An investigation of mycotoxin induced damage and remediation strategies in porcine intestinal cells

A thesis submitted for the degree of Doctor of Philosophy

by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of **Doctor of Philosophy** is entirely my own work, and that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of work.

Signed: Ashmita Thapa

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Mischief Managed!

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Abbreviations

α	Alpha
α-ZOL	α-Zearalenol
15–ADON	15-Acetyldeoxynivalenol
3-ADON	3-Acetyldeoxynivalenol
AB	Alamar blue
AF	Aflatoxins
AFB1	Alfatoxin B1
AFB2	Alfatoxin B2
AFG1	Alfatoxin G1
AFG2	Alfatoxin G1
ALA	α-Linolenic acid
ARA	Arachidonic acid
ARS	Agriculture Research Service
β	Beta
β-ZOL	β- Zearalenol

C	Carbon
CAST	Council for Agricultural Science and Technology
Cd	Cadmium
CFDA-AM	5-Carboxyfluorescein diacetate acetoxymethyl ester
CHI	Chitosan
CI	Combination index
CMC	Carboxymethylcellulose
CSREES	Cooperative State Research Education and Extension Service
DON-3G	Deoxynivalenol-3-glucoside
DGLA	Dihomo-y-linolenic acid
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
DOM-1	Deepoxy-deoxynivalenol
DON	Deoxynivalenol
EC	European Commission
EFSA	European Food Safety Authority
EndoIII	Endonuclease III

EPA	Eicosapentaenoic acid
ER	Oestrogen receptors
ESCs	Endometrial stromal cells
EtOH	Ethanol
EU	European Union
FA	Fatty acids
FAO	Food and Agriculture Organisation
FDA	Food and Drug Administration
FHB	Fusarium head blight
FPG	Formamidopyrimidine DNA glycosylase
FX	Fusarenon X
GSH	Glutathione
GSHPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
H_2Se	Dihydrogen selenide
HCl	Hydrochloric acid
Hg	Mercury

HPMC	Hydroxypropyl methylcellulose
IARC	International Agency for Research on Cancer
IC (50)	Half maximal inhibitory concentration
IECs	Intestinal epithelial cells
LA	Linoleic acid
LDH	Lactate dehydrogenase
MCC	Microcrystalline cellulose
MDA	Malondialdehyde
mRNA	Messenger ribonucleic acid
My-A+	Mycosorb A+
n-3	Omega 3
n-6	Omega 6
NAC	N-acetylcysteine
NaOH	Sodium hydroxide
NIV	Nivalenol
OTA	Ochratoxin A
OTB	Ochratoxin B

OTC	Ochratoxin C
PACA	Partnership for Aflatoxin Control in Africa
Pb	Lead
pH	Log of the hydrogen ion concentration
PUFAs	Polyunsaturated fatty acids
ROS	Reactive oxygen species
SCGE	Single-cell gel electrophoresis
Se	Selenium
Se-C	Selenocysteine
Se-M	Selenomethionine
Se-Na	Sodium selenate
Se-Ni	Sodium selenite
Se-Y	Selenium Yeast
TEER	Trans-epithelial electrical resistance
TrXR	Thioredoxin reductase
TUNEL	Terminal deoxynucleotidyl tranferase nick-end labelling
US	United States

USDA	United States Department of Agriculture
WHO	World Health Organisation
YCW	Yeast cell wall
ZEN	Zearalenone

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Abstract

An investigation of mycotoxin induced damage and remediation strategies in porcine intestinal cells

Asmita Thapa

Mycotoxins are naturally occurring secondary metabolites, produced by fungal species, and can be toxic to both humans and animals when consumed. Deoxynivalenol (DON) is one of the most commonly occurring mycotoxins and is found to be a common contaminant of cereal grains that are consumed by humans and animals. Consumption of DON contaminated feed can result in vomiting, refusal of feed and reduced weight gain. Zearalenone (ZEN) is an oestrogenic mycotoxin that has been shown to have a negative effect on the reproductive function of animals. The structure of ZEN resembles that of naturally occurring oestrogens, which allows it to bind to oestrogenic receptors, resulting in hormonal disturbances. It has been shown that pigs are most susceptible to both DON and ZEN toxicity through their feeds. The European Food Safety Authority (EFSA) has suggested that the maximum level DON in pig feed should not exceed 0.9 ppm and ZEN in feed for sows and fattening pigs should not exceed 0.25 ppm. DON and ZEN are commonly found to co-occur as both are produced by the *Fusarium* species. Their common co-occurrence makes it a critical issue in the agriculture industry. Organic selenium yeasts are frequently used as an animal feed supplement as it has a positive impact on animal health. Mycotoxin binders that reduce the amount of mycotoxin absorbed by animals are also used as supplements to animal feeds. In this thesis, the effect of DON and ZEN, individually and combined, on the cell viability, DNA damage and apoptosis of porcine intestinal epithelial (IPEC-J2) cells was studied. Additionally, the potential ameliorative effects of organic selenium yeast and polyunsaturated fatty acids from a mycotoxin binder (Mycosorb A+) against mycotoxin-induced damage was investigated. This research illustrates the damaging effects of the co-occurring mycotoxins and a potential mitigation strategy against such damage.

Chapter 1: Introduction

1.1 Introduction to mycotoxins

1.1.1 What are mycotoxins?

It has been estimated by the Food and Agriculture Organisation (FAO) of the United Nations that at least 25% of the world's crops are contaminated by mycotoxins each year [1]. This is of global concern, as ingestion of mycotoxin or mycotoxin contaminated food can result in the accumulation of the toxin in organs or tissues which is a cause of concern due to their immunosuppressive and carcinogenic effects [2]. Mycotoxins are structurally diverse, low molecular mass, toxic fungal metabolites [3,4]. They are naturally occurring secondary metabolites produced by moulds and are further defined as toxins produced by fungi that are harmful in low concentrations [3–5]. They are often found in many staple foods including maize, cereals and nuts. Mycotoxins make their way easily into the food chain as they contaminate and have toxic effects on animal feed, raw materials for these feeds and human food [3,5,6] .

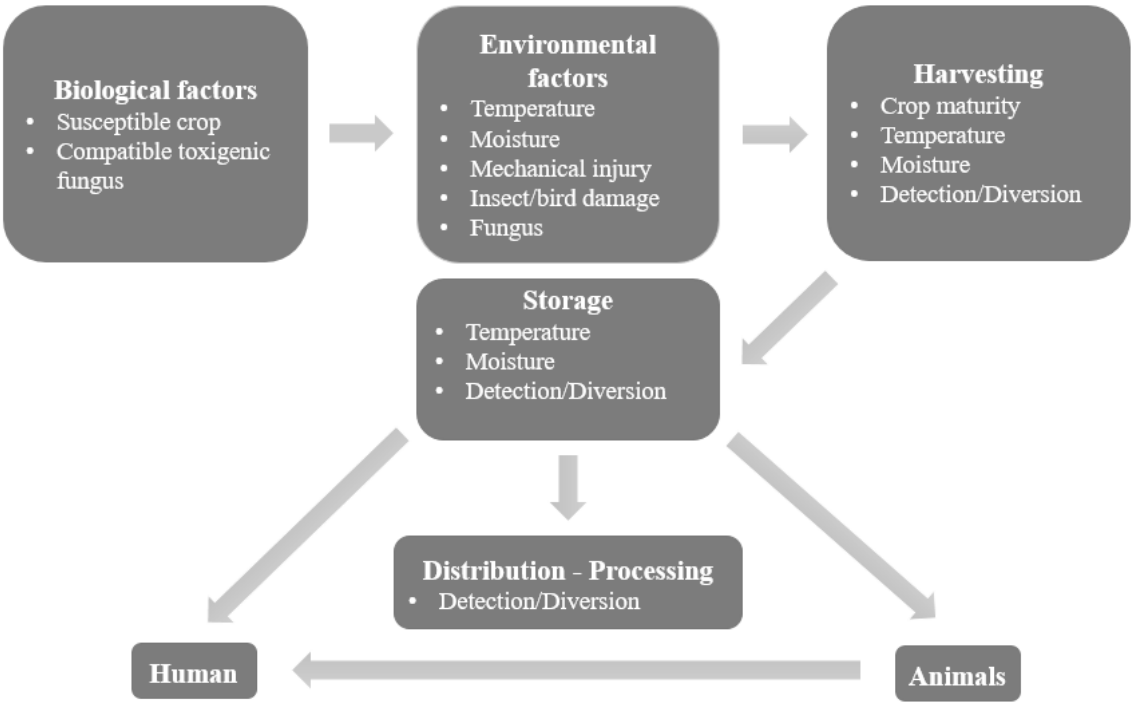

Figure 1.1: *Factors that affect the occurrence of mycotoxins in food and feedstuff adapted from Pestka and Casale 1990 and Bryden 2012* [7,8]

Chemical, physical and biological factors can affect the growth and production of mycotoxins. Physical factors include moisture content, rainfall and temperature, meaning that weather plays a big a role in the growth of mould and the resultant production of

mycotoxins. Chemical factors can include carbon dioxide, oxygen content and pesticide concentrations. Plant variety, stress and insects are some of the biological factors that may play a role in mycotoxin production [7]. Production of mycotoxin can occur during harvest, in the field and even during storage if the right conditions are met [9]. Figure 1.1 illustrates some of the factors that affect the occurrence of mycotoxins in food and feedstuff [7].

1.1.2 Mycotoxin impacts

Mycotoxin contamination of food, feed and raw products is a growing cause of concern due to their negative effects on animals and human health [4,10,11]. It has been estimated by the FAO that around 25% of the world's food crops are contaminated with mycotoxins [1]. Some of these mycotoxins are known carcinogens such as aflatoxin B1 (AFB1) which is carcinogenic in humans [12].

As well as detrimental health impacts, economic losses are also a huge issue as a result of mycotoxin contamination. Economic losses include different types of costs such as loss of contaminated feed, equipment required for the cleaning and sorting to reduce mycotoxins, health costs and reduction of animal productivity [7]. It was announced by the Partnership for Aflatoxin Control in Africa (PACA) that in Africa there is a loss of $670 million annually due to aflatoxin contamination [13].

In the United States (US), it was estimated by the Food and Drug Administration (FDA) in 2003 that the average economic costs of crop losses due to aflatoxins, fumonisins and deoxynivalenol to be $932 million annually [14]. In the Council for Agricultural Science and Technology (CAST) 2003 report, it was estimated that the annual losses in the USA ranged from $0.5 million to over $1.5 billion due to aflatoxin (AF) contamination in corns and peanuts, fumonisin in corn and deoxynivalenol (DON) in wheat [15]. In wheat, the majority of the loss is due to fusarium head blight (FHB) resulting from the presence of DON. In 1933, there was a loss of $200 to $400 million due to the infection. In 1996 again based on US data, $300 million was lost to farmers again due to this infection [16].

Costs are also incurred due to investments in research programs to prevent and mitigate mycotoxin contamination in crops. In the year 2000, approximately $17.7 million was spent on research focused towards the prevention of mycotoxin production in crops by the United States Department of Agriculture (USDA) Agricultural research service

(ARS). According to the USDA's Cooperative State Research Education and Extension Service (CSREES) report, $4.7 million was spent on mycotoxin research in 2003 [15,16].

The values above demonstrate the impact of mycotoxin in the world. It shows that it is important to address this issue and that more research is required in mitigating this problem. The importance of the issue has been highlighted in a press release by the International Agency for Research on Cancer (IARC) and World Health Organisation (WHO) in 2016. The chair of the IARC working group stated that due to mycotoxins "more than 160 million children younger than 5 years are stunted" [17].

1.1.3 Characterisation of mycotoxins by fungal species source

There are many different ways to group mycotoxins, for example, they can be grouped by the organs that they affect or the illness that they cause. Most commonly, they are grouped by the fungus from which they are produced [18]. The main types of fungi that produce mycotoxins, namely *Aspergillus*, *Fusarium* and *Penicillium*, are shown in Table 1.1 [19].

Table 1.1: *Mycotoxins, fungal species that produce them and their health effects* [20–24]

Mycotoxin	Fungal Species	Human health effects
Deoxynivalenol	*Fusarium*	Growth stunt, reproductive disorder, vomiting, feed refusal
Zearalenone	*Fusarium*	Hormonal imbalance, oestrogenic effects
Aflatoxins	*Aspergillus*	Haemorrhage, liver damage carcinogenesis, gastrointestinal dysfunction, anaemia, jaundice, reduced reproductivity
Ochratoxins	*Aspergillus*, *Penicillium*	Carcinogenesis, nephrotoxicity

Mycotoxins can infect crops both during, before and after harvesting. *Fusarium* is a field fungus, producing mycotoxins on field crops before harvesting [25]. Fusarium head blight is one of the main fungal diseases that affects wheat, barley, maize and other small grains. As well as producing trichothecenes, a major group of mycotoxins, *fusarium* is also responsible for the production of Zearalenone (ZEN), T-2 and HT-2 toxins [26–28]. *Fusarium* contamination of cereal grains and animal feed is a cause of concern as ingestion of contaminated feed can result in mycotoxicoses [25]. *F. gremanarium* is the predominant FHB species and produces the mycotoxins zearalenone and deoxynivalenol [26,29].

In contrast, *Aspergillus* is a problematic fungus during storage, as it infects crops after they have been harvested [25]. Members of the *Aspergillus* family, *A. flavus and A. parasiticus* produce one of the most toxic mycotoxins, aflatoxin [30]. Aflatoxin has been identified as harmful since the 1960s, after the death of 100,000 turkeys, in an incident that has become known as the turkey X disease. The turkeys died after the consumption of groundnuts that were contaminated with *A. flavus*, which resulted in an acute necrosis of the liver [18,31,32].

The mycotoxins ochratoxin A (OTA) and patulin are produced by members of the *Aspergillus* and *Penicillium* genus, and can contaminate grains both prior to harvest or more commonly during storage [33,34]. OTA, which is mainly found in wheat, barley and rye, is produced by *P. verrucosum* and A. *ochraceus*, [35]. *P. expansum* and *P. griseofulvum* are the producers of the neurotoxic and immunotoxic mycotoxin Patulin [36].

1.2 Chemistry and effects of some major mycotoxins

There are hundreds of known mycotoxins [11,37]. From these, AFs, ochratoxins, ZEN and trichothecenes such as DON are commonly found to contaminate cereal grains, thus resulting in their presence in animal feed [37,38]. These mycotoxins can vary in their chemistry, occurrence and effects they can have on humans and animals [11].

1.2.1 Aflatoxins

AFs are toxic metabolites produced from the fungal species *A. flavus* and *A. parasiticus* and are potent liver toxins [3,32]. These toxins infect cereals crops, nuts, spices and oilseeds [39,40]. Infections can occur during harvest or storage. Warm conditions are favoured for the production of aflatoxins, with the optimum temperature for growth being 33 °C [41]. There are four major aflatoxins, denoted by AFB1, AFB2, AFG1 and AFG2 and these are known by their fluorescing properties. AFB1 and AFB2 fluoresce blue under ultraviolet light, whereas AFG1 and AFG2 fluoresce yellow-green [19]. Their structures are shown in Figure 1.2. AFB1 and AFG1 have been identified as being carcinogenic and more toxic than the other two, with AFB1 being more toxic than AFG1 [42].

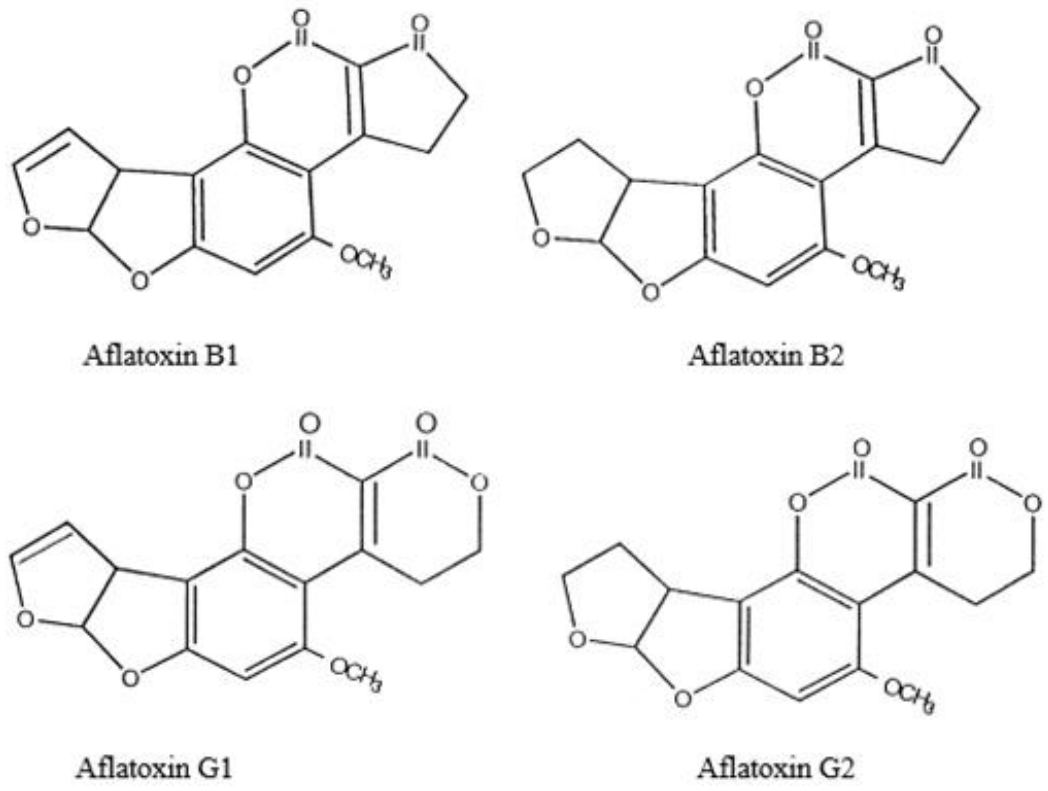

Figure 1.2: *Structures of the four major aflatoxins; AFB1, AFB2, AFG1 and AFG2* [42].

