtures but it was significant only in the case of the ternary one. After 48h, DZ and DFZ exerted an antagonistic effect, being significant only for DFZ; DF was synergistic, while FZ showed no interaction. After 72h antagonism was exerted by all four mixtures but was significant only for DF. Occasionally interactions vary among different exposure times like in the case of DF mixture which exerted antagonistic effects after 24h and 72h and synergistic effects after 48h of exposure.

Since the DNA damage observed was only slightly higher than the normal range for control cells, following the proposition of Gopalan et al. (2011), higher

concentrations were used (25, 0.21 and 10 μg/ml for FB_1, DON and ZEN, respectively) in order to confirm/reject the observed interactions (figure 8).

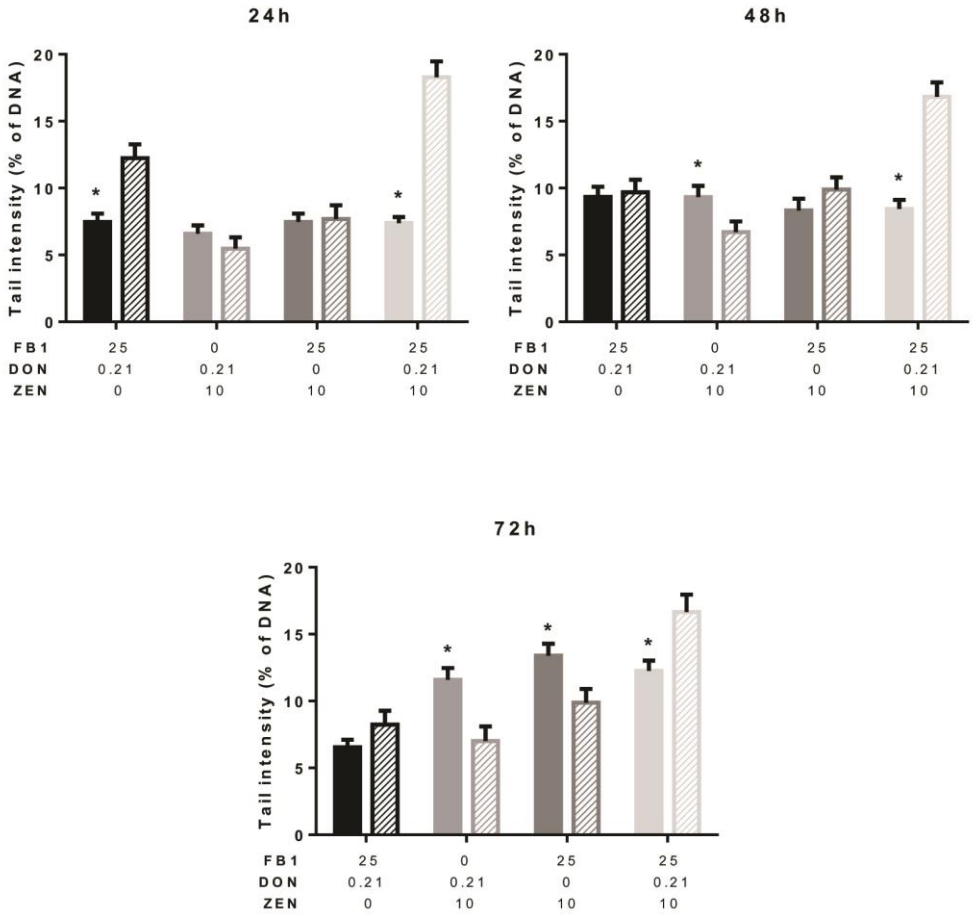


Figure 8: Genotoxic effects of binary and ternary mixtures in higher concentrations (μg/ml) of DON, FB_1 and ZEN on porcine lymphocytes after 24h, 48h and 72h of incubation (sketched bars represent the expected values, *: indicates the statistically significant difference of observed and expected values, P<0.05).

The antagonistic effect of DFZ was confirmed. After 24h, a significant antagonism was expressed by the mixtures DF and DFZ, while DZ exerted synergism which was not significant 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 DF and DFZ (not significant and significant respectively). It has been observed that the antagonism exerted by the combinations of mycotoxins in lower concentrations can convert to synergism in

higher concentrations (Alassane-Kpembi et al., 2015). Likewise, the effects of DZ and FZ were synergistic in a time-dependent manner. Like in the experiment performed with lower concentrations, DF mixture showed inconsistency among different exposure periods. It can be concluded that ZEN acts synergistically with DON and FB_1, especially after a prolonged exposure (72h). However, this synergism was not exerted when all three mycotoxins were combined. This hypothesis should be further investigated since there is no confirmed data on the mechanism of genotoxicity for any of the three toxins on porcine lymphocytes.

Yang et al. (2014) investigated the expression of a heme oxygenase gene (HO-1) in human lymphocytes after exposure to DON. A reduction in the expression of HO-1 was observed and it was suggested that this could probably be related to the mechanism of action. Human lymphocytes which were exposed to FB_1 showed DNA strand breaks only at the highest concentration (i.e. 20 μg/ml) (Domijan et al., 2015). These results suggest that FB_1 exerts weak genotoxicity, which is in accordance with our results since FB_1 reached a maximum tail intensity of 12.8 %. Although it was stated that the exact mechanisms responsible for the genotoxicity of FB_1 are not clear, the authors suggested that oxidative stress could account for the genotoxic effects at least partially (Domijan et al., 2015). Oxidative stress mediated genotoxicity has been suggested by Theumer et al., (2010) but the authors could not explain the exact mechanisms for the observed antagonistic effect of FB_1 against aflatoxin B1. According to several authors, ZEN induces DNA damage through oxidative stress (Abid-Essefi et al., 2003; Bouaziz et al., 2013). However, Gao et al. (2013) suggested that oxidative stress does not play a key role in ZEN-induced DNA strand breaks on human embryonic kidney cell line, and they showed that the lysosome is the main determining factor for the ZEN-induced DNA strand breaks.

In conclusion, the mixtures of DON, FB_1 and ZEN exert mostly antagonistic effects on porcine lymphocytes, with the interactions being more evident after 72h of exposure. Further studies with additional endpoints (e.g. DNA fragmentation, protein synthesis) should be performed in order to elucidate the mechanisms underlying the interactions observed. Furthermore, an antioxidant agent such as vitamin E could be used in combination with these mycotoxins in order to assess whether oxidative damage plays a significant role in genotoxicity.

5. CONCLUSIONS AND RECOMMENDATIONS

Mycotoxins can affect farm animals in an adverse manner. Among the 400 metabolites characterised so far, *Fusarium* mycotoxins are the most frequently occurring worldwide. The combined effects of FB_1, 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 FB_1, DON and ZEN, only a few have investigated their ternary mixtures.

In our multi-toxic (FB_1, 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 FB_1 acted antagonistic against DON+ZEN on feed intake, lipid-peroxidation, and genotoxicity. All mycotoxins provoked moderate lipid-peroxidation and exerted slight genotoxicity.

Swine is the most sensitive species to DON, but little is known for rabbits. One of the main targets of mycotoxins is the liver thus a hepatoprotective 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 evident. Even though DON is known to possess immunomodulatory properties, these were not demonstrated as strongly (see later) as it has been reported. Gut cytokines and the phagocytic activity of the macrophages did not differ significantly. In agreement with the IARC classification of DON as non-carcinogenic to humans, DON did not exhibit genotoxicity effects. DON did not alter small intestine's morphology.

Despite the fact that in our study it was demonstrated that rabbits are resistant to DON, histopathological findings, haematological parameters and caecal

fermentation were negatively affected. The mild fibrosis induced in the DON-treated animals was inhibited by *C. marianus*, confirming thus its hepatoprotective effects. *C. marianus* was shown to exert spleen-protective effects as well, inhibiting the morphological changes induced by DON. As aforementioned, DON did affect slightly the immune system although these changes were not depicted in the overall health of the animals. In the gut-associated lymphoid tissue, the ratio of lymphoblast proliferation and simultaneous apoptosis shifted towards apoptosis. The percentage of some of the mononuclear cells was significantly different in the toxin-fed group.