AFs are highly liposoluble compounds [43] and due to this are difficult to remove by washing [44]. They are generally toxic to the liver and AFB1 is the most harmful. They are also teratogenic, carcinogenic and immunosuppressive compounds [38].

1.2.2 Ochratoxins

Ochratoxins are a group of mycotoxins produced by *Aspergillus* and *Penicillium* and are found in maize, dry beans, wheat, barley, oats and grapes [2,34].The main forms of this group are OTA, ochratoxin B (OTB) and ochratoxin C (OTC), from which OTA is both the most common and the most toxic [2,45]. It is a highly stable compound that can withstand temperatures as high as 250 °C and therefore is not entirely removed during everyday food preparation methods [2,46]. OTA is known for its nephrotoxic effects to both human and animals and is also classified as a carcinogen in animals and a Group 2b possible carcinogen in humans by the IARC [35,47,48]. OTB is an unchlorinated form of OTA and OTC is an ethyl ester of OTA [45]. OTB is less toxic than OTA but they often co-occur [49]. Their structures are shown in Figure 1.3.

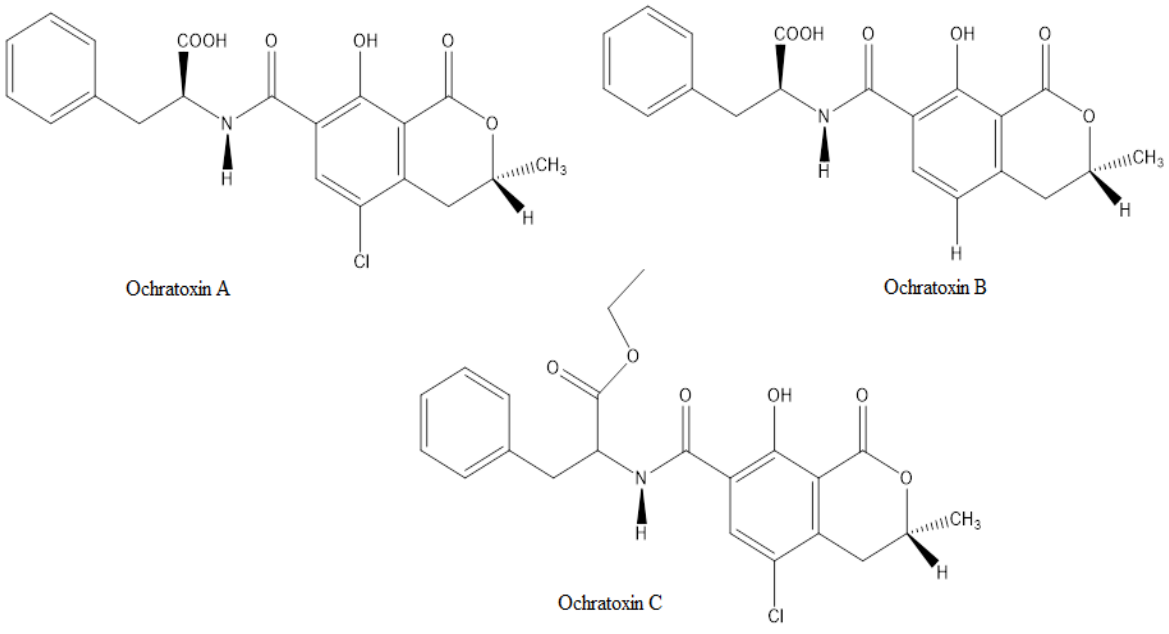

Figure 1.3: *The structure of the different types of Ochratoxins; OTA, OTB and OTC* [50].

1.2.3 Trichothecenes

Trichothecenes are one of the biggest groups of mycotoxins and are produced by various fungal species, including *Fusarium, Trichothecium, Myrothecium* and *Stachybotrys* [21,51]. They are a group of structurally related mycotoxins and all have a tetracyclic

sesquiterpenoid 12,13-epoxytrichothec-9-ene ring. There are four types of trichothecenes, shown in Figure 1.4, divided by their chemical structures and the functional groups present, type A, B, C and D. Trichothecenes all have a C9,10 double bond and an epoxide group at C12,13 in common [52].

Type A trichothecene includes T-2 toxin and HT-2 toxin amongst others [51,53]. Structurally, type A trichothecenes differ from type B as they do not contain a carbonyl in the C-8 position. Type A trichothecenes are highly toxic, especially T-2 toxin, which is amongst the most toxic. [53]. It can have harmful effects on poultry, ruminants and swine resulting in weight loss, haemorrhage, necrosis, decreased blood cell count, deoxyribonucleic acid (DNA) damage and induction of apoptosis [54].

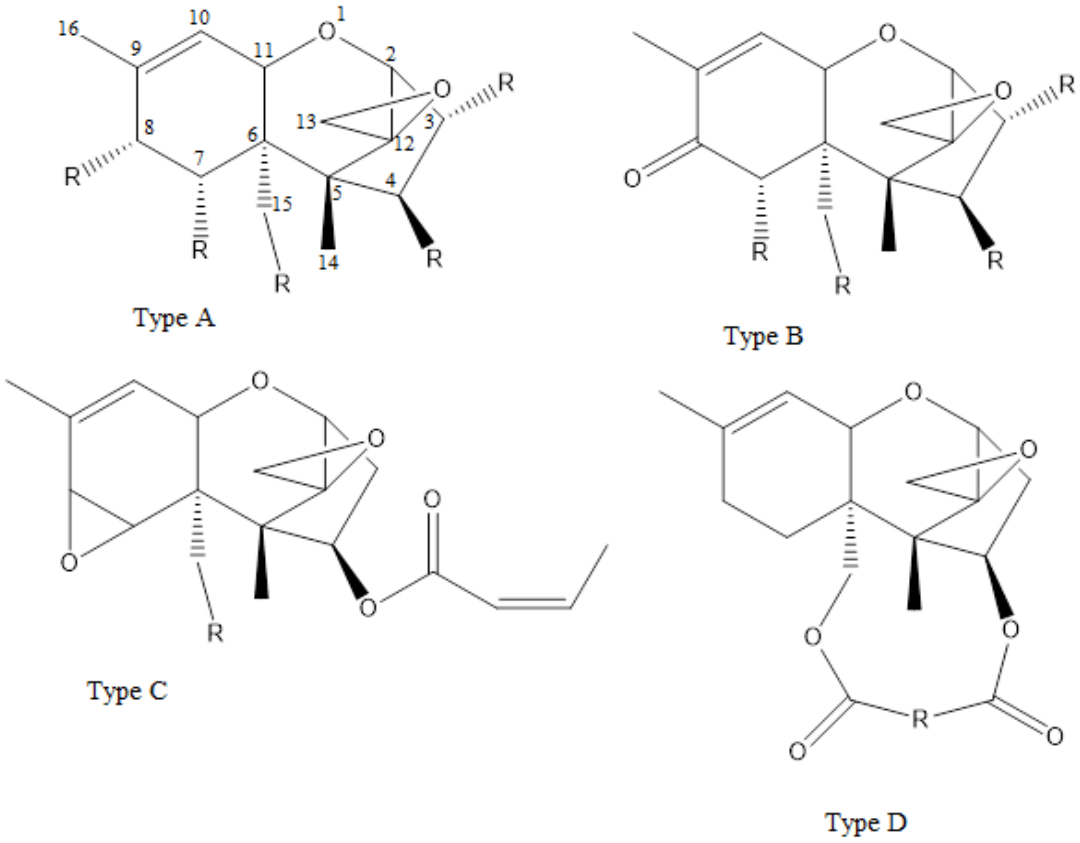

Figure 1.4: *The structure of the four types of trichothecenes Type A, B, C and D. Type A does not have a carbonyl group that is present in the C-8 position of type B trichothecene. Type C has an additional epoxide at C7/C8 and Type D has a ring at the C4/C15 position* [51].

A keto-carbonyl group is present at the C8 position of type B trichothecenes. *Fusarium* type B compounds have a hydroxyl group in the C-7 position, represented mainly by DON and nivalenol (NIV) [51,55]. These mycotoxins have many toxicological effects such as depression, nausea, feed refusal, vomiting and skin irritability [56].

Type C trichothecenes are non-*Fusarium* trichothecenes and have a second epoxide at the C7/C8 position. An example of a Type C trichothecene, which is less common than the other types, is crotocin [51,53]. A macrocyclic ring linking the C4 to the C15 position makes the Type D trichothecene more distinguishable from the others [51]. Satratoxins, verrucarins, myrotoxins and roridins are some examples of Type D trichothecenes [51,53].

ZEN and DON are two commonly occurring mycotoxins [11,38]. They will be discussed in more detail in the next section 1.3 and 1.4 as they are the focus of this thesis. ZEN and DON were chosen for this project as they have often been found to contaminate animal feed and can result in detrimental effects on animal health. Additionally, as they are both produced by the same *Fusarium* species, they are often found to co-occur and co-contaminate grains [19,38]. Thus, these mycotoxins were chosen for this study in order to investigate their individual and combined effects on porcine intestinal epithelial cells.

1.3 Zearalenone

1.3.1 Structure and mode of action

ZEN, shown in Figure 1.5, is one of the most important *Fusarium* mycotoxins, produced by several species including *F. graminearum, F. culmorum, F. cerealis* and *F. equiseti* [57,58]. It is a non-steroidal oestrogenic mycotoxin found mainly in corn, wheat, oats, barley and sesame seeds [57,59]. The production of ZEN is greatest at cool temperatures and high humidity [14]. It is a stable compound that does not degrade during storage and food preparation or at high temperatures [60].

ZEN has a resorcyclic acid lactone structure and is similar to that of naturally occurring oestrogens such as 17-β-oestradiol. As a consequence, ZEN mimics the activity of oestrogens and binds to mammalian oestrogen receptors (ER) and is therefore classified as a xenoestrogen [61]. Due to its ability to bind to ERs, consumption of feed contaminated with ZEN results in the disruption of hormonal balance and perturbance of the reproductive system in farm animals [23,62]. ZEN is also known to be hepatotoxic, genotoxic and can cause immunosuppression [63]. It has been classified as group 3 (‘*not classifiable as to its carcinogenicity to humans*) by the IARC [64].

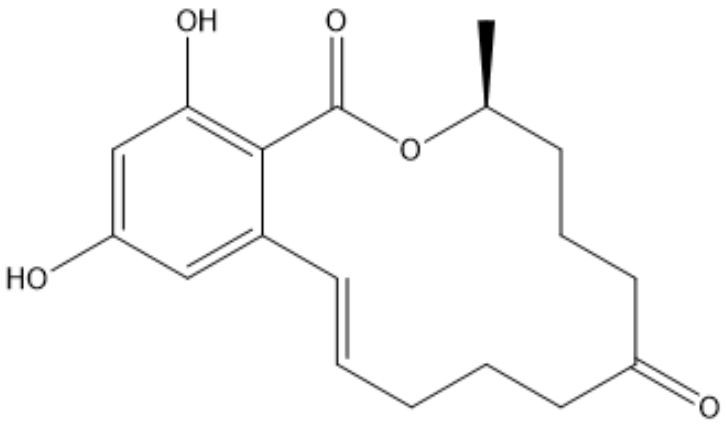

Figure 1.5: *The chemical structure of zearalenone* [19]

Following oral exposure, ZEN is absorbed rapidly and is metabolised in the liver. There are two major pathways for the biotransformation of ZEN by animals [65]. Biotransformation via hydroxylation results in the formation of the two major metabolites of ZEN, namely α-zearalenol (α-ZOL) and β- zearalenol (β-ZOL), which are believed to be catalysed by 3α- and 3β-hydroxysteroid dehydrogenases. The second pathway is the

conjugation of ZEN and its reduced metabolites with glucuronic acid and sulphate resulting in the formation of the metabolites ZEA-14-O-glucoside, ZEA-16-O-glucoside and ZEA-14-sulphate [23,59,62,63,65].

α-ZOL and β-ZOL, are also produced by *Fusarium* at much lower concentrations than ZEN and differ in their binding affinities to ERs [66]. α-ZOL is known to have a higher affinity for ERs than ZEN and its other metabolites [67]. Due to this ZEN is more reactive and toxic in species where the biotransformation to α-ZOL is preferred [68]. The metabolism of ZEN is highly dependent on the animal species. Pigs are more susceptible to ZEN toxicity than any other domestic animals due to the fact that the toxin is mainly metabolised to α-ZOL. In contrast, ZEN is mostly converted to β-ZOL in cattle thus making them less susceptible to its toxicity [66,69].

1.3.2 Occurrence of ZEN

ZEN is commonly found contaminating cereals in warm and temperate climates. In the Republic of Serbia, ZEN was detected in 12%, 37%, 100% and 53% of maize samples in 2012, 2013, 2014 and 2015, respectively. The weather conditions for each year were also analysed in the same study and it was found that the detection of ZEN in all of the 2014 samples correlated with extreme wet weather conditions that year [70]. Another study also showed the presence of ZEN in Romanian wheat was found to be more elevated with higher amounts of rainfall [71]. In a ten-year study (2008 – 2017), samples of feed and feed raw materials were collected from 100 countries and analysed for the presence of mycotoxins. The study showed a presence of ZEN in 45% of the total 61,413 samples tested. The study also showed that there was a variation in ZEN concentrations each year and it was suggested that the variation might have corresponded with the amount of rainfall [72].

The occurrence of ZEN in various food products has been noted in several studies. In a Turkish study, ZEN was found in 4% of 50 wheat samples, 20% of 15 maize samples, 55% of 20 paddy rice samples and 4% of 50 wheat flour samples [73]. The occurrence of ZEN in corn meal that was produced and commercialised in Brazil, found that of the 84 samples that were examined, 78.6% (66) tested positive for ZEN [74]. In India, samples of corn, rice, wheat and oats from local markets were tested for ZEN. It was found that 84% of the 117 samples were contaminated with ZEN, with 33% of the samples exceeding the EU permissible limit (Table 1.2) [75]. In another study, 43% of maize kernel samples from

Poland (2011 and 2012) tested positive for ZEN [76]. The limits for the presence of ZEN within the European Union (EU) are described by the Commission Regulation (EC) and these values are highlighted in Table 1.2. The European Food Safety Authority (EFSA) collects and examines the occurrence data on mycotoxins in both food and feed. They provide scientific advice on setting the maximum levels in food and feed by evaluating the risk posed to humans and animals. Exposure of mycotoxins to specific populations and groups of humans (children, infants or adults) and animals (different species) are also analysed by appointed expert panels. The EU commission then sets maximum levels for mycotoxins in food and feed following the advice and information provided [77].

Table 1.2: *Maximum levels of ZEN in various foodstuffs. Table adapted from Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs* [78]

Foodstuffs	Maximum level (µg/kg)
Unprocessed cereals other than maize	100
Unprocessed maize except for unprocessed maize intended to be processed by wet milling	350
Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption	75
Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize-snacks and maize-based breakfast cereals	50
Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	100
Processed cereal based foods and baby foods for infants and young children	20

1.3.3 Toxic effects of ZEN

The toxicity of ZEN has been studied on various cell lines, especially its cytotoxicity, genotoxicity and induction of apoptosis. A study investigated the effect of ZEN on IPEC-J2 cells by incubating cells with ZEN for 24 hours. The results showed that there was a decrease in cell viability at ZEN concentrations of 10 -100 µM. ZEN was also shown to inhibit cell proliferation at 40 µM resulting in decreased cell count [79]. A concentration dependent decrease of cell viability was also observed when Caco-2 cells were incubated with ZEN (1-150 µM) for 72 h. The neutral red test and the MTT assay was used to

measure cell viability in this study and it was found that ZEN has an effect on both the membrane integrity and the mitochondrial metabolism of Caco-2 cells [80]. These results correlate with another study where the cytotoxicity of ZEN was analysed on HepG2 cells. The cells were incubated with increasing concentrations of ZEN (1-200 µM) for 24 h and the MTT assay was used to measure cell viability in this study. The results of the assay showed a dose-dependent decrease in cell viability where the cell viability was 48% at 100 µM and 11% at 200 µM ZEN [81]. HepG2 cells were used in a different study where the cytotoxicity of ZEN and its two metabolites α-ZOL, and β-ZOL was analysed by the neutral red assay. The results of this study also showed a dose-dependent decrease of cell viability and β-ZOL was found to be the most toxic, based on the half maximal inhibitory concentration (IC50) values, followed by ZEN and α-ZOL [82].