Rabbits seem to be 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 spleen as well as the caecal fermentation.

In our series of experiments the cytotoxic and genotoxic effects of FB_1, DON and ZEN were investigated.

The IC_{50} value for each toxin after 24, 48 and 72h of incubation was determined. DON proved to be the more potent while FB_1 was the least, and 50% viability was achieved only after 72h of exposure.

The main interaction observed in the cytotoxicity experiments was antagonism. In the case of the ternary mixture, it was evident for all exposure times whereas for the binary mixture the interactions were either not consistent or time-dependent.

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 was antagonism. However, at higher concentrations, the antagonism was confirmed only for DFZ whereas synergism was observed for DZ and FZ. Interactions of DF were inconsistent in different exposure periods in both series of experiments. 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 FB_1 and/or DON.

Further studies with additional endpoints should be performed (e.g. DNA fragmentation, protein synthesis) in order to elucidate the mechanisms underlying the interactions observed.

6. NEW SCIENTIFIC RESULTS

1. The interactive effects of FB_1 (5 mg/kg), DON and ZEN (1 mg/kg + 0.25 mg/kg) in the feed on 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 FB_1 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 mg/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 DON in the feed.
4. The IC_{50} values of FB_1, 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 FB_1 50% viability was reached only after 72h and thus only one IC_{50} value -101.15 μg/ml- could be calculated. As it is depicted by the IC_{50} values, DON was the most cytotoxic whereas FB_1 was the least.
5. The interaction of FB_1 (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 72 h) manner.
6. The interaction of FB_1, DON and ZEN as investigated by the means of comet assay revealed antagonism in lower concentration (5 μg/ml for FB_1 and ZEN, while 0.07 μg/ml for DON) whereas for higher concentrations (25, 0.21 and 10 μg/ml for FB_1, DON and ZEN, respectively) a synergistic effect was revealed for two of the binary mixtures.

7. SUMMARY

Mycotoxins, the secondary metabolites of filamentous fungi pose a serious problem in animal farming. The main *Fusarium* mycotoxins include trichothecenes (T-2 toxin [T-2], deoxynivalenol [DON], nivalenol [NIV]), fumonisins (e.g. fumonisin B_1 [FB_1]) and zearalenone (ZEN). These fusariotoxins are very likely to co-occur but despite this fact their mixtures have not been studied as widely as the mixtures of aflatoxin B1 (AFB_1) or ochratoxin A (OTA).

A vast amount of studies investigating the effects of mycotoxins both *in vivo* and *in vitro* have been conducted. Legislation has been enacted around the globe setting maximum limits or guidance levels on single mycotoxins. However, it is a fact that single mycotoxin exposure is rather the exception than the rule. Co-occurrence is a fact due to global trade, compound feed that is fed to animals and the ability of several fungi to produce more than one mycotoxin.

In order to counteract the adverse effects of mycotoxins various strategies have been implemented. In traditional medicine plants have been widely used for thousands of years worldwide (e.g. China, India, South Africa). Nowadays, medicinal plants are often used to improve the health status of animals, especially after the banning of antibiotics in European Union. Oxidative stress is one of the main pathways for cell damage (cytotoxicity, genotoxicity, immunotoxicity) thus many plants that possess antioxidant properties have been used in studies investigating their possible protective effect against mycotoxins. *Carduus marianus*, a member of Asteraceae family, is known for its hepatoprotective as well as for its antioxidant effect.

The objectives of my studies were to assess the single and combined effects of FB_1, DON and ZEN *in vivo* (on rabbits) and *in vitro* (on porcine lymphocytes). Moreover, the possible protective effect of the medicinal plant *Carduus marianus* was assessed against DON.

Reproductive toxicity of *Fusarium* toxins were determined in rabbit bucks. The mycotoxins were administered orally at subchronic doses. The four treatments were: control (C, toxin-free diet), F (5 mg/kg FB_1), DZ (1 mg/kg DON+ 0.25 mg/kg ZEN), FDZ (5 mg/kg FB_1+ 1 mg/kg DON+0.25 mg/kg ZEN) for 65 days

(n=15/treatment). The doses were pre-determined according the EU limits in finished feed for young pig (in the absence of limits for rabbits' feed in case of DON and ZEN; based on the European Commission Recommendation 2006/576/EC).

The most pronounced effects of the toxins were exerted on the reproductive processes. The ratio of spermatozoa showing progressive forward motility decreased from 80% to 67% in the FDZ group by day 60. Differences were found between the groups DZ (66.3% ± 23.7) and C (80.2% ± 11.2) in spermatozoa morphology. GnRH treated animals produced less testosterone in FDZ animals, compared to the other three groups. In the comet assay the individual fumonisin treatment resulted in significantly less 0 comets (intact cells), compared to all others. Based on the prevalence of score, lower damage was observed in FDZ group, as compared to F and DZ. Among the mycotoxins studied, additive or less than additive effect was found in case of spermatogenesis and sperm cell morphology and synergism in testosterone production.

All mycotoxins provoked moderate lipid-peroxidation and exerted slight genotoxicity based on comet assay, FB_1 being antagonistic towards DON+ZEN. In F, DZ and FDZ animals the intensity of spermatogenesis decreased by 43, 31 and 64%, respectively, which was reflected by 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.

Results indicate that a prolonged low-dose mycotoxin exposure may adversely affect male reproduction. Reproductive processes show higher sensitivity to toxic effects, shown by the different parameters examined.

The aim of the second *in vivo* study was to investigate the effects of dietary deoxynivalenol fed at a high level (10 mg/kg of feed) solely or with the medicinal plant *Carduus marianus* (5 and 10 g/kg of feed) in growing rabbits. The examined parameters were productive performance, blood indices, immunological variables, histopathological changes, genotoxicity, caecal microbiota and fermentation.

After one week adaptation following weaning at 35 days of age rabbits received the following diets: control (C) diet, which was a non-supplemented basal

diet, control diet supplemented with the herb (*C. marianus*) in a concentration of 5 g/kg (H1) and 10 g/kg (H2), respectively. After 3 weeks each group was subdivided to two groups, and diet in every second group was supplemented with DON at 10 mg/kg (C, CT, H1, H1T, H2, H2T).

Despite its high concentration, DON did not affect any of the productive parameters. Liver and kidney function was not affected, as shown by the clinical chemistry indices. Conversely, in three rabbits the toxin caused mild fibrosis of the liver, without degenerative changes of the hepatocytes. No genotoxicity could be observed either. Gut cytokines and the phagocytic activity of the macrophages did not differ significantly. The percentage of neutrophils was significantly lower, whereas that of eosinophils was significantly higher in the toxin-fed group. DON did not cause significant changes in gut and villus morphology. In 4 out of the 6 DON treated animals, the ratio of lymphoblast proliferation and simultaneous apoptosis shifted towards apoptosis in the gut-associated lymphoid tissue. In the central part of the lymphoid follicles of the spleen, lymphocyte depletion and follicular atrophy could be detected. Regarding caecal fermentation there was no significant difference in total or individual volatile fatty acids. The number of the anaerobic bacteria was significantly higher in the toxin-treated groups (regardless the plant supplementation) in comparison to non-toxin groups. *C. marianus* exerted protective effects against the DON-induced liver fibrosis and lymphocyte depletion and follicular atrophy in spleen, whereas it showed no protective effect against apoptosis in Peyer's plaque.

It can be concluded that rabbits are less sensitive to DON, but the findings confirm that this *Fusarium* toxin is capable of modulating the immune response. In addition, the protective effect of *C. marianus* exerted in liver was confirmed and it was demonstrated that it can exert protective effects to the lymphoid organs.