Elsewhere, it was shown that ZEN was cytotoxic at high concentrations but at low concentrations, it increased cell proliferation. Human colon carcinoma cell line HCT116 was treated with increasing concentrations of ZEN for 120 h and cell proliferation was measured. After 120 h of exposure, ZEN enhanced cell proliferation at low concentrations between 1 nM to 1 µM. To measure cell viability, the cells were treated with ZEN (0 - 320 µM) for 48 h. The blue methylene assay showed that there was a dose-dependent inhibition of cell viability with concentrations higher than 20 µM [83]. A similar trend was observed in another study looking at the effect of ZEN in IPEC-J2 cells. After 48 hour incubation with ZEN, there was a significant increase in cell viability at 10 µM, which was followed by a significant decrease in viability when the concentration of the toxin was increased to 40 µM [84]. These studies have shown that the low doses of ZEN can result in increased cell proliferation but high doses of the mycotoxin can result in decreased cell viability and cell death.

The genotoxicity of ZEN has been studied on various cell lines. In a study investigating DNA damage induced by ZEN, HEK293 cells were exposed increasing concentrations of ZEN (0 -20 µM) for 2 h. The comet assay was used to detect DNA strand breaks in the cells. The results of the assay showed that there was an increase in DNA damage, indicated by the percentage of DNA in the tail of the comet, with increasing concentrations of ZEN. Percentage tail DNA for cells incubated with 0, 10 and 20 µM ZEN was 2.27 ± 1.17, 5.44 ± 4.64 and 14.64 ± 4.25 respectively [85]. In another study the comet assay and the gel electrophoresis assay was used to examine the induction of DNA

damage by ZEN on the SH-SY5Y human neuroblastoma cell line. The tail length of the comet was used to measure the level of DNA damage in the cell. An increase in tail length was found with increasing concentration of ZEN. The cells exposed to 0, 25 and 100 µM ZEN had a tail length of 10.0 ± 1.5, 31 ± 2.6 and 83 ± 5.3 µm respectively. The gel electrophoresis assay used in the study also showed a ladder pattern as an indication of DNA fragmentation [86]. Similarly, in another study it was found that ZEN induced oxidative DNA damage in HepG2 cells by using the comet assay with repair enzymes [87].

Apoptosis is defined as programmed cell death and ZEN has been shown to induce cell apoptosis. Induction of apoptosis by ZEN was detected by flow cytometric analysis in a bovine mammary epithelial cell line. Cells were exposed to ZEN for 24 hours and the results showed that there was an increase in the number of apoptotic cells in the ZEN treated cells when compared to the control cells [88]. Elsewhere ZEN was found to induce apoptosis in porcine granulosa cells. The cells were exposed to 0 (control), 10 and 30 µM ZEN for 48 h and an increase in early and late apoptotic cells was seen with increasing concentration. The percentage of early apoptotic cells for the control, 10 and 30 µM cells were 3.28%, 4.72% and 5.59% respectively and the percentage of late apoptotic cells for the same groups were 10%, 14.3% and 29.3% respectively [89]. Similar results were found when rat Sertoli cells were treated with 0, 5, 10 and 20 µM ZEN. An increase in the apoptosis rate of the cells was detected, by flow cytometry, with an increase in ZEN concentration. The upregulation of the pro-apoptotic proteins, Bid and Bax, and the down-regulation of the anti-apoptotic protein, Bcl-2, was also detected [90]. In another study, the effect of ZEN in the intestine of juvenile grass carp was investigated. Six different concentrations of ZEN between 0-2500 µg/kg was used to contaminate the fish feed. It was found that the diet with ZEN concentration greater than or equal to 1548 µg/kg resulted in the induction of apoptosis by up-regulation of apoptosis promoters caspase-2, caspase-8 and caspase-9 as well as apoptosis executers caspase-3, caspase-7 and pro-apoptotic Apaf-1, Bax, and FasL. It should be considered, however that the concentration of ZEN used in this study is significantly higher than the limits set by EFSA for maximum levels of ZEN in feeding stuff (Table 1.3). It also lead to the decrease of gene expressions of anti-apoptotic Bcl-2and Mcl-2. The results showed that ZEN induced apoptosis in the fish intestine [91].

Table 1.3: *Guidance values provided by the Environmental Food Safety Authority (EFSA) for limit of ZEN in feeding stuff, adapted from Commission Regulation (EC) 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding* [92]

Feed products	**Guidance value in ppm relative to a feeding-stuff with a moisture content of 12%**
Cereals and cereal products with the exception of maize by-products	2
Maize by-products	3
Complementary and complete feedingstuffs for piglets and gilts (young sows)	0.1
Complementary and complete feedingstuffs for sows and fattening pigs	0.25
Complementary and complete feeding stuff for calves, dairy cattle, sheep (including lamb) and goats (including kids)	0.5

Due to the toxicity of ZEN, EFSA recommends a maximum of 2 ppm ZEN in cereal and cereal products and 3 ppm for maize by-products for use in feed materials, as shown in Table 1.3. The limit for ZEN in feedstuff for piglets and gilts is 0.1 ppm and for sows and fattening pigs is 0.25 ppm [92].

1.4 Deoxynivalenol

1.4.1 Structure and mode of action

DON is a mycotoxin produced in grains infected with *F. graminearum and F. culmorum.* It is to be found mostly in wheat, barley, rye and corn. DON is the most commonly found type B trichothecene. Its structure is shown in Figure 1.6 [93]. DON has two secondary and one primary hydroxyl groups present along with an epoxide and a conjugated ketone, either of which may be associated with DON's toxicity [94].

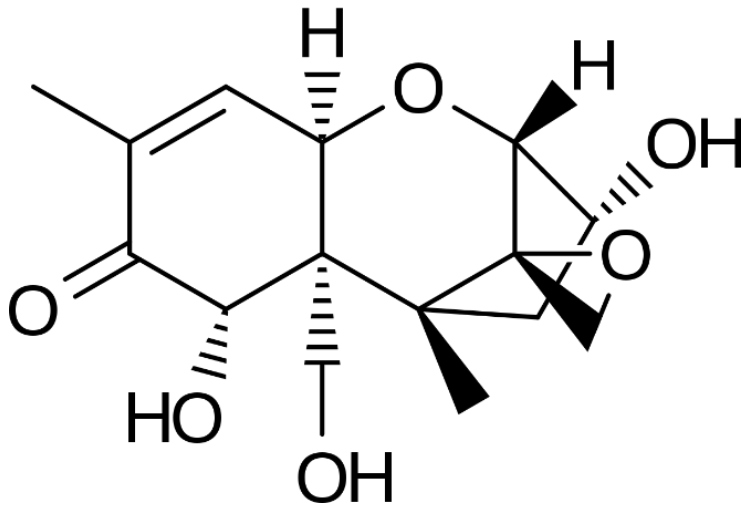

Figure 1.6: *The chemical structure of deoxynivalenol* [94].

The epoxide on the C12/13 position of DON is considered essential for its toxicity, as it is necessary for the inhibition of protein synthesis, which is the main reported mode of action of DON [95–97]. It has been shown that the opening of the epoxide results in a loss of toxic activity of DON [98]. The role that the group plays in DON toxicity has been demonstrated by studies that have shown that the de-epoxy metabolites of DON are less toxic than DON itself [99–101]. In one study the cytotoxicity of DON and another trichothecene, NIV and their de-epoxy metabolite was compared using a BrdU bioassay. The results illustrated that the de-epoxy metabolites of DON and NIV were 54 and 55 times less toxic, respectively, than the toxins with the epoxide ring [99]. Similar results were obtained in another study where the cytotoxicity of DON and deepoxy-deoxynivalenol (DOM-1) were compared and in which the lack of DOM-1 toxicity was confirmed using six different cytotoxicity assays [101]. Thus, it can be concluded that the 12, 13-epoxide ring plays an important role in DON toxicity.

The presence of the hydroxyl groups may also be associated with the toxicity of trichothecenes. In a study by Shima *et al*., the hydroxyl group in the C3 position was transformed to oxygen by oxidative biotransformation, resulting in the formation of 3-keto-DON, illustrated in Figure 1.7. It was found that this compound displayed a reduced immunosuppressive toxicity in comparison to DON [102]. NIV, a type B trichothecene, is structurally very similar to DON, the only difference being the hydroxyl group at the C4 position of NIV. However, NIV is found to be more toxic than DON, which shows that the position of a hydroxyl group in trichothecenes is relevant [103]. In a study investigating the toxicity of NIV and DON on jejunum mucosa, acute exposure to NIV was found to be more toxic than DON. Exposure to NIV resulted in mucosal changes at lower concentrations than DON [104]. In a study comparing the toxicity of various DON family mycotoxins, NIV was found to be the most toxic mycotoxin, followed by fusarenon-X and DON. The acetyl derivatives of DON were found to be the least toxic, with 15-acetyldeoxynivalenol (15-ADON) more toxic than 3-acetyldeoxynivalenol (3-ADON) [105].

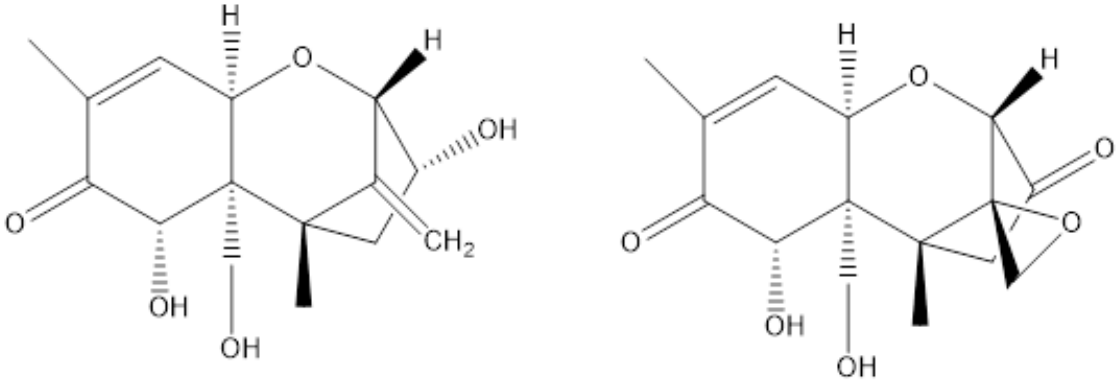

Figure 1.7: *Structure of de-epoxy DON and 3-keto DON, which are both less toxic than DON due to their missing epoxide and hydroxyl groups respectively* [106,107].

The primary mode of action of DON and other trichothecenes is the inhibition of protein synthesis. An intact C12-13 epoxide and a double bond at C9-10 position is essential for this inhibitory activity. DON binds to the 60 S subunit of the eukaryotic ribosome and interferes with the action of peptidyl transferase [108,109]. In a study investigating the effect of DON on protein content and synthesis on human intestinal epithelial Caco-2 cells derived from colorectal carcinoma tissue, it was found that there was a concentration-dependent effect of DON on the total protein content of the cells. A similar trend was also observed in terms of protein synthesis in mature Caco-2 cells [110]. In a study by

Ghareeb *et al*. the mRNA levels of IFN-γ, IL-1β and TGFBR1 were down regulated by DON in jejunal tissues of broiler chickens [111]. Another study was carried out to investigate the effect of DON on protein synthesis in pig tissues. Pigs were fed a DON contaminated diet where the DON concentration used were 2 µg/kg (control group), 77 µg/kg (chronic oral), 83 µg/kg (acute oral) and 53 µg/kg (acute intravenous). The results showed a significant reduction in overall protein synthesis in the kidneys, spleen and ileum of DON exposed pigs [112].

1.4.2 Occurrence of DON

Although DON is not the most toxic trichothecene, it is one of the most commonly found. Studies have shown that it can be globally present in wheat, barley, maize and rice, amongst others [19,113]. A review by Tola and Kebede suggested that there are five mycotoxins that occur more often in food than others and DON was one of the five listed along with the fumonisins, aflatoxins, OTA and ZEN [114]. In a three-year study conducted in America, Europe and Asia between 2009 and 2011, 7049 samples were analysed for mycotoxins. The samples included soybean, wheat, dried distillers grains with soluble and finished feed. The study showed that DON was present in over half of the samples analysed (59%) [115]. The common occurrence of DON in food and animal feed is clearly a problem in both the food industry and the livestock industry [116]. The limits for the presence of DON within the EU are highlighted in Table 1.4.

Table 1.4: *Maximum levels of DON in various foodstuffs. Table adapted from Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs* [78].

Foodstuff	Maximum level (µg/kg)
Unprocessed cereals except durum wheat, oats and maize	1250
Unprocessed durum wheat and oats	1750
Unprocessed maize except for unprocessed maize intended to be processed by wet milling	1750
Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption	750
Dry pasta	750
Bread, pastries, biscuits, cereal snacks and breakfast cereal	500
Processed cereal based foods and baby foods for infants and young children	200

The common occurrence of DON is a cause of concern, as it is a very stable compound, both in storage and during the food preparation stage [117]. It has been found that low temperature along with high humidity and heavy rainfall can increase the concentration of DON [118,119]. A study by Hoogenboom *et al.* involving wheat samples collected in 2003 and 2004 from the Netherlands revealed levels of DON found in the 2003 samples was below 1000 µg/kg, below the maximum level for the EU. However, for the samples collected in August 2004, after a period of heavy rainfall, the level of DON found was as high as 11,000 µg/kg, by far in exceedance of maximum EC levels [120].

In Luxemburg (2007 and 2008), 75% of 33 fields of winter wheat sampled were found to have DON contamination. Nine percent of these exceeded the recommended EU maximum level of DON for unprocessed wheat (Table 1.4) [121]. In a study in The Netherlands, 57 out of 86 samples that were collected from winter wheat fields in 2009 tested positive for DON. The maximum concentration of DON found in these samples was 2524 µg/kg [122]. In Argentina, 120 samples of freshly harvested wheat from nine locations in the Northern Buenos Aires Province (2004) confirmed that 85% of the samples were contaminated with DON. The maximum level of DON found in the samples was 2788 µg/kg [123]. In China in the Jiangsu province 74.4% of 180 wheat samples harvested in 2010 – 2012 tested positive for DON [124]. In another study, 16 out of 23 samples from the latter half of 2006 from South Africa were contaminated with DON [125]. These studies highlight the global occurrence of DON and therefore the importance of efforts to mitigate the effect of the toxin [113].

DON often co-occurs with other *fusarium* toxins such as ZEA and NIV. A study investigated wheat samples from seven states of Brazil and DON was found to co-occur with NIV and ZEA in 43% of the 745 samples [126]. In Portugal, 307 samples of plant crops were taken and ZEA and DON co-occurrence was found in 46 of those samples with a mean ZEA concentration of 0.17 mg/kg and mean DON concentration of 0.07 mg/kg [127]. It is also sometimes found with lower concentrations of its two acetylated forms, 3-ADON and 15-ADON [52,105,109]. In Lithuania, 103 samples of spring wheat grain were tested for the presence and co-occurrence of DON, 3-ADON and 15-ADON. The samples were grouped as per the production system – organic, sustainable and intensive. It was shown that 81% of samples from the intensive production system was contaminated with a combination of all three mycotoxins. DON was found on its own in 10% of the samples, and 5% was contaminated with a combination of DON and 15-ADON and 1% with a combination of DON and 3-ADON [128]. Out of 370 wheat samples tested for Fusarium mycotoxins in China, it was found that 368 samples were contaminated with more than one *Fusarium* mycotoxins. The results showed that DON was found in co-occurrence with six other mycotoxins: 3-ADON, 15-ADON, NIV, ZEN, fusarenon-X (FX) and DON-3G [129].

1.4.3 Toxic effects of DON

DON is toxic to both humans and animals when ingested. It mainly affects the immune system and the gastrointestinal tract and can result in nausea, diarrhoea and vomiting [10,110]. Among animals, pigs are the most sensitive to DON toxicity and the main effect of its consumption is feed refusal or decreased intake of feed [130]. A study carried out by Waché *et al*. showed that consumption of DON contaminated feed (at 2800 μg/kg) resulted in decreases in both feed intake and weight gain in pigs [130].