In order to investigate the effects of FB_1, DON and ZEN on a cellular level, several *in vitro* experiments were conducted. The aims of these studies were to determine the IC_{50} (half maximal inhibitory concentration) values for each mycotoxin (after 24, 48 and 72h) and to investigate their combined effects in binary

(DON+ZEN: DZ, DON+FB_1: DF, FB_1+ZEN: FZ) and ternary (DFZ) mixtures using as endpoints cyto- and genotoxicity on porcine lymphocytes.

The potency of cytotoxicity of the three toxins in an increasing order was FB_1<ZEN<DON. The IC values' range were depending on the period of exposure 0.31-0.42 μg/ml and 16.6-22.9 μg/ml for DON and ZEN, respectively, while 101.15 μg/ml for FB_1 (50% of viability was reached only after 72h). The main interaction observed was antagonism regarding cytotoxicity.

Lower and higher sets of concentrations were used for the genotoxicity (comet assay) experiments. When lower concentrations were used antagonism was again the main interaction observed. However, at higher concentrations the antagonism was confirmed only for DFZ whereas synergism was observed for DZ and FZ. Interactions of DF were inconsistent in different exposure periods in both series of experiments.

Further studies with additional endpoints should be performed (e.g. DNA fragmentation, protein synthesis) in order to elucidate the mechanisms underlying the interactions observed.

8. ÖSSZEFOGLALÁS (Summary in Hungarian)

A mikotoxinok, amelyek a penészgombák másodlagos metabolitjai, súlyos állategészségügyi problémákkal hozhatók összefüggésbe. A fő *Fuzárium* mikotoxinok közé soroljuk a trichotecéneket (T-2 toxin [T-2], deoxinivalenol [DON], nivalenol [NIV]), a fumonizineket (pl. a fumonizin B1 [FB_1]) és a zearalenont (ZEN). Ezek a *Fuzárium* toxinok nagyon gyakran együttesen is előfordulnak, ennek ellenére az együttes hatásukról ismereteink nem olyan széleskörűek, mint az aflatoxin B_1 (AFB_1) vagy az ochratoxin A (OTA) esetében.

Nagyszámú tanulmány foglalkozik a mikotoxinok hatásaival, mind *in vivo*, mind pedig *in vitro* vonatkozásban. A törvényi szabályozás az egyes mikotoxinokra egyedileg érvényesek (önálló előfordulást feltételezve), meghatározva azok maximum határértékeit vagy az ajánlati értéküket, ugyanakkor ma már egyértelmű, hogy az együttes előfordulásuk sokkal gyakoribb. A mikotoxinok együttes előfordulását elősegíti a globális kereskedelem, a keveréktakarmányok alkalmazása az állattartásban és az a tény is, hogy számos penészgombafaj képes egyidejűleg több mikotoxint is termelni.

A mikotoxinok káros hatását számos módszerrel próbálták kivédeni. A tradicionális gyógynövények évezredek óta széleskörben használatosak világszerte (pl. Kína, India, Dél-Afrika). Manapság a gyógynövényeket gyakran alkalmazzák az állatok egészségügyi állapotának javítása érdekében is, különösen miután jelentősen korlátozták az antibiotikumok használatát az Európai Unióban. Az oxidatív stressz az egyik fő oka a mikotoxinok okozta sejtkárosodás (citotoxicitás, genotoxicitás, immunotoxicitás) kialakulásának, ezért számos, antioxidáns tulajdonságú növény protektív hatását vizsgálták már. A *Carduus marianus* az Asteraceae család tagja, ismert májvédő, illetve antioxidás hatású gyógynövény.

A kutatómunka során kitűzött céljaim a következők voltak: megállapítani a FB_1, DON és ZEN önálló, illetve kombinált hatását *in vivo* (házinyúlban) és *in vitro* (sertés limfocitákon). Továbbá célom volt meghatározni a *Carduus marianus* gyógynövény lehetséges pozitív hatását DON expozíciónak kitett nyulakban.