Elsewhere Kolf-Clauw *et al.* exposed pig jejunal cells to DON and recorded time and dose dependent toxicity responses from observations of cell morphology following treatments in which up to 5 μM DON was used over 4 hours [131]. Pig jejunal cells were also used by Awad *et al.* to assess DON cytotoxicity. Here, total cell counts and a lactate dehydrogenase (LDH) release assay were used to show time-dose toxicity dependent effects by DON over a concentration range of 0 – 10 μM, over 24 to 48 hours. They showed a 62% increase in LDH release, which correlates with reduced membrane integrity and decreased cell viability, when the cells were exposed to 10 μM DON for 48 h [132].

Genotoxic effects of DON have also been described. In a study by Zhang *et al*., the comet assay was used to investigate DNA damage due to DON in human liver (HepG2) carcinoma cells. The results of the study showed that DNA damage was induced in a dose dependent manner following exposure to DON for 1 hour [133]. These findings are consistent with another study, where the comet assay was carried out to measure the genotoxicity of DON on Caco-2 cells. DON concentration range of 0.01 – 0.5 μM was used and cells were exposed to DON concentration range of 0.01 – 0.5 μM for 24 to 72 hours. The results showed that exposure to DON caused DNA damage in a time and dose dependent manner. The average tail moment, which represents the extent of DNA damage, for healthy differentiated Caco-2 cells was 1.23±0.73; this value increased to 4.11±1.53 when the cells were incubated with 0.1 μM DON for 24 hours. When the cells were exposed to 0 and 0.1 μM DON for 72 hours, the average tail moment was 1.09±0.31 and 4.6±0.81 respectively [134]. Both studies showed a dose dependent response in DNA damage on exposure to DON.

Elsewhere, DON-associated genotoxicity was investigated *in vivo* in seven mouse organs (duodenum, colon, blood, liver, spleen, kidney, bone marrow), following a 3-day oral administration of DON. The results of the comet assay failed to show evidence of DNA damage at up to 53.9 µM DON. The same study also used human lymphoblast TK6 cells exposed to DON at concentrations of up to 25 µM for 3 and 24 h. Again, the results showed that there was no increase in DNA damage at both exposure times [135]. This data is clearly in contrast to that observed with HepG2 illustrating the extent to which DON toxicity may be dependent on the cell type or animal used.

The intestine is a major site of DON absorption as it is exposed to contaminated feed. The intestinal epithelium acts as a frontier barrier to the external environment including harmful toxins [136,137]. However, DON has been shown to alter the intestinal barrier function by affecting the trans-epithelial electrical resistance (TEER), a good indicator of its integrity and permeability [137,138]. A significant time and dose dependent reduction of the TEER value of Caco-2 cells was observed following treatment with 5 µM DON (a decrease of 19% after a 14 day exposure). Higher DON concentrations had more drastic negative effects on TEER values [139]. A similar trend was observed for two porcine intestinal epithelial cells, IPEC-1 and IPEC-J2. For IPEC-1 cells, there was a 25% and 60% decrease in the TEER value when the cells were exposed to 10 and 50µM DON respectively over 24 h. After exposure for 14 days, the TEER decrease was 58% and 97% for 10 and 50µM respectively [139]. For IPEC-J2 cells, there was a significant decrease in TEER value with 20 µM DON at 4, 8, 12 and 24 hours [140]. All three cell lines showed a decrease in TEER value with increasing DON concentrations and exposure times. These studies indicate that DON is effective in disrupting the integrity of the intestinal epithelium. Overall, it can be concluded that DON exhibits dose and time dependent toxicity across a range of cells and tissues with additional evidence of cell type dependent genotoxicity.

Table 1.5: *Guidance values provided by the EFSA for limit of DON in feeding stuff, adapted from Commission Regulation (EC) 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding* [92]

Feed products	**Guidance value in ppm relative to a feeding-stuff with a moisture content of 12%**
Cereals and cereal products with the exception of maize by-products	8
Maize by-products	12
Complementary and complete feeding stuff (not including the ones listed below)	5
Complementary and complete feeding stuff for pigs	0.9
Complementary and complete feeding stuff for calves, younger than 4 months, lambs and kids	2

Due to the toxicity of DON, EFSA recommends a maximum of 8 ppm DON in cereal and cereal products and 12 ppm for maize by-products, as shown in Table 1.5. The limit for animal feedstuff is 5 ppm except for calves, lambs and kids where the limit is set at 2 ppm. However, as pigs are the most sensitive to DON toxicity, the limit for DON in pig feedstuff is set at 0.9 ppm [92].

1.5 Toxicity following co-exposure to DON and ZEN

Given the extent to which DON and ZEN have been shown to co-occur, it is important to study and understand the effect that these combined mycotoxins can have. The effect of the combined mycotoxins are generally classified as synergistic, antagonistic or additive. The combinatorial toxicity of these mycotoxins have been studied both *in vivo* and *in vitro*. Various cell types have been used for *in vitro* studies including liver cells, kidney cells, intestinal cells, fish cells and white blood cells, including lymphocytes and monocytes, amongst others. Rats, mice and piglets have been used for *in vivo* studies.

1.5.1 In Vitro studies on co-exposure to DON and ZEN

Due to the importance of mycotoxin interaction with the liver, there are a number of studies evaluating the impact of the combination of DON and ZEN on liver cells, but even within this cell type, results were not consistent. Some studies have shown a synergistic impact while others showed an antagonistic impact on the cells.

Of the studies, where the combination of DON + ZEN have shown to have a synergistic effect, one such study investigated the individual and combined effect of the fusariotoxins using HepaRG human hepatic cells. Cell viability results showed that the combination of DON (0.2 – 10 μM) and ZEN (1.5 – 75 μM) resulted in synergism after 48 hour exposure as assessed by the MTS assay. Cell apoptosis and necrosis was also analysed using the Annexin V-FITC/PI staining followed by flow cytometry analysis. The results showed that at lower exposure times of 3 h and 12 h, the combination of DON (7.35 μM) and ZEN (55.1 μM) resulted in the induction of significant cell mortality, which was not observed with the individual mycotoxins, thus indicating a synergistic effect. However, following 18 h incubation, ZEN was shown to induce significant apoptosis and necrosis but there was no significant cell mortality observed at that exposure time with DON alone (7.35 μM). Once the cells were exposed to the combined mycotoxins, the combinatorial effect was similar to that of ZEN alone [141].

In another study also using the HepaRG cells, the individual and combined effect of non-cytotoxic concentrations of DON (0.2 μM) and ZEN (20 μM) on the proteome of the cells at 1 h and 24 h was investigated. The results showed that even with a low exposure time of 1 h to DON, ZEN and DON + ZEN, a significant change in the proteomes of the cells was observed, and that the changes were not consistent between mycotoxin exposures.

At 1 h, there were more proteins affected by the combined mycotoxins than by DON and ZEN individually, thus indicating a synergistic effect of the mixture. However, an antagonistic effect was observed when the cells were exposed to the mycotoxins for a longer exposure time of 24 h. It was also found that the cellular response to ZEN induced stress, following 24 h exposure, was reduced when it was combined with DON. It was suggested that the observed antagonistic effect following a longer exposure time might have been due to the possible mitigation by the hepatocytes [142]. Elsewhere, the chronic effect of DON, ZEN and DON + ZEN was explored on HepaRG cells. Here the cells were exposed to the maximum level of DON (2.5 μM) and ZEN (0.24 μM) permitted by the European regulation in cereals intended for direct human consumption. The cells were exposed to the toxins for 14, 28 and 42 days. DON was found to be cytotoxic after 14 days exposure but ZEN did not have a significant effect on the cell viability even after exposure for 42 days. When the mycotoxins were combined, the effect was found to be the same as for exposure to DON alone, indicating no antagonistic or synergistic effect. As ZEN did not induce cytotoxicity, the effect observed was described to be additive [143].

All three studies used the HepaRG cells to study the combinatorial effect of DON and ZEN, however, different results were obtained in each study. These studies clearly demonstrate that the individual effect of the mycotoxins cannot predict the effect of the mycotoxins in combination. Although the same cell line was used in each of the studies, the concentrations of the toxins and the exposure times were different in each case. However, even when the cells were treated with the same exposure time of 24 h, synergistic effect was observed in the first study and an antagonistic effect in the second. This may be because of the different types of toxicity explored in the two studies; cell viability and apoptosis in the first and effect on the proteome of the cell in the second. It is also interesting to note that in the first study DON (7.35 μM) did not induce cell death and only ZEN (55.1 μM) did following 18 h incubation. However in the third study DON (2.5 μM) alone was cytotoxic following a chronic exposure for 14 days, whereas ZEN (0.24 μM) was not. This further highlights that the effect of the mycotoxin on cells is largely dependent on the concentration and the exposure time.

Liver cells were used in another study where the effect of mycotoxins were analysed on human hepatocellular carcinoma (BEL-7402) cells. A cell based electrochemical sensor was developed to assess the individual and combined toxicity of DON (ranging between

0.37 – 16.9 μM) and ZEN (0.31 – 31.4 μM) after 24 h exposure. The results of the study showed that an additive effect was observed when the cells were exposed to DON and ZEN together [144]. Another study developing a cell based electrochemical sensor, measured the effect of combined mycotoxins on human liver carcinoma HepG2 cells. The concentration range of DON and ZEN used in this study is higher with the concentration of DON ranging between 0.034 – 67.5 μM and the concentration of ZEN between 15.7 – 157 μM but the exposure time was the same as the previous study at 24 h. The results showed that the different concentration combinations of the mycotoxins had a synergistic effect. The results of the sensor was validated by the CCK-8 cell viability assay [145]. HepG2 cells were also used in another study that again showed synergism in its cell viability when the two fusarium mycotoxins were combined. In this study mixtures of 0.02 - 2 μM DON and 0.28 – 34.5 μM ZEN were used to treat the cells for 48 h. The same study also measured the cytotoxicity of the mixture on RAW 246.7 (murine leukaemia virus-induced tumour) cells. These cells were treated with mixtures of 0.0027 – 0.34 μM DON and 028 – 37.69 μM ZEN for 48 h. As with the HepG2 cells, the mixture resulted in a synergistic effect in the cytotoxicity [146].

All three studies above used human liver carcinoma cells and the effects were found to be similar, with synergistic effects observed in each case for combination exposure. In the first two studies, the cells were exposed to the mycotoxins for 24 h. Two studies used HepG2 cells for their research, but with different concentrations and exposure time. The first of those studies used a higher range of concentration with 24 h exposure time and the other studies used lower concentrations with a higher exposure time of 48 h. A synergistic effect in the cell viability was observed in both.

The toxic effects DON and ZEN have on the liver have also been studied *in vivo*. A study used mice to investigate the hepatotoxic effect of ZEN + DON. In this study, the antioxidant capacity and the concentration of malondialdehyde (MDA) was measured to investigate the oxidative stress induced by the mycotoxins. The results of the study showed that when mice were injected with ZEN (5000 μg/kg bodyweight) + DON (5000 μg/kg bodyweight), for 2 weeks, the combined exposure had an antagonistic effect on the antioxidant capacity and the MDA content, which shows that the mycotoxins induced more oxidative stress individually than they did when combined. However, in contrast, it was found that ZEN + DON exhibited a synergistic effect on mRNA expression of the

pro-apoptotic protein Bax and apoptotic enzyme Caspase-3 in the liver. Thus it was concluded that the underlying mechanism of the mycotoxins needs to be further studied [147].

The liver is one of the most important organs in the metabolic pathway of the mycotoxins. The detoxification of DON occurs in the liver with the formation of DON-glucuronide in both humans and animals [148]. The biotransformation of ZEN to its major metabolites α-ZOL and β-ZOL also occurs in the liver [23]. Due to this, the effect of the combined mycotoxins have been investigated using various liver cell both *in vitro* and *in vivo*. These studies have shown that the toxicity of the combined mycotoxins on liver cells cannot be predicted by their individual effects. The concentration of the mycotoxins, exposure time and the cell line used can all have an effect on toxicity of the mixture. In addition, results can vary depending on the parameter being measured, such as cell viability, apoptosis and metabolism.

After the consumption of contaminated food or feed, the intestinal epithelium can be exposed to mycotoxins, as it is the first barrier against foreign matter [149–151]. Being the first point of contact for food and feed contaminants, the toxicity of mycotoxins on various intestinal epithelial cells have been studied. A study by Ji *et al*. investigated the individual and combined cytotoxic effect of the two mycotoxins on human intestinal epithelial cells Caco-2 cells. The CCK-2 assay was used to measure the viability of the cells. The cells were incubated with DON (3.3 -16.7 μM) and ZEN (10 – 50 μM) in a 1:3 ratio for 24 h. It was found that each combination of the mycotoxins showed a strong antagonistic effect as the toxicity of the mycotoxins combined was lower than that of DON and ZEN individually [152]. The antagonistic effect of DON + ZEN was also observed in HCT116 human colon carcinoma cells. Cells were treated with 100 μM DON and 40 μM ZEN for 24 hours and cytotoxicity and mitochondrial apoptosis was measured. For cell cycle analysis, the cells were treated at the above concentrations for 48 h. Each of the analysis showed that the combined mycotoxins were less toxic to the cells than the individual treatments, thus resulting in an antagonistic response [153].

Porcine intestinal epithelial IPEC-J2 cells were used in another study to investigate the combinatorial effect of DON and ZEN. In this study, the cells were exposed to cytotoxic concentrations (2 μM DON and 40 μM ZEN) and non-cytotoxic concentrations (0.5 μM DON and 10 μM ZEN) for 48 h. The results showed a synergistic effect at the non-

cytotoxic concentration as this mixture of the mycotoxins was found to be cytotoxic [84]. However, there is some ambiguity in the results of the cytotoxic concentrations. The combined interaction was described to be non-additive and it was concluded that in mixtures containing DON, "there were no increases in overall cytotoxicity of such mixtures" but in mixtures containing ZEN "all of the mixtures were more cytotoxic" than ZEN individually. This study has been cited by others as DON and ZEN having a synergistic effect [147,154,155], synergistic at cytotoxic concentrations [141,156], synergistic at cytotoxic concentrations and antagonistic at non-cytotoxic concentrations [157], antagonistic [158] and antagonistic at the lower dose [159]. The different interpretation of the results further proves the ambiguity of the results and makes it difficult to conclude.

As with the liver cells, both synergistic and antagonistic effects were observed in the studies using different intestinal cells discussed above. The combined mycotoxins resulted in an antagonistic effect in the cytotoxicity of Caco-2 and HCT116 cells. In both studies the exposure time was 24 h but the concentration used varied. Most studies show that DON is more toxic than ZEN but interestingly, HCT116 cells were more sensitive to ZEN than to DON and therefore a higher concentration of DON was used due to the higher IC_{50} value. With IPEC-J2 cells, a different trend again was observed, as synergistic effects were observed with lower concentrations. This shows that varying the concentrations of the mycotoxin mixtures can have an effect on the toxicity of the combined mycotoxins.

In addition to liver and intestinal damage, some mycotoxins have also been shown to induce nephrotoxicity. PK15 (porcine kidney) cells were used to investigate the effect of 24 h exposure to the combined mycotoxins (DON 0.25 µM and ZEN 20 µM). The results of the study showed that a higher level of ROS production was found when the cells were treated with the combination than those treated with individual toxins. The combined mycotoxins also induced more apoptosis than the mycotoxins individually as evidenced by the increased expression of Bax and caspase-3 [160]. The nephrotoxicity of DON + ZEN was also studied *in vivo* in the kidney of mice. In this study, female mice were injected with DON (1500, 2500 µg/kg body weight) and ZEN (20,000, 30,000 µg/kg body weight), individually and combined for 12 days. It was found that DON and/or ZEN was able to induce oxidative stress and renal apoptosis but the nephrotoxicity of the combined mycotoxins was antagonistic [161].

Studies of DON + ZEN toxicity have also been carried out on white blood cells including lymphocytes, monocytes and macrophages. In a study by Ren *et al,* the induction of apoptosis and oxidative injury by DON, ZEN and DON + ZEN was studied on porcine splenic lymphocytes. The cells were exposed to the DON and ZEN (0.2 μM and 0.25 μM, 1 μM and 1.26 μM, 5.1 μM and 6.28 μM) for 48 hours. It was found that DON and ZEN induced apoptosis and oxidative injury in a dose dependent manner and the combination of the two toxins resulted in a synergistic effect [162]. Another study used lymphocytes from *venae cava cranialis* of pigs to study the combined effects of DON and ZEN. The results showed that the combined mycotoxins had an antagonistic effect on the cell viability after 48 and 72 h of incubation. The genotoxicity of the compounds was also studied using the comet assay. When low doses of the combined mycotoxins were used (0.24 μM and 15.7 μM DON and ZEN respectively), an antagonistic effect was observed following 24, 48 and 72 h exposure, but the antagonism was not statistically significant. However, when higher doses were used (0.71 μM and 31.4 μM DON and ZEN respectively), after 72 hour exposure, a significant synergism was observed [159]. In another study, mouse primary spleen T lymphocytes were used to investigate the toxic effect of ZEN and DON alone and combined. The cells were activated by concanavalin and it was found that ZEN and DON, both individually and combined inhibited the activation of the cells. By cell counting it was shown that ZEN and DON decreased the cell viability in a dose dependent manner. It was also found that 24 h exposure to the combination of ZEN (10 μM, 20 μM, 40 μM) and DON (0.5 μM, 1 μM, 2 μM) was more cytotoxic to the cells resulting in a synergistic effect. The immune-related function of the activated cells were also inhibited by the mycotoxin synergistically [156]. The first two studies here both used porcine lymphocytes to investigate the effects of DON + ZEN. Although a synergistic effect was observed in the first study following a 48 h incubation, in the second study an antagonistic effect was observed at the same exposure time. The differences observed may be due to the variation in the concentration used in the study, along with the difference in the lymphocytes used. In the third study, where mice spleen lymphocytes were used, a synergistic effect was observed in the cell viability, comparable to the first study, which also used lymphocytes from the spleen but from pigs.

A study used human leukaemia monocytic THP-1 cells to measure effect of DON and ZEN on its cell viability, using the MTS test. Cells were treated with DON (0.1, 0.8, 2, 4, 10 μM) and ZEN (2, 16, 40, 80, 100 μM) in a 1:20 ratio. The results showed an

antagonistic effect on cell viability with each of the combinations following 48 hr exposure [163]. A study by Ji *et al* tested the effects of DON + ZEN on ANA-1 (mice macrophage) cells. The cells treated with 3.4 μM DON, for 24 h, had a viability of 74% and the cells treated with 25.1 μM ZEN had a cell viability of 92%. However, the cell viability was decreased to 50% when DON and ZEN were combined at those concentrations, exhibiting a significant synergistic effect. Similar effects were observed in terms of cell apoptosis. DON and ZEN alone exhibited early apoptotic effect but the combination of the toxins resulted in late stage apoptosis. However, the metabolism of the cells that were treated with DON + ZEN showed a decrease in estrogenic effects, thus suggesting the inhibition of the estrogenic effects of ZEN by DON [164]. These two studies further demonstrate that the combination of DON and ZEN can have different effects on different cells.

Another study used a fish cell line BF-2 (*in vitro*) and zebrafish larvae (*in vivo*) to study the individual and combined effect of AFB1, DON and ZEN. Resazurin assay was used to measure the cytotoxicity of the cells and for the combined effects, DON concentration 0.13 – 16.2 μM and ZEN concentration 1.33 – 120.3 μM was used. The results showed that DON and ZEN, with 48 h exposure, were more cytotoxic to the fish cell line individually than they were when combined, thus exhibiting an antagonistic effect. This antagonistic effect was also observed with oxidative stress induced cell death in BF-2 cells. Zebrafish larvae were also exposed to the mycotoxins for 72 h and it was found that DON was unable to induce embryo mortality and in contrast, ZEN induced embryo mortality in a dose dependent manner. An antagonistic effect was observed with the combined mycotoxins (67.5 μM DON and 6.28 μM ZEN) as they were also unable to induce embryo mortality [154].

In order to reflect *in vivo* conditions, the individual and combined cytotoxicity of DON and ZEN was tested on three different cell lines, human intestinal epithelial cells Caco-2, human lukemia monocytic cells THP-1 and HepaRG, co-cultured in bi- and tri- culture systems. The IC_{10} and IC_{30} concentrations of the mycotoxins for the cells in the luminal compartment (cells growing in the hanging inserts) were used in this experiment. In the bi-culture system, when the IC_{10} concentrations were used there was no cytotoxicity observed. When the IC_{30} concentrations were used, a synergistic effect was observed as the cytotoxicity of the combined mycotoxins was higher than that of the mycotoxins alone. However, in the tri-culture system the mycotoxins, both individually and

combined, did not induce significant cytotoxicity on the cells. These results show that the cytotoxicity of the combined and individual mycotoxin was reduced by the increase in cell number and the cell-to-cell interactions in the tri-culture [165]. These results from the various *in vitro* studies demonstrate that the toxic effect of the mycotoxins DON and ZEN can vary depending on the types of cells, concentration of the toxins and the exposure times used.

1.5.2 In vivo studies on the co-exposure to DON and ZEN

In vitro studies can provide important information on the interaction of mycotoxins with specific cell types. However, it is essential to understand the effect of mycotoxin interaction with the whole animal and this can only be obtained by *in vivo* studies. In order to understand the effect of DON + ZEN mixture, animals including rats, mice and pigs have been fed/injected with contaminated feed and studied. In a 14-day study, rats were used to investigate the chronic effects of DON and ZEN. The animals were fed with 15µg/animal/day ZEN, 30 µg/animal/day DON and 150 µg/animal/day fumonisin B1, individually and in combinations. The study showed an antagonistic effect between DON and ZEN on the absolute liver weight of the rats. As well as that DON + ZEN also resulted in an antagonistic effect on the level of GSH in the liver samples and the concentration of MDA in the kidney [166]. In another study by the same group, the effect of lower concentrations and shorter treatment time was evaluated. In this study, the rats were injected with the mycotoxins daily for 5 days with 12.75µg/animal/day ZEN, 16.5 µg/animal/day DON and 9 µg/animal/day fumonisin B1, individually and in combinations. However, in this study it was found that the total glutathione (GSH) and the glutathione peroxidase (GSHPx) activity were increased in the liver of the rats treated with DON + ZEN but not in the rats treated with DON and ZEN individually. This shows that the combination of DON and ZEN resulted in a synergistic effect [167]. These two studies show that varying the treatment dose and time can influence whether the combinatorial effects are synergistic or antagonistic.

Mice were used in another study where they were treated with 2,000 µg/kg DON, 20,000 µg/kg ZEN, and DON + ZEN combined with the same doses for 21 days. Here, the metabolic profiling on the liver and serum of the mice was carried out. It was found that there was an obvious antagonistic effect observed, as the toxic effect of DON and ZEN individually was higher than when combined [168]. Another study by the same group again

used mice to investigate the effects of combined mycotoxins using urinary metabolic profiling. Five-week-old mice were exposed to 2,000 μg/kg DON and 20,000 μg/kg ZEN, and their mixture for three weeks through intragastric administration. The urine samples showed that the combined mycotoxins had an antagonistic effect as the individual mycotoxins effected the metabolic pathway of the mice more than when the toxins were combined [169]. The two studies discussed here used the same concentration and exposure time of the mixture of DON and ZEN. Under these conditions, antagonistic effects were observed in both the metabolic profiling on the liver and serum and in urinary metabolic profiling.

When the combined effect of DON and ZEN was analysed on the brain of mice a synergistic effect was observed. In this study, 360 mice were treated by intraperitoneal injection with DON (1500, 2500 μg/kg body weight) and ZEN (20,000, 30,000 μg/kg body weight), both individually and combined, every 24 h for 4 days. It was found that DON + ZEN resulted in a reduced antioxidant system activity in the brain, a reduction in brain protein levels, as well as an increase in apoptotic cells in a dose-dependent and synergistic manner [155].

In a study by Jia *et al*, 21-day-old female piglets with DON (1000.6 μg/kg), ZEN (269.1 μg/kg) or DON + ZEN (1007.5 + 265.4 μg/kg) for three weeks followed by normal toxin-free diet for 2 weeks. The body weight gain and the average daily feed intake was not significantly impacted by DON or ZEN individually, but the DON + ZEN fed piglets showed a significantly lower body weight gain and average daily feed intake. The results also showed that the combined mycotoxin disrupted intestinal functions of the piglets and caused systematic inflammation synergistically [158]. As with the *in vitro* studies, the *in vivo* studies also show that the resulting combinatorial effects of DON and ZEN can be different depending on the animals used in the study as well as the parameter measured.

The effect of the combined mycotoxins DON and ZEN have been studied both *in vivo* and *in vitro.* The above studies show that it is not possible to predict the toxic effect of DON and ZEN combined based on their individual toxicity. The toxicity of the combined mycotoxin is hugely effected by various factors including cell type, mycotoxin concentration and exposure time. This makes it difficult to compare between the different results. The studies showed that even when the same cell type was used in different studies, the effect of DON + ZEN varied between additive, synergistic or antagonistic.

As *in vitro* studies can only provide information of specific cells, *in vivo* studies have also been carried out and similar results were obtained. In conclusion, due to the different species, cells, concentrations, exposure time and administration methods it is difficult to predict and compare the combinatorial effects of the co-occurring mycotoxins DON and ZEN. Thus, further research is required to understand the interactions of the combined mycotoxins.

Table 1.6: *Combinatorial interactions between DON and ZEN*

Cell Line used	Toxin Levels	Exposure Time and Route	Conclusions	Observations	Comment	References
HepaRG	DON 0.2–10 µM ZEN 1.5–75 µM	48 h	Synergistic at 48 h cell viability	Cell viability	Doses correspond to IC50 values after 48 h	141
	DON 7.35 µM ZEN 55.1 µM	18 h	Additive at 18 h cell mortality	Cell mortality		
HepaRG	DON 0.2 µM ZEN 20 µM	1 and 24 h	Synergistic at 1 h Antagonistic at 24 h	Cell proteome	Doses correspond to IC10 values after 48 h	142
HepaRG	DON 2.5 µM ZEN 0.24 µM	14 days 28 days 42 days	Additive	Cell viability	Doses correspond to maximum levels permitted in cereals for humans	143
BEL-7402	DON 0.37–16.9 µM ZEN 0.31–31.4 µM	24 h	Additive	Cell viability	Mixtures used DON + ZEN 0.37 + 0.31 µM 0.68 + 0.63 µM 1.69 + 1.57 µM 3.7 + 3.1 µM 6.8 + 15.7 µM 16.9 + 31.4 µM	144
HepG2	DON 0.34–67.5 µM ZEN	24 h	Synergistic	Cell viability	Mixtures used DON + ZEN 0.034 + 15.7 µM	145

	15.7–157 µM				1.69 + 47.1 µM 3.7 + 62.8 µM 37 + 126 µM 67.5 + 157 µM	
HepG2	DON 0.02–2 µM ZEN 0.28–34.5 µM	48 h	Synergistic	Cell viability	DON + ZEN 0.02 + 0.28 µM 0.03 + 0.53 µM 0.07 + 1.1 µM 0.14 + 3 µM 0.27 + 4.4 µM 0.51 + 8.6 µM 1 + 17.3 µM 2 + 34.5 µM	146
RAW 246.7	DON 0.0027– 0.34µM ZEN 028–37.69 µM	48 h	Synergistic	Cell viability	DON + ZEN 0.0027 + 0.28 µM 0.0054 + 0.6 µM 0.01 + 1.19 µM 0.02 + 2.36 µM 0.04 + 4.71 µM 0.08 + 9.42 µM 0.17 + 18.8 µM 0.34 + 37.69 µM	146
Caco-2	DON 3.3–16.7 µM ZEN 10–50 µM	24 h	Antagonistic	Cell viability	DON and ZEN combination in 1:3 ratio	152
HCT116	DON 100 µM ZEN 40 µM	24 h 48 h	Antagonistic	Cytotoxicity, mitochondrial apoptosis Cell cycle analysis	Doses correspond to IC30 values after 24 h	153

IPEC-J2	Cytotoxic concentration DON–2 µM ZEN–40 µM Non-cytotoxic concentration DON–0.5 µM ZEN –10 µM	48 hr	Cytotoxic concentration Reported as non-additive Non-cytotoxic concentration Synergistic	Cell viability	Dose correspond to cytotoxic and non-cytotoxic concentrations	84
PK15	DON 0.25 µM ZEN 20 µM	24 h	Synergistic	ROS levels Apoptosis	Doses used are concentrations close to IC10 concentration which were 0.157 and 27.583 µM for DON and ZEN respectively	160
Porcine splenic lymphocytes	DON + ZEN 0.2 + 0.25 µM 1 + 1.26 µM 5.1 + 6.28 µM	48 h	Synergistic	Apoptosis Oxidative injury		162
Porcine lymphocytes	DON + ZEN 0.24 + 15.7 µM 0.71 + 31.4 µM	24/48/72 h	Antagonistic Antagonistic at lower concentration Synergistic at 72 h at higher concentration	Cell viability Genotoxicity	Doses used were below IC50 concentration after 24, 48, 72 h exposure	159
THP-1	DON 0.1–10 µM ZEN 2–100 µM	48 h	Antagonistic	Cell viability	DON + ZEN + 2 µM 0.8 + 16 µM 2 + 40 µM 4 + 80 µM 10 + 100 µM	163

ANA-1	DON 0–33.7 µM ZEN 0–37.7 µM	24 h	Synergistic Antagonistic	Cell viability and apoptosis Cell metabolism	DON + ZEN concentration used for apoptosis and metabolism study 0.34 + 25.1 µM	164
BF-2	DON 0–16.2 µM ZEN 0–120.3 µM	48 h	Antagonism	Cell viability fish Oxidative stress fish	DON + ZEN 0.13 + 1.33 0.25 + 2.66 0.51 + 5.32 1.01 + 10.64 2.02 + 21.29 4.05 + 42.58 8.1 + 85.15 16.2 + 170.3	154
Caco-2, HepaRG and THP-1 Co-culture	DON + ZEN Concentration used when Caco-2 cells were in luminal compartment: 1.6 + 24 µM 3 + 31 µM Concentration used when HepaRG cells were in luminal compartment: +20 µM 2.3 + 33 µM	48 h	No cytotoxicity with low concentration and in tri-culture Synergistic effect with higher concentration in bi-culture system	Cell viability		165

Animal used	Toxin levels	Exposure time and route	Conclusions	Observations	Comment	References
Zebrafish larvae	DON 67.5 µM ZEN 6.28 µM	72 h	Antagonistic	Cell mortality		154
Mice	DON–1500, 2500 µg/kg bodyweight ZEN–20,000, 30,000 µg/kg bodyweight	12 days Intraperitoneal injection	Antagonistic	Oxidative stress Renal apoptosis	DON + ZEN 1500 + 20,000 1500 + 30,000 2500 + 20,000 25 + 30,000 µg/kg bodyweight	161
Mice	DON–5000 µg/kg bodyweight ZEN–5000 µg/kg bodyweight	2 weeks Oral administration	Antagonistic Synergistic	Oxidative stress Apoptosis	No change observed on liver weight	147
Rats	DON 30 µg/animal/day ZEN 15 µg/animal/day	14 days Administered as a gavage dose	Antagonistic	Liver weight Glutathione level in liver Malondialdehyde level in kidney	Doses are according to EU limits in finished feed for young pigs	166
Mice	DON 0.5–2 µM ZEN 10–40 µM	24 h	Synergistic	Cell viability Immune function	DON and ZEN combined 1:20	156
Rats	DON 16.5 µg/animal/day ZEN	5 days Intraperitoneal administration	Synergistic	Glutathione and glutathione peroxidase	Doses correspond to 1mg/kg diet for DON and 1.5 mg/kg diet for	167

	12.75 µg/animal/day			activity in the liver	ZEN which are close to EU limits in finished feed for young pigs	
Mice	DON 2000 mg/kg ZEN 20,000 mg/kg	21 days Intragastric administration	Antagonistic	Metabolic profiling of liver and serum		168
Mice	DON 2000 mg/kg ZEN 20,000 mg/kg	3 weeks Intragastric administration	Antagonistic	Metabolic pathway		169
Mice	DON 1500, 2500 µg/kg body weight ZEN 20,000, 30,000 µg/kg body weight	4 days Intraperitoneal injection	Synergistic	Apoptosis Antioxidant levels	DON + ZEN 1500 + 20,000 1500 + 30,000 2500 + 20,000 2500 + 30,000	155
Female piglets	DON 1000.6 µg/kg ZEN 269.1 µg/kg DON + ZEN 1007.5 + 265.4 µg/kg	3 weeks *Ad libitum* feeding	Synergistic	Body weight gain Average daily feed intake Intestinal functions	Barley naturally contaminated with DON and corn naturally contaminated with ZEN was used to manufacture the feed	158

1.6 Mycotoxin remediation strategies

Many mycotoxin decontamination strategies have been developed to reduce the amount of toxin that is ultimately consumed, including cleaning, sorting, heating and adsorption. Biotransformation of mycotoxins has also been explored for the reduction of toxin levels in contaminated feed. Chemical strategies include the conversion of mycotoxins into less toxic end products by methods such as alkaline hydrolysis, peroxidation and the use of bisulphites [170]. In the section below, each of these strategies is explored in further detail.