Vizsgáltuk három *Fuzárium* mikotoxin interakcióját és baknyulak reprodukciós teljesítményére kifejtett hatását. A mikotoxinok expozíciója szájon át,

szubkrónikus dózisban történt. Négy kezelést alkalmaztunk: kontrol (C, toxinnal nem szennyezett takarmány), F (5 mg/kg FB_1), DZ (1 mg/kg DON + 0,25 mg/kg ZEN), FDZ (5 mg/kg FB_1 + 1 mg/kg DON + 0,25 mg/kg ZEN), 65 napon keresztül (n=15/kezelés).

Az alkalmazott dózisok a DON és a ZEN esetében választott malacok takarmányára vonatkozó EU ajánlati értékek (2006/576/EC) alapján kerültek meghatározásra, tekintettel arra, hogy nyulakra vonatkozóan nincsen határérték ezekre a toxinokra. A FB1 esetében a nyúltakarmányokra megszabott 5 mg/kg ajánlott határértéket vettük figyelembe.

A progresszív előrehaladó motilitást mutató spermiumok aránya 80%-ról 67%-ra csökkent az FDZ csoportban a 60. napon. Különbség volt tapasztalható a DZ (66,3% ± 23,7) és C (80,2% ± 11,2) csoportok között a normál morfológiát mutató spermiumok arányában. GnRH kezelést követően az állatok alacsonyabb tesztoszteron szintet mutattak az FDZ csoportban, összehasonlítva azt a többi csoporttal. Comet assay vizsgálat (genotoxicitás) során az önálló fumonizin kezelés szignifikánsan kevesebb 0 értéket (sértetlen sejt) eredményezett, összehasonlítva a többi csoporttal. A mikotoxinok között additív, valamint az additív hatásnál gyengébb összefüggést igazoltunk a spermatogenezis és a sejtmorfológia esetében, míg szinergista hatást mutattunk ki a tesztoszteron szintézisnél.

Mindegyik mikotoxin mérsékelt lipid peroxidációt és enyhe genotoxicitást indukált (comet assay), az FB_1 és a DON+ZEN pedig antagonistáknak bizonyultak. Az F, DZ és FDZ csoportokban a spermatogenezis intenzitása 43, 31, illetve 64%-kal csökkent, melyet a differenciálódott spermiumok kisebb aránya, az elvékonyodott csírahám és a sokmagvú óriássejtek megjelenése jelzett.

Az eredmények egyértelműen igazolták, hogy egy viszonylag hosszú ideig tartó, alacsony dózisú mikotoxin expozíció negatívan befolyásolja a hím ivarú állatok szaporodási folyamatait, valamint, hogy az egyes mikotoxinok közötti kölcsönhatások változóak és nehezen előrejelezhetőek.

Egy másik *in vivo* kísérlet célja a magas dózisú (10 mg/kg takarmány) deoxinivalenol hatásának meghatározása volt, illetve annak vizsgálata, hogy a *Carduus marianus* gyógynövény (5 and 10 g/kg takarmány koncentrációban) kivédi-e a DON káros hatását választott nyulakon. A megvizsgált paraméterek a

következők voltak: termelési paraméterek, vér és immunológiai paraméterek, hisztopatológiás elváltozások, genotoxicitás, illetve vakbél mikrobióta és fermentációs paraméterek.

Egy héttel a 35 napos választást követően az alábbi takarmányozási csoportokat alakítottuk ki: kontroll (C) takarmányozási csoport, amely a korra jellemző toxinmentes alaptakarmányt fogyasztott, a kontroll takarmány kiegészítve *C. marianus* növénnyel 5 g/kg (H1), illetve 10 g/kg (H2) dózisban. Ezeket a takarmányokat az állatok 3 hétig fogyasztották, majd ezt követően minden csoportot további két csoportra osztottunk: az egyes alcsoportokban a takarmányt 10 mg/kg DON toxinnal egészítettünk ki (C, CT, H1, H1T, H2, H2T).