1.6.1 Cleaning and sorting

The processing steps of cereals are important from a food production perspective, but they can also play a role in the reduction of mycotoxins in the final product. Physical processes such as cleaning and sorting can reduce mycotoxin contamination as these steps remove contaminated and broken kernels, dirt and dust materials, in which much of the toxins can accumulate [171]. A study showed that cleaning of wheat grain samples before milling resulted in a DON reduction by 50, 55, 41 and 47% from four different samples. The samples were cleaned by sieving, scouring and polishing methods and the outer layers of the bran was removed [172]. Another study investigated the effect of washing and drying naturally contaminated wheat samples. A reduction of 30.3% DON and 21.1% ZEN was observed in wheat grains pressure washed with water for 1 min followed by oven drying. When the washing time was increased to 2 min, a reduction of 33.9% and 24.7% of DON and ZEN was observed [173].

One study used an air screen cleaner to clean two samples of wheat cultivars from Brazil, followed by a gravity separation method. Once the cleaning steps were complete, the wheat samples were milled. The cleaning and milling method resulted in a DON reduction of 57% in the finished flour for both cultivars. [174]. Elsewhere, DON reduction of 48 to 86% was observed in hard red winter wheat following cleaning, with the reduction achieved being dependent on DON concentration. The wheat was also milled and the flour was shown to have lower concentration of DON when compared to cleaned wheat [175]. Milling can reduce mycotoxin levels in flour by fractionation [176,177]. The effect of sorting was studied by the removal of small kernel fraction from oat grains. A reduction of 56% was observed in T2+HT2 toxins in the samples from 2015 and 32% in the 2018 samples. The variation in the reduction levels between samples from 2015 and 2018 may have been be due to the considerably higher levels of T2+HT2 in the 2015 samples, thus

allowing a more efficient removal by sorting in these samples. Additionally, a reduction of DON by 24% was also observed in the 2018 samples. [178]. These studies show that cleaning and sorting methods are important for the reduction of mycotoxin contamination but are not sufficient for their complete removal.

1.6.2 Thermal processes

Thermal processes are commonly applied in order to preserve and heat food. These processes include methods such as boiling, frying, baking and steaming. Studies have been carried out to investigate the effect of heating on mycotoxin degradation. In one study, the reduction of AFB1 and OTA levels in rice was observed by cooking it in three different ways. In rice cooked normally, a reduction of 82.3% AFB1 and 83.1% OTA was observed. Rice cooked in excess water resulted in the removal of 89.1% AFB1 and 86.7% OTA. Rice cooked in the microwave resulted in the removal of over 72.5% AFB1 and 82.4% OTA [179]. In another study, roasting pistachio nuts spiked with AFB1 at 150°C for 120 min resulted in the degradation of 95% of the mycotoxin, although the resulting product was inedible. However, it was also shown that naturally occurring AFs were more heat resistant [180]. When heated at 150 °C for 60 min at pH 7, ZEN degradation of 34% was observed. This value was increased to 68% at pH 4 and pH 10. A complete reduction of ZEN was seen in less than 30 min at 225 °C regardless of the pH [181].

DON is a highly stable compound and has a melting point of 151-153 °C [182]. The effect of heat and pH on DON concentration was investigated in one study. It was found that DON was stable when it was heated at 100 °C and 120 °C for 60 min at pH 4 and 7. Some degradation was observed at a more alkaline pH of 10, where the concentration of DON was reduced from around 2.25 to 1.1 ppm at 100 °C. Under alkaline conditions at 120 °C, some degradation was observed after 15 min of heating and full degradation was observed after 30 min. At the highest temperature used in the experiment, 170 °C, full degradation was observed at pH 10 and pH 7 at 15 and 60 min, respectively. The results show that DON is stable at high temperatures and at neutral or acidic pH. Alkaline treatments were found to be more effective for reducing DON concentrations [183]. The stability of DON at high temperatures was also observed in a study by Lancova *et al.* who showed that there was no significant reduction of DON concentration with a bread baking process of 210 °C for 14 min [172]. This is similar to the results from another study where the effect of baking on DON contaminated flour was investigated by the making of

Egyptian bread. The bread was baked at 350 °C and there was no significant change in DON concentration following this treatment [184]. DON contaminated wheat from Canada was baked for 30 min at a temperature of 205 °C in another study investigating DON degradation with heat. It was found that DON was not destroyed upon making the bread [185]. The mycotoxin's stability at high temperatures and low pH is concerning and suggests that the usual cooking and baking processes associated with grains are not sufficient to completely remove mycotoxin contamination and highlights the need for more effective means of mitigation.

1.6.3 Physical adsorption

While cleaning and heating are processes carried out on grains prior to consumption, physical adsorption methods take place in the intestine of the animal during feed consumption, by use of adsorbent materials as feed additives, and are designed to bind to the mycotoxins and prevent their ingestion by the animal [186]. Adsorbent materials are materials with a large molecular weight and they can bind to the mycotoxins in contaminated feed without interfering with the gastrointestinal tract of the animals. Aluminosilicates, bentonites, zeolite, activated carbons and yeast cell walls are just some of the materials that have been used as mycotoxin adsorbent [187].

One *in vitro* study found that a mixture of activated charcoal and yeast cell wall (75:25) was highly efficient in the adsorption of ZEN (97.6 – 99.7%) [188]. Another study analysed the adsorbent capacities of chitosan (CHI), hydroxypropyl methylcellulose (HPMC), sodium carboxymethylcellulose (CMC) and microcrystalline cellulose (MCC) against various mycotoxins. The results showed that all four materials demonstrated a high adsorption capacity for ZEN with 89.7% as the highest (MCC) and 75.6% as the lowest (CHI). Each of the adsorbent materials showed the lowest adsorption percentage for DON, with the highest of these being 36.3% with CMC [189]. Elsewhere, the binding of OTA and ZEN by *Saccharomyces cerevisiae* yeast strains was analysed *in vitro*. All of the yeast strains were able to adsorb both OTA and ZEN but the binding levels varied between the strains. OTA binding levels varied between 71.3% ± 1.33 and 82.3% ± 6.02 and ZEN binding levels varied between 59.19% ± 4.29 and 97.8% ± 1.48 [190].

In a study by Sabater-Vilar *et al.*, the binding affinity of various smectites, humic substances and yeast cell walls on DON was investigated *in vitro*. It was found that there was no significant difference in the adsorption of DON with the materials tested when

compared to the control (with no binder). Only activated charcoal, which was the positive control, resulted in 90% adsorption of DON [191]. These results also comply with a study by Avantaggiato *et al*. where it was demonstrated that out of the 21 non-nutritive adsorbent materials that were tested *in vitro*, only activated charcoal was able to adsorb DON [192]. DON is a non-ionisable and hydrophilic molecule and has a bulky epoxy group, which along with its lack of polar groups could be the reason that it is not adsorbed efficiently [187,191].

These studies show that these feed supplements have the potential to adsorb and reduce the effects of mycotoxins on animals. However, the binding affinity of the different materials can vary depending on the mycotoxins and studies have been predominantly carried out *in vitro*, with fewer studies demonstrating comparable binding affinities *in vivo*.

1.6.4 Biotransformation

Biodegradation is another method of mycotoxin remediation. This method includes using biotransforming agents such as bacteria, fungi, yeasts and enzymes to transform toxic compounds into their less or non-toxic metabolites. Like the mycotoxin adsorbents, these agents are also added as feed additives in order to degrade the mycotoxins in the digestive tracts [187].

In order to detoxify DON it is important to target the group that is essential for its toxicity, the epoxide group. It was found that DON can be transformed into its de-epoxy form DOM-1 using microorganisms from catfish digesta microbial culture C133. DON in full medium with the culture was 100% transformed after 96 h incubation [193]. The bacterial *Coriobacteriaceae* strain BBSH 797 also biotransforms DON into the metabolite DOM-1 through enzymatic deactivation. BBSH 797 was isolated from bovine rumen fluid and it produces de-epoxidase enzymes. BBSH 797 is now sold commercially by Biomin as the ‘first and only microorganism authorized in Europe’ as a feed additive to reduce DON contamination of feed [186,187,194]. Deepoxidation of DON was also observed when the biodegradation of DON by a chicken intestinal microorganism was studied by Young *et al.* In this investigation, sub-cultures of the single colony microbial isolates LS100 (from the large intestine) and SS3 (from the small intestine) were used for the biodegradation of DON. As with the C133 and BBSH 797, these microorganisms also degraded of DON

by deepoxidation [187,195]. The deepoxy metabolite DOM-1 has been shown to be 500 times less toxic than DON due to the loss of the toxic epoxide group [100,101,187].

ZEN was biotransformed into its two main metabolites α- and β- ZOL by a mixed culture of *Candida tropicalis*, *Zygosaccharomyces rouxii* and seven *Saccharomyces* strains [196]. ZEN was also biotransformed into its metabolites and conjugates by members of the *Rhizopus* and *Aspergillus* genera. ZEN was added to liquid cultures of these fungal species and incubated for 3 days. Seven *Rhizopus* and two *Aspergillus* species converted ZEN into its various metabolites including ZEN-O-14-glucoside, ZEN-O-16-glucoside, ZEN-14-sulfate and α-zearalenol sulfate. The formation of α-ZOL was also observed [197]. However, α-ZOL has been reported to have a higher binding affinity to ERs and thus can be more toxic than ZEN [67]. Biotransformation agents could potentially be used as a remediation strategy for mycotoxin contamination in animal feed. However, these agents need to meet some requirements in order to be used as a feed additive. They must be safe to ingest and need to be able to degrade mycotoxins quickly and effectively into their non-toxic metabolites [187].

In summary, mycotoxin management is a major issue in the agri-food industries. Cleaning, sorting and heating are amongst the remediation strategies that have been studied. Although cleaning and sorting can somewhat reduce the amount of mycotoxins in grains, it cannot be relied on for the complete removal of mycotoxins. Due to the heat stability of the mycotoxins, heating is also not a reliable method of removal. The use of feed additives are increasingly being used for the removal toxins from the digestive tract of the animals. However, it has been shown that the binding capacity of such materials can vary with mycotoxins, therefore further research efforts in this area is required. Feed additives can also be used for the degradation of mycotoxins into their non-toxic metabolites in the digestive tract of the animals. However, further study is required here, as some biotransformation agents have been shown to transform mycotoxins into their more toxic metabolites. Although various mycotoxin remediation strategies exist, more work is still required in this field before it can be determined that mycotoxins can be effectively managed in grains. More specifically with feed additives, as *in vitro* experiments are increasingly popular, it is still important to compare and confirm the efficacy of these materials *in vivo.*

1.7 Thesis aims

ZEN is an oestrogenic mycotoxin that has been shown to have a negative effect on the reproductive function of animals. The structure of ZEN resembles that of naturally occurring oestrogens, whereby it to bind to oestrogenic receptors, resulting in hormonal disturbances. Contamination of animal feed with DON is also of particular concern for livestock production. Consumption of DON-contaminated feed can result in vomiting, diarrhoea, refusal of feed and reduced weight gain in animals. DON is commonly known as 'vomitoxin' due to its emetic effects upon ingestion. The main mode of action of this mycotoxin is the inhibition of protein synthesis by binding to the eukaryotic 60S ribosomal subunit. As ZEN and DON are both produced by the same fungal species, they are often found to co-occur in nature. It has been shown that pigs are most susceptible to both DON and ZEN toxicity. EFSA has suggested that the maximum level of DON in pig feed should not exceed 0.9 ppm and ZEN in feed for sows and fattening pigs should not exceed 0.25 ppm. It is important to understand the effects of the mycotoxins when combined as well as their individual effects. Here in particular there is a limited understanding of the combined impacts of ZEN and DON in pigs.

The negative health and economic impact of mycotoxins are of worldwide concern, with an estimated 25% of the world's crops being contaminated by these toxins annually, which makes it a major issue in the agri-food industry. There are remediation strategies, which partially protect against mycotoxins including cleaning and sorting, heating, physical adsorption and biotransformation. However, it is difficult to achieve complete removal of mycotoxins from contaminated feed. Feed supplements added to animal feed are one of the most promising avenues to ameliorate negative mycotoxin impacts, with *in vitro* and *in vitro* studies emerging that are aimed at both elucidating the molecular modes of action and determining the extent to which supplemental regimes are effective.

The first aim of this project was aims to investigate the effect of ZEN on IPEC-J2 cells at the maximum EFSA recommended level for pig feed of 0.25 ppm (Chapter 2). Following this, the ameliorative effect of the animal feed supplements Sel-Plex (Se-Y) and Mycosorb A+ (My-A+) against ZEN induced damage on IPEC-J2 cells was investigated. Se-Y is a commercially available selenium enriched yeast feed supplement. Selenium is known to have antioxidant properties and selenium enriched yeast is often used for its protective effects against various toxins. My-A+ is a commercially available

mycotoxin binder made up of algae that has been enriched with polyunsaturated fatty acids (PUFAs) which have antioxidant properties. It was found that pre-incubating the cells for 48 h with these supplements prior to exposure to mycotoxin resulted in a reduction of ZEN induced toxicity. The second aim was to study the effects of DON on IPEC-J2 cells at or below the EFSA recommended maximum value for pig feed of 0.9 ppm and the results obtained are shown in Chapter 3. The protective effects of Se-Y and My-A+ against DON induced toxicity was also examined here. The third aim of the study was to investigate the effects of co-exposure of IPEC-J2 cells to ZEN and DON (Chapter 4). The protective effects of the feed supplements against damage induced by the combined mycotoxins was also investigated.

As summarised in this chapter, both ZEN and DON have been shown to induce DNA damage in various cell types. Additionally, Se-Y and PUFAs have both been shown to mitigate against DNA damage due to their anti-oxidant properties. Thus, in order to investigate the protective effects of the feed supplements, there is a focus on the DNA damaging effects of the mycotoxins in this project.

Overall, this research contributes to our understanding of the toxic effects of ZEN and DON, both individually and when combined, on porcine intestinal epithelial cells and it provides evidence that supplementation with feed additives such as Se-Y and My-A+ may mitigate the effects of DON and ZEN.

1.8 References

(1) Rice, L. G.; Ross, P. F. Methods for Detection and Quantitation of Fumonisins in Corn, Cereal Products and Animal Excreta. *J Food Prot* **1994**, *57* (6), 536–540. https://doi.org/10.4315/0362-028X-57.6.536.

(2) Marin, S.; Ramos, A. J.; Cano-Sancho, G.; Sanchis, V. Mycotoxins: Occurrence, Toxicology, and Exposure Assessment. *Food Chem Toxicol* **2013**, *60*, 218–237. https://doi.org/10.1016/j.fct.2013.07.047.

(3) Wang, J.-S.; Groopman, J. D. DNA Damage by Mycotoxins. *Mutat Res* **1999**, *424* (1–2), 167–181. https://doi.org/10.1016/S0027-5107(99)00017-2.

(4) Peraica, M.; Radić, B.; Lucić, A.; Pavlović, M. Toxic Effects of Mycotoxins in Humans. *Bull World Health Organ* **1999**, *77* (9), 754–766.

(5) Hussein, H. Toxicity, Metabolism, and Impact of Mycotoxins on Humans and Animals. *Toxicology* **2001**, *167* (2), 101–134. https://doi.org/10.1016/S0300-483X(01)00471-1.

(6) Streit, E.; Naehrer, K.; Rodrigues, I.; Schatzmayr, G. Mycotoxin Occurrence in Feed and Feed Raw Materials Worldwide: Long-Term Analysis with Special Focus on Europe and Asia: Mycotoxin Occurrence in Feed and Feed Raw Materials Worldwide. *J Sci Food Agric* **2013**, *93* (12), 2892–2899. https://doi.org/10.1002/jsfa.6225.

(7) Bryden, W. L. Mycotoxin Contamination of the Feed Supply Chain: Implications for Animal Productivity and Feed Security. *Anim Feed Sci Tech* **2012**, *173* (1), 134–158. https://doi.org/10.1016/j.anifeedsci.2011.12.014.

(8) Pestka, J.; Casale, W. Naturally Occurring Fungal Toxins. *Environ Sci Technol* **1990**, *23*, 613–638.

(9) Jackson, L. S.; Bullerman, L. B. Effect of Processing on Fusarium Mycotoxins. In *Impact of Processing on Food Safety*; Jackson, L. S., Knize, M. G., Morgan, J. N., Eds.; Springer US: Boston, MA, 1999; Vol. 459, pp 243–261. https://doi.org/10.1007/978-1-4615-4853-9_16.

(10) Bennett, J. W.; Klich, M.; Mycotoxins, M. Mycotoxins. *Clinical microbiology reviews* **2003**, *16* (3), 497–516. https://doi.org/10.1128/CMR.16.3.497.

(11) Omotayo, O. P.; Omotayo, A. O.; Mwanza, M.; Babalola, O. O. Prevalence of Mycotoxins and Their Consequences on Human Health. *Toxicol Res* **2019**, *35* (1), 1–7. https://doi.org/10.5487/TR.2019.35.1.001.