A viszonylag magas koncentráció ellenére a DON toxin nem okozott csökkenést egyetlen termelési paraméter esetében sem. A toxinnak nem volt negatív hatása a máj és vese funkcióira, amelyet a fiziológiás klinikai-kémiai paraméterek támasztottak alá. Ellenben három állat esetében a toxin enyhe fokú fibrózist okozott a májban, a májsejtek degeneratív elváltozása nélkül. Comet assay-vel az izolált limfocitákat vizsgálva nem volt génkárosodás megfigyelhető. A bél citokinek és a makrofágok fagocitáló aktivitása szintén nem mutatott szignifikáns különbséget.

A neutrofil granulociták aránya szignifikánsan alacsonyabb, míg az eozinofil granulociták hányada szignifikánsan magasabb értéket mutatott a toxinnal kezelt csoportban. A DON toxin nem okozott szignifikáns elváltozásokat a bélben és a bélbolyhok morfológiájában. A 6 DON toxinnal kezelt állat közül négyben a bélben a limfoblaszt proliferációjának és egyidejű apoptózisának aránya az apoptózis irányában tolódott el. A lép centrális limfoid állományában limfocita kiürülés és atrófia volt megfigyelhető. A *C. marianus* protektív hatását figyeltük meg a DON indukált máj fibrózis és a lép limfocita kiürülése, illetve az atrófia esetében. A jótékony hatása a Peyer-plakkok apoptózisában nem volt kimutatható.

A vakbél fermentáció során keletkező összes, illetve az egyes illó zsírsavak mennyisége és aránya nem változott szignifikánsan a kezelések hatására. Az anaerob baktériumok száma szignifikánsan magasabb volt a toxinnal kezelt csoportokban, összevetve a toxin-mentes csoportokkal.

Az eredmények alapján arra a következtetésre jutottunk, hogy a házinyúl kevésbé szenzitív a DON toxinra, de csökkentheti az állat immunválasz készségét. A *C. marianus* protektív hatást fejt ki a DON-nal szemben a lépben.

Számos *in vitro* kísérletet végeztem annak céljából, hogy meghatározzam a FB_1, a DON és a ZEN azon koncentrációját, amely 50%-kal csökkenti az élő sejtek számát (IC_{50} érték) 24, 48 és 72 órás expozíciót követően; továbbá hogy megvizsgáljam két toxin (DON+ZEN: DZ, DON+FB_1: DF, FB_1+ZEN: FZ), majd a három toxin (DFZ) kombinált hatását, sertés limfocitákon végzett cito-és genotoxicitás tesztekben.

A három toxin citotoxicitásának tendenciája emelkedő sorrendben a következő volt: FB_1<ZEN<DON. Az IC értékek az expozíciós idő függvényében is változtak; 0,31-0,42 μg/ml és 16,6-22,9 μg/ml értéket tudtunk meghatározni a DON és a ZEN esetében, külön-külön, míg 101,15 μg/ml koncentrációt a FB_1 tekintetében (50%-os életképesség csökkenés csak 72 h expozíciót követően volt elérhető).

A koncentrációk alacsonyabb, majd magasabb tartományú sorozatát vizsgáltam a genotoxicitásra irányuló kísérletekben (comet assay). Alacsonyabb tartományú koncentrációk alkalmazása során a mikotoxinok között antagonizmust tapasztaltam, míg magasabb koncentrációk esetében az antagonizmus csak a DFZ csoportban volt megfigyelhető; míg a DZ és FZ kezelésekben szinergizmust mutattam ki. A DF esetében az interakciók nem voltak következetesek a különböző expozíciós periódusokban és a különböző koncentráció tartományokban.

További vizsgálatok javasoltak (pl. DNS fragmentáció, protein szintézis) annak érdekében, hogy tisztázzuk az interakciókat meghatározó mechanizmusokat.

9. ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor Professor Melinda Kovács for trusting this project with me and for the opportunities she gave me to visit other research laboratories in Europe and Africa. Without her, this PhD and its completion would not have been a reality. Furthermore, I would like to thank my co-supervisor Dr Judit Szabó-Fodor for her invaluable help and advice in experiments, publishing, and at a personal level as well. I would also like to thank Mrs Zsófia Bodnár Blochné for training me and helping me in practical matters of laboratory work. Another colleague that I would like to thank is Dr Katalin Horvatovich who also helped me in laboratory work with her suggestions or in practice. Dr Bónai András was another important person who helped me a lot with statistical analysis, translations of emails in Hungarian but also with his psychological support.

I would like to give special thanks to colleagues from the Norwegian Institute for Air Research, Professor Dusinska Maria and Dr El Yamani Naouale for training me and advising me whenever I needed it. Furthermore, I woould like to express my gratitude to Professor Segvic-Klaric Maja and Dr Jaksic Daniela from the University of Zagern for accepting my visit and share with me invaluable knowledge and resources without which I would not be able to complete my Thesis.

I would like to thank the Hungarian governmet for giving me the opportunity to fulfill my PhD studies by supporting me throughtout the three years with the state scholarship. I would also like to thank the Hungarian Academy of Sciences for the financial support of my research in frame of the MTA-KE Mycotoxins in the Food Chain Research Group. The research and publications were partly supported also by the GINOP-2.2.1-15-2016-00021 and EFOP-3.6.3-VEKOP-16-2017-00008 projects. The projects were co-financed by the European Union and the European Social Fund.

In this journey, I had the opportunity to meet wonderful people from many countries all over the world who helped me and supported me during my hard times here. I cannot mention all their names so instead, I am going to refer to their countries. In alphabetical order I am thanking my brothers and sisters from Algeria,

China, Ghana, Greece, Italy, Kazakhstan, Kenya, Namibia, Philippines, Spain, Sudan, Tunisia, Turkey, Ukraine and Vietnam.

I would like to thank my family who has supported during my PhD studies like the rest of my life and was encouraging me whenever I was losing faith to myself. I could not do it without you Sabrin, Froso and Chedli. Last but not least I want to thank my future husband Aris, for his encouragement and support by being there with me literally every day.

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

11.1 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)

11.2 Peer-reviewed papers published in Hungarian scientific journal 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 B_1 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 the *Fusarium* mycotoxins, fumonisin B_1, deoxynivalenol and zearalenone: A review. Állattenyésztés és Takarmányozás, 66, 3: 181-195.

11.3 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, 25th May, 2016, pp. 71-76. (ISBN:978-615-5599-30-9)

11.4 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.

12. OTHER PUBLICATIONS

12.1 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 B_1 in different matrices after *in vitro* digestion. World Mycotoxin Journal, 8 (3), 261-267.

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12.2 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.

12.3 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 B_1 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.

12.4 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.

13. CURRICULUM VITAE

I was born in Athens on the 10th of December in 1988 but attended nursery and partly primary school in the home town of my mother, Paramithia. I obtained my High School Diploma in 2006 form the 1st General High School of Igoumenitsa, Thesprotia, Greece.

Through the national exams, I was admitted at the Agricultural University of Athens. In 2012, I visited Kaposvár University as an exchange student within the frame of Erasmus exchange program in order to carry out a 3-month practical training at the Department of Physiology, Hygiene and Animal Health. On June of 2013, I obtained my degree in Agricultural/Animal Science with a grade of 7.61 out of 10. On September of the same year, I took the entrance examination to the Doctoral School of Animal Science of Kaposvár University.

Since 2013, I have pursued my doctoral studies at the Faculty of Agricultural and Environmental Sciences of Kaposvár University in the framework of a PhD programme in Animal Science. In 2014 I spent three months in South Africa, 2 months at the University of Johannesburg and one month at Rhodes University. In 2015, I spent 3 weeks at the Norwegian Institute for Air Researcgh. Last but not least, I spent one week at the University of Zagreb in November of 2015. I obtained my final pre-degree certificate in November of 2016.

In December of 2017 I successfully carried out the pre-defense.

I am working as a research assistant of the Mycotoxins in the Food Chain Research Group since September 2016.