(12) Bedard, L. L.; Massey, T. E. Aflatoxin B1-Induced DNA Damage and Its Repair. *Cancer Letters* **2006**, *241* (2), 174–183. https://doi.org/10.1016/j.canlet.2005.11.018.

(13) Wu, F.; Guclu, H. Aflatoxin Regulations in a Network of Global Maize Trade. *PLoS One* **2012**, *7* (9), e45151. https://doi.org/10.1371/journal.pone.0045151.

(14) Mycotoxins: Risks in Plant, Animal, and Human Systems. *Task Force Report* **2003**, *139*, 1–199.

(15) Robens, J.; Cardwell, K. The Costs of Mycotoxin Management to the USA: Management of Aflatoxins in the United States. *J Toxicol. Toxin Rev* **2003**, *22* (2–3), 139–152. https://doi.org/10.1081/TXR-120024089.

(16) Cardwell, K. F.; Desjardins, A.; Munkvold, G.; Robens, J. Mycotoxins: The Cost of Achieving Food Security and Food Quality. *APS Feature Articles* **2001**. https://doi.org/10.1094/APSnetFeature-2001-0901.

(17) International Agency for Research on Cancer. *New IARC Report Urges Action against Widespread Mycotoxin Contamination in Developing Countries*; Press Release 242; World Health Organisation, 2016; p 2.

(18) Zain, M. E. Impact of Mycotoxins on Humans and Animals. *J Saudi Chem Soc* **2011**, *15* (2), 129–144. https://doi.org/10.1016/j.jscs.2010.06.006.

(19) Bennett, J. W.; Klich, M. Mycotoxins. *Clin Microbiol Rev* **2003**, *16* (3), 497–516. https://doi.org/10.1128/CMR.16.3.497-516.2003.

(20) Sarma, U. P.; Bhetaria, P. J.; Devi, P.; Varma, A. Aflatoxins: Implications on Health. *Ind J Clin Biochem* **2017**, *32* (2), 124–133. https://doi.org/10.1007/s12291-017-0649-2.

(21) Rocha, O.; Ansari, K.; Doohan, F. M. Effects of Trichothecene Mycotoxins on Eukaryotic Cells: A Review. *Food Addit Contam* **2005**, *22* (4), 369–378. https://doi.org/10.1080/02652030500058403.

(22) Bui-Klimke, T. R.; Wu, F. Ochratoxin A and Human Health Risk: A Review of the Evidence. *Crit Rev Food Sci Nutr* **2015**, *55* (13), 1860–1869. https://doi.org/10.1080/10408398.2012.724480.

(23) Rogowska, A.; Pomastowski, P.; Sagandykova, G.; Buszewski, B. Zearalenone and Its Metabolites: Effect on Human Health, Metabolism and Neutralisation Methods. *Toxicon* **2019**, *162*, 46–56. https://doi.org/10.1016/j.toxicon.2019.03.004.

(24) Hueza, I. M.; Raspantini, P. C. F.; Raspantini, L. E. R.; Latorre, A. O.; Górniak, S. L. Zearalenone, an Estrogenic Mycotoxin, Is an Immunotoxic Compound. *Toxins (Basel)* **2014**, *6* (3), 1080–1095. https://doi.org/10.3390/toxins6031080.

(25) Antonissen, G.; Martel, A.; Pasmans, F.; Ducatelle, R.; Verbrugghe, E.; Vandenbroucke, V.; Li, S.; Haesebrouck, F.; Van Immerseel, F.; Croubels, S. The Impact of Fusarium Mycotoxins on Human and Animal Host Susceptibility to Infectious Diseases. *Toxins (Basel)* **2014**, *6* (2), 430–452. https://doi.org/10.3390/toxins6020430.

(26) McMullen, M.; Jones, R.; Gallenberg, D. Scab of Wheat and Barley: A Re-Emerging Disease of Devastating Impact. *Plant Dis* **1997**, *81* (12), 1340–1348. https://doi.org/10.1094/PDIS.1997.81.12.1340.

(27) Dweba, C. C.; Figlan, S.; Shimelis, H. A.; Motaung, T. E.; Sydenham, S.; Mwadzingeni, L.; Tsilo, T. J. Fusarium Head Blight of Wheat: Pathogenesis and Control Strategies. *Crop Prot* **2017**, *91*, 114–122. https://doi.org/10.1016/j.cropro.2016.10.002.

(28) Pleadin, J.; Vahčić, N.; Perši, N.; Ševelj, D.; Markov, K.; Frece, J. Fusarium Mycotoxins' Occurrence in Cereals Harvested from Croatian Fields. *Food Control* **2013**, *32* (1), 49–54. https://doi.org/10.1016/j.foodcont.2012.12.002.

(29) Lofgren, L. A.; LeBlanc, N. R.; Certano, A. K.; Nachtigall, J.; LaBine, K. M.; Riddle, J.; Broz, K.; Dong, Y.; Bethan, B.; Kafer, C. W.; Kistler, H. C. Fusarium Graminearum: Pathogen or Endophyte of North American Grasses? *New Phytol.* **2018**, *217* (3), 1203–1212. https://doi.org/10.1111/nph.14894.

(30) Yin, H.-B.; Chen, C.-H.; Kollanoor-Johny, A.; Darre, M. J.; Venkitanarayanan, K. Controlling *Aspergillus Flavus* and *Aspergillus Parasiticus* Growth and Aflatoxin Production in Poultry Feed Using Carvacrol and *Trans* - Cinnamaldehyde. *Poult Sci* **2015**, *94* (9), 2183–2190. https://doi.org/10.3382/ps/pev207.

(31) Abbas, H.; Wilkinson, J.; Zablotowicz, R.; Accinelli, C.; Abel, C.; Bruns, H.; Weaver, M. Ecology of *Aspergillus Flavus*, Regulation of Aflatoxin Production, and Management Strategies to Reduce Aflatoxin Contamination of Corn. *Toxin Rev.* **2009**, *28* (2–3), 142–153. https://doi.org/10.1080/15569540903081590.

(32) D'Mello, J. P. F.; Macdonald, A. M. C. Mycotoxins. *Anim Feed Sci Tech* **1997**, *69* (1), 155–166. https://doi.org/10.1016/S0377-8401(97)81630-6.

(33) Food Safety Authority of Irelan (FSAI). Mycotoxins - Contamination of Foodstuffs https://www.fsai.ie/legislation/food_legislation/contamination_of_foodstuffs/mycotoxins.html (accessed 2021 -04 -19).

(34) Reddy, L.; Bhoola, K. Ochratoxins—Food Contaminants: Impact on Human Health. *Toxins (Basel)* **2010**, *2* (4), 771–779. https://doi.org/10.3390/toxins2040771.

(35) Cabañes, F. J.; Bragulat, M. R.; Castellá, G. Ochratoxin A Producing Species in the Genus Penicillium. *Toxins (Basel)* **2010**, *2* (5), 1111–1120. https://doi.org/10.3390/toxins2051111.

(36) Welke, J. E.; Hoeltz, M.; Dottori, H. A.; Noll, I. B. Patulin Accumulation In Apples During Storage by Penicillium Expansum and Penicillium Griseofulvum Strains. *Braz J Microbiol* **2011**, *42* (1), 172–180. https://doi.org/10.1590/S1517-83822011000100021.

(37) Ismaiel, A. A.; Papenbrock, J. Mycotoxins: Producing Fungi and Mechanisms of Phytotoxicity. *Agriculture* **2015**, *5* (3), 492–537. https://doi.org/10.3390/agriculture5030492.

(38) Richard, J. L. Some Major Mycotoxins and Their Mycotoxicoses—An Overview. *Int J Food Microbiol* **2007**, *119* (1–2), 3–10. https://doi.org/10.1016/j.ijfoodmicro.2007.07.019.

(39) Kumar, P.; Mahato, D. K.; Kamle, M.; Mohanta, T. K.; Kang, S. G. Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management. *Front Microbiol.* **2017**, *07*, 2170. https://doi.org/10.3389/fmicb.2016.02170.

(40) Paterson, R. R. M.; Lima, N. How Will Climate Change Affect Mycotoxins in Food? *Food Res Int* **2010**, *43* (7), 1902–1914. https://doi.org/10.1016/j.foodres.2009.07.010.

(41) Milani, J. Ecological Conditions Affecting Mycotoxin Production in Cereals: A Review. *Vet Med (Praha)* **2013**, *58* (No. 8), 405–411. https://doi.org/10.17221/6979-VETMED.

(42) Jaimez, J.; Fente, C. A.; Vazquez, B. I.; Franco, C. M.; Cepeda, A.; Mahuzier, G.; Prognon, P. Application of the Assay of Aflatoxins by Liquid Chromatography with Fluorescence Detection in Food Analysis. *J Chromatogr A.* **2000**, *882* (1–2), 1–10. https://doi.org/10.1016/S0021-9673(00)00212-0.

(43) Adam, A.; Adam, M.; Tabana, Y. M.; Musa, K. B.; Sandai, D. A. Effects of Different Mycotoxins on Humans, Cell Genome and Their Involvement in Cancer (Review). *Oncol Rep.* **2017**, *37* (3), 1321–1336. https://doi.org/10.3892/or.2017.5424.

(44) Jalili, M. A Review on Aflatoxins Reduction in Food. *Iran J Health Saf Environ.* **2016**, *3* (1), 15. http://ijhse.ir/index.php/IJHSE/article/view/136.

(45) Heussner, A. H.; Bingle, L. E. H. Comparative Ochratoxin Toxicity: A Review of the Available Data. *Toxins (Basel)* **2015**, *7* (10), 4253–4282. https://doi.org/10.3390/toxins7104253.

(46) El Khoury, A.; Atoui, A. Ochratoxin A: General Overview and Actual Molecular Status. *Toxins (Basel)* **2010**, *2* (4), 461–493. https://doi.org/10.3390/toxins2040461.

(47) IARC (International Agency For Research on Cancer). *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*; International Agency for Research on Cancer, Ed.; Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins: views and expert opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, which met in Lyon, 9 - 16 June 1992; Lyon, 1993; Vol. 56.

(48) Hope, J. H.; Hope, B. E. A Review of the Diagnosis and Treatment of Ochratoxin A Inhalational Exposure Associated with Human Illness and Kidney Disease Including Focal Segmental Glomerulosclerosis. *J Environ Public Health* **2011**, *2012*, 835059. https://doi.org/10.1155/2012/835059.

(49) Limay-Rios, V.; Miller, J. D.; Schaafsma, A. W. Occurrence of Penicillium Verrucosum, Ochratoxin A, Ochratoxin B and Citrinin in on-Farm Stored Winter Wheat from the Canadian Great Lakes Region. *PLoS One* **2017**, *12* (7), e0181239. https://doi.org/10.1371/journal.pone.0181239.

(50) Gupta, R. C.; Srivastava, A.; Lall, R. Chapter 72 - Ochratoxins and Citrinin. In *Veterinary Toxicology (Third Edition)*; Gupta, R. C., Ed.; Academic Press, 2018; pp 1019–1027. https://doi.org/10.1016/B978-0-12-811410-0.00072-6.

(51) McCormick, S. P.; Stanley, A. M.; Stover, N. A.; Alexander, N. J. Trichothecenes: From Simple to Complex Mycotoxins. *Toxins (Basel)* **2011**, *3* (7), 802–814. https://doi.org/10.3390/toxins3070802.

(52) Pestka, J. J. Mechanisms of Deoxynivalenol-Induced Gene Expression and Apoptosis. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **2008**, *25* (9), 1128–1140. https://doi.org/10.1080/02652030802056626.

(53) Kimura, M.; Tokai, T.; Takahashi-Ando, N.; Ohsato, S.; Fujimura, M. Molecular and Genetic Studies of Fusarium Trichothecene Biosynthesis: Pathways, Genes,

and Evolution. *Biosci Biotechnol Biochem* **2007**, *71* (9), 2105–2123. https://doi.org/10.1271/bbb.70183.

(54) Adhikari, M.; Negi, B.; Kaushik, N.; Adhikari, A.; Al-Khedhairy, A. A.; Kaushik, N. K.; Choi, E. H. T-2 Mycotoxin: Toxicological Effects and Decontamination Strategies. *Oncotarget* **2017**, *8* (20), 33933–33952. https://doi.org/10.18632/oncotarget.15422.

(55) Foroud, N. A.; Eudes, F. Trichothecenes in Cereal Grains. *Int J Mol Sci* **2009**, *10* (1), 147–173. https://doi.org/10.3390/ijms10010147.

(56) Llorens, A.; Mateo, R.; Hinojo, M. J.; Valle-Algarra, F. M.; Jiménez, M. Influence of Environmental Factors on the Biosynthesis of Type B Trichothecenes by Isolates of Fusarium Spp. from Spanish Crops. *Int J Food Microbiol* **2004**, *94* (1), 43–54. https://doi.org/10.1016/j.ijfoodmicro.2003.12.017.

(57) Beev, G.; Denev, S.; Bakalova, D. Zearalenone - Producing Activity of Fusarium Graminearum and Fusarium Oxysporum Isolated from Bulgarian Wheat. *Bulg J Agric Sci* **2013**, *19*, 255–259.

(58) Kumar, V.; Basu, M. S.; Rajendran, T. P. Mycotoxin Research and Mycoflora in Some Commercially Important Agricultural Commodities. *Crop Prot* **2008**, *27* (6), 891–905. https://doi.org/10.1016/j.cropro.2007.12.011.

(59) Marin, D. E.; Taranu, I.; Burlacu, R.; Tudor, D. S. Effects of Zearalenone and Its Derivatives on the Innate Immune Response of Swine. *Toxicon* **2010**, *56* (6), 956–963. https://doi.org/10.1016/j.toxicon.2010.06.020.

(60) Yazar, S.; Omurtag, G. Z. Fumonisins, Trichothecenes and Zearalenone in Cereals. *Int J Mol Sci* **2008**, *9* (11), 2062–2090. https://doi.org/10.3390/ijms9112062.

(61) Tatay, E.; Espín, S.; García-Fernández, A.-J.; Ruiz, M.-J. Estrogenic Activity of Zearalenone, α-Zearalenol and β-Zearalenol Assessed Using the E-Screen Assay in MCF-7 Cells. *Toxicol Mech Methods* **2018**, *28* (4), 239–242. https://doi.org/10.1080/15376516.2017.1395501.

(62) Minervini, F.; Dell'Aquila, M. E. Zearalenone and Reproductive Function in Farm Animals. *Int J Mol Sci* **2008**, *9* (12), 2570–2584. https://doi.org/10.3390/ijms9122570.

(63) Zinedine, A.; Soriano, J. M.; Moltó, J. C.; Mañes, J. Review on the Toxicity, Occurrence, Metabolism, Detoxification, Regulations and Intake of Zearalenone: An Oestrogenic Mycotoxin. *Food Chem Toxicol* **2007**, *45* (1), 1–18. https://doi.org/10.1016/j.fct.2006.07.030.

(64) IARC (International Agency For Research on Cancer). List of Classifications – IARC Monographs on the Identification of Carcinogenic Hazards to Humans https://monographs.iarc.fr/list-of-classifications/ (accessed 2020 -06 -03).

(65) Olsen, M.; Pettersson, H.; Kiessling, K.-H. Reduction of Zearalenone to Zearalenol in Female Rat Liver by 3α-Hydroxysteroid Dehydrogenase. *Acta Pharmacol Toxicol (Copenh)* **1981**, *48* (2), 157–161. https://doi.org/10.1111/j.1600-0773.1981.tb01602.x.

(66) Zhang, G.-L.; Feng, Y.-L.; Song, J.-L.; Zhou, X.-S. Zearalenone: A Mycotoxin With Different Toxic Effect in Domestic and Laboratory Animals' Granulosa Cells. *Front Genet.* **2018**, *9*, 667. https://doi.org/10.3389/fgene.2018.00667.

(67) Shier, W. T.; Shier, A. C.; Xie, W.; Mirocha, C. J. Structure-Activity Relationships for Human Estrogenic Activity in Zearalenone Mycotoxins. *Toxicon* **2001**, *39* (9), 1435–1438. https://doi.org/10.1016/S0041-0101(00)00259-2.

(68) Yang, C.; Song, G.; Lim, W. Effects of Mycotoxin-Contaminated Feed on Farm Animals. *J Hazard Mater* **2020**, *389*, 122087. https://doi.org/10.1016/j.jhazmat.2020.122087.

(69) Malekinejad, H.; Maas-Bakker, R.; Fink-Gremmels, J. Species Differences in the Hepatic Biotransformation of Zearalenone. *Vet J* **2006**, *172* (1), 96–102. https://doi.org/10.1016/j.tvjl.2005.03.004.

(70) Kos, J.; Janić Hajnal, E.; Malachová, A.; Steiner, D.; Stranska, M.; Krska, R.; Poschmaier, B.; Sulyok, M. Mycotoxins in Maize Harvested in Republic of Serbia in the Period 2012–2015. Part 1: Regulated Mycotoxins and Its Derivatives.

Food Chem **2020**, *312*, 126034. https://doi.org/10.1016/j.foodchem.2019.126034.

(71) Stanciu, O.; Juan, C.; Berrada, H.; Miere, D.; Loghin, F.; Mañes, J. Study on Trichothecene and Zearalenone Presence in Romanian Wheat Relative to Weather Conditions. *Toxins* **2019**, *11* (3), 163. https://doi.org/10.3390/toxins11030163.

(72) Gruber-Dorninger, C.; Jenkins, T.; Schatzmayr, G. Global Mycotoxin Occurrence in Feed: A Ten-Year Survey. *Toxins* **2019**, *11* (7), 375. https://doi.org/10.3390/toxins11070375.

(73) Golge, O.; Kabak, B. Occurrence of Deoxynivalenol and Zearalenone in Cereals and Cereal Products from Turkey. *Food Control* **2020**, *110*, 106982. https://doi.org/10.1016/j.foodcont.2019.106982.

(74) Pante, G. C.; Silva, M. V.; Romoli, J. C. Z.; Rocha, G. H. O.; Bando, E.; Nerilo, S. B.; Mossini, S. A. G.; Machinski, M. Occurrence of Zearalenone in Corn Meal Commercialized in South Region of Brazil and Daily Intake Estimates in the Brazilian Population. *J Food Saf* **2019**, *39* (5), e12672. https://doi.org/10.1111/jfs.12672.

(75) Rai, A.; Dixit, S.; Singh, S. P.; Gautam, N. K.; Das, M.; Tripathi, A. Presence of Zearalenone in Cereal Grains and Its Exposure Risk Assessment in Indian Population. *J Food Sci* **2018**, *83* (12), 3126–3133. https://doi.org/10.1111/1750-3841.14404.

(76) Czembor, E.; Stępień, Ł.; Waśkiewicz, A. Effect of Environmental Factors on Fusarium Species and Associated Mycotoxins in Maize Grain Grown in Poland. *PLoS One* **2015**, *10* (7), e0133644. https://doi.org/10.1371/journal.pone.0133644.

(77) European Food Safety Authority. Mycotoxins | European Food Safety Authority https://www.efsa.europa.eu/en/topics/topic/mycotoxins (accessed 2021 -04 -04).

(78) European Commission. *Commission Regulation (EC) No 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs*; L 364/5; 2006.

(79) Wang, X.; Yu, H.; Fang, H.; Zhao, Y.; Jin, Y.; Shen, J.; Zhou, C.; Zhou, Y.; Fu, Y.; Wang, J.; Zhang, J. Transcriptional Profiling of Zearalenone-Induced Inhibition of IPEC-J2 Cell Proliferation. *Toxicon* **2019**, *172*, 8–14. https://doi.org/10.1016/j.toxicon.2019.10.004.

(80) Kouadio, J. H.; Mobio, T. A.; Baudrimont, I.; Moukha, S.; Dano, S. D.; Creppy, E. E. Comparative Study of Cytotoxicity and Oxidative Stress Induced by Deoxynivalenol, Zearalenone or Fumonisin B1 in Human Intestinal Cell Line Caco-2. *Toxicology* **2005**, *213* (1), 56–65. https://doi.org/10.1016/j.tox.2005.05.010.

(81) Hassen, W.; Golli, E. E.; Baudrimont, I.; Mobio, A. T.; Ladjimi, M. M.; Creppy, E. E.; Bacha, H. Cytotoxicity and Hsp 70 Induction in Hep G2 Cells in Response to Zearalenone and Cytoprotection by Sub-Lethal Heat Shock. *Toxicology* **2005**, *207* (2), 293–301. https://doi.org/10.1016/j.tox.2004.10.001.

(82) Marin, D. E.; Pistol, G. C.; Bulgaru, C. V.; Taranu, I. Cytotoxic and Inflammatory Effects of Individual and Combined Exposure of HepG2 Cells to Zearalenone and Its Metabolites. *Naunyn-Schmiedeberg's Arch Pharmacol* **2019**, *392* (8), 937–947. https://doi.org/10.1007/s00210-019-01644-z.

(83) Abassi, H.; Ayed-Boussema, I.; Shirley, S.; Abid, S.; Bacha, H.; Micheau, O. The Mycotoxin Zearalenone Enhances Cell Proliferation, Colony Formation and Promotes Cell Migration in the Human Colon Carcinoma Cell Line HCT116. *Toxicol Lett* **2016**, *254*, 1–7. https://doi.org/10.1016/j.toxlet.2016.04.012.

(84) Wan, L. Y. M.; Turner, P. C.; El-Nezami, H. Individual and Combined Cytotoxic Effects of Fusarium Toxins (Deoxynivalenol, Nivalenol, Zearalenone and Fumonisins B1) on Swine Jejunal Epithelial Cells. *Food Chem Toxicol* **2013**, *57*, 276–283. https://doi.org/10.1016/j.fct.2013.03.034.

(85) Gao, F.; Jiang, L.; Chen, M.; Geng, C.; Yang, G.; Ji, F.; Zhong, L.; Liu, X. Genotoxic Effects Induced by Zearalenone in a Human Embryonic Kidney Cell Line. *Mutat Res.* **2013**, *755* (1), 6–10. https://doi.org/10.1016/j.mrgentox.2013.04.009.

(86) Venkataramana, M.; Chandra Nayaka, S.; Anand, T.; Rajesh, R.; Aiyaz, M.; Divakara, S. T.; Murali, H. S.; Prakash, H. S.; Lakshmana Rao, P. V. Zearalenone Induced Toxicity in SHSY-5Y Cells: The Role of Oxidative Stress Evidenced by N-Acetyl Cysteine. *Food Chem Toxicol* **2014**, *65*, 335–342. https://doi.org/10.1016/j.fct.2013.12.042.

(87) Hassen, W.; Ayed-Boussema, I.; Oscoz, A. A.; De Cerain Lopez, A.; Bacha, H. The Role of Oxidative Stress in Zearalenone-Mediated Toxicity in Hep G2 Cells: Oxidative DNA Damage, Gluthatione Depletion and Stress Proteins Induction. *Toxicology* **2007**, *232* (3), 294–302. https://doi.org/10.1016/j.tox.2007.01.015.

(88) Fu, Y.; Jin, Y.; Zhao, Y.; Shan, A.; Fang, H.; Shen, J.; Zhou, C.; Yu, H.; Zhou, Y. F.; Wang, X.; Wang, J.; Li, R.; Wang, R.; Zhang, J. Zearalenone Induces Apoptosis in Bovine Mammary Epithelial Cells by Activating Endoplasmic Reticulum Stress. *J Dairy Sci.* **2019**, *102* (11), 10543–10553. https://doi.org/10.3168/jds.2018-16216.

(89) Zhang, F.-L.; Li, N.; Wang, H.; Ma, J.-M.; Shen, W.; Li, L. Zearalenone Exposure Induces the Apoptosis of Porcine Granulosa Cells and Changes Long Noncoding RNA Expression To Promote Antiapoptosis by Activating the JAK2–STAT3 Pathway. *J Agric Food Chem* **2019**, *67* (43), 12117–12128. https://doi.org/10.1021/acs.jafc.9b05189.

(90) Cai, G.; Si, M.; Li, X.; Zou, H.; Gu, J.; Yuan, Y.; Liu, X.; Liu, Z.; Bian, J. Zearalenone Induces Apoptosis of Rat Sertoli Cells through Fas-Fas Ligand and Mitochondrial Pathway. *Enrivon Toxicol* **2019**, *34* (4), 424–433. https://doi.org/10.1002/tox.22696.

(91) Wang, Y.-L.; Zhou, X.-Q.; Jiang, W.-D.; Wu, P.; Liu, Y.; Jiang, J.; Wang, S.-W.; Kuang, S.-Y.; Tang, L.; Feng, L. Effects of Dietary Zearalenone on Oxidative Stress, Cell Apoptosis, and Tight Junction in the Intestine of Juvenile Grass Carp

(Ctenopharyngodon Idella). *Toxins (Basel)* **2019**, *11* (6), 333. https://doi.org/10.3390/toxins11060333.

(92) European Commission. *Commission Recommendation of 17 August 2006 on the Presence of Deoxynivalenol, Zearalenone, Ochratoxin A, T-2 and HT-2 and Fumonisins in Products Intended for Animal Feeding*; Official J. Eur. Union 32006H0576; 2006; Vol. 229.

(93) Pestka, J. J. Deoxynivalenol: Toxicity, Mechanisms and Animal Health Risks. *Animal Feed Science and Technology* **2007**, *137* (3–4), 283–298. https://doi.org/10.1016/j.anifeedsci.2007.06.006.

(94) Nagy, C. M.; Fejer, S. N.; Berek, L.; Molnar, J.; Viskolcz, B. Hydrogen Bondings in Deoxynivalenol (DON) Conformations—a Density Functional Study. *J Mol Struct* **2005**, *726* (1–3), 55–59. https://doi.org/10.1016/j.theochem.2005.02.079.

(95) Tian, Y.; Tan, Y.; Liu, N.; Yan, Z.; Liao, Y.; Chen, J.; De Saeger, S.; Yang, H.; Zhang, Q.; Wu, A. Detoxification of Deoxynivalenol via Glycosylation Represents Novel Insights on Antagonistic Activities of Trichoderma When Confronted with Fusarium Graminearum. *Toxins* **2016**, *8* (11). https://doi.org/10.3390/toxins8110335.

(96) Ueno, Y.; Nakajima, M.; Sakai, K.; Ishii, K.; Sato, N.; Shimada, N. Comparative Toxicology of Trichothec Mycotoxins: Inhibition of Protein Synthesis in Animal Cells. *J Biochem* **1973**, *74* (2), 285–296.

(97) Karlovsky, P. Biological Detoxification of the Mycotoxin Deoxynivalenol and Its Use in Genetically Engineered Crops and Feed Additives. *Appl Microbiol Biotechnol* **2011**, *91* (3), 491–504. https://doi.org/10.1007/s00253-011-3401-5.

(98) Wu, Q.; Dohnal, V.; Kuca, K.; Yuan, Z. Trichothecenes: Structure-Toxic Activity Relationships. *Curr Drug Metab* **2013**, *14* (6), 641–660. https://doi.org/10.2174/1389200211314060002.

(99) Sundstøl Eriksen, G.; Pettersson, H.; Lundh, T. Comparative Cytotoxicity of Deoxynivalenol, Nivalenol, Their Acetylated Derivatives and de-Epoxy Metabolites. *Food Chem Toxicol* **2004**, *42* (4), 619–624. https://doi.org/10.1016/j.fct.2003.11.006.

(100) Mayer, E.; Novak, B.; Springler, A.; Schwartz-Zimmermann, H. E.; Nagl, V.; Reisinger, N.; Hessenberger, S.; Schatzmayr, G. Effects of Deoxynivalenol (DON) and Its Microbial Biotransformation Product Deepoxy-Deoxynivalenol (DOM-1) on a Trout, Pig, Mouse, and Human Cell Line. *Mycotoxin Res* **2017**, *33* (4), 297–308. https://doi.org/10.1007/s12550-017-0289-7.

(101) Springler, A.; Hessenberger, S.; Reisinger, N.; Kern, C.; Nagl, V.; Schatzmayr, G.; Mayer, E. Deoxynivalenol and Its Metabolite Deepoxy-Deoxynivalenol: Multi-Parameter Analysis for the Evaluation of Cytotoxicity and Cellular Effects. *Mycotoxin Res* **2017**, *33* (1), 25–37. https://doi.org/10.1007/s12550-016-0260-z.

(102) Shima, J.; Takase, S.; Takahashi, Y.; Iwai, Y.; Fujimoto, H.; Yamazaki, M.; Ochi, K. Novel Detoxification of the Trichothecene Mycotoxin Deoxynivalenol by a Soil Bacterium Isolated by Enrichment Culture. *Appl Environ Microbiol* **1997**, *63* (10), 3825–3830. https://doi.org/10.1128/aem.63.10.3825-3830.1997.

(103) Betina, V. Structure-Activity Relationships among Mycotoxins. *Chem Biol Interact* **1989**, *71* (2–3), 105–146. https://doi.org/10.1016/0009-2797(89)90030-6.

(104) Cheat, S.; Gerez, J. R.; Cognié, J.; Alassane-Kpembi, I.; Bracarense, A. P. F. L.; Raymond-Letron, I.; Oswald, I. P.; Kolf-Clauw, M. Nivalenol Has a Greater Impact than Deoxynivalenol on Pig Jejunum Mucosa in Vitro on Explants and in Vivo on Intestinal Loops. *Toxins (Basel)* **2015**, *7* (6), 1945–1961. https://doi.org/10.3390/toxins7061945.

(105) Yang, Y.; Yu, S.; Tan, Y.; Liu, N.; Wu, A. Individual and Combined Cytotoxic Effects of Co-Occurring Deoxynivalenol Family Mycotoxins on Human Gastric Epithelial Cells. *Toxins (Basel)* **2017**, *9* (3), 96. https://doi.org/10.3390/toxins9030096.

(106) Wu, Q.; Dohnal, V.; Huang, L.; Kuča, K.; Yuan, Z. Metabolic Pathways of Trichothecenes. *Drug Metab Rev* **2010**, *42* (2), 250–267. https://doi.org/10.3109/03602530903125807.

(107) He, J. W.; Bondy, G. S.; Zhou, T.; Caldwell, D.; Boland, G. J.; Scott, P. M. Toxicology of 3-Epi-Deoxynivalenol, a Deoxynivalenol-Transformation Product by Devosia Mutans 17-2-E-8. *Food Chem Toxicol.* **2015**, *84*, 250–259. https://doi.org/10.1016/j.fct.2015.09.003.

(108) Ehrlich, K. C.; Daigle, K. W. Protein Synthesis Inhibition by 8-Oxo-12,13-Epoxytrichothecenes. *Biochim Biophys Acta* . **1987**, *923* (2), 206–213. https://doi.org/10.1016/0304-4165(87)90005-5.

(109) EFSA. Opinion of the Scientific Panel on Contaminants in the Food Chain [CONTAM] Related to Deoxynivalenol (DON) as Undesirable Substance in Animal Feed. *EFSA J* **2004**, *2* (6), 73. https://doi.org/10.2903/j.efsa.2004.73.

(110) De Walle, J. V.; Sergent, T.; Piront, N.; Toussaint, O.; Schneider, Y.-J.; Larondelle, Y. Deoxynivalenol Affects in Vitro Intestinal Epithelial Cell Barrier Integrity through Inhibition of Protein Synthesis. *Toxicol Appl Pharmacol* **2010**, *245* (3), 291–298. https://doi.org/10.1016/j.taap.2010.03.012.

(111) Ghareeb, K.; Awad, W. A.; Soodoi, C.; Sasgary, S.; Strasser, A.; Böhm, J. Effects of Feed Contaminant Deoxynivalenol on Plasma Cytokines and MRNA Expression of Immune Genes in the Intestine of Broiler Chickens. *PLoS One* **2013**, *8* (8), e71492. https://doi.org/10.1371/journal.pone.0071492.

(112) Dänicke, S.; Goyarts, T.; Döll, S.; Grove, N.; Spolders, M.; Flachowsky, G. Effects of the Fusarium Toxin Deoxynivalenol on Tissue Protein Synthesis in Pigs. *Toxicol Lett* **2006**, *165* (3), 297–311. https://doi.org/10.1016/j.toxlet.2006.05.006.

(113) Mishra, S.; Srivastava, S.; Dewangan, J.; Divakar, A.; Kumar Rath, S. Global Occurrence of Deoxynivalenol in Food Commodities and Exposure Risk Assessment in Humans in the Last Decade: A Survey. *Crit Rev Food Sci Nutr* **2019**, *60* (8), 1346–1374. https://doi.org/10.1080/10408398.2019.1571479.

(114) Tola, M.; Kebede, B. Occurrence, Importance and Control of Mycotoxins: A Review. *Cogent Food Agric* **2016**, *2* (1), 1191103. https://doi.org/10.1080/23311932.2016.1191103.

(115) Rodrigues, I.; Naehrer, K. A Three-Year Survey on the Worldwide Occurrence of Mycotoxins in Feedstuffs and Feed. *Toxins (Basel)* **2012**, *4* (9), 663–675. https://doi.org/10.3390/toxins4090663.

(116) Park, J.; Chang, H.; Kim, D.; Chung, S.; Lee, C. Long-Term Occurrence of Deoxynivalenol in Feed and Feed Raw Materials with a Special Focus on South Korea. *Toxins (Basel)* **2018**, *10* (3), 127. https://doi.org/10.3390/toxins10030127.

(117) Rotter, B. A.; Prelusky, D. B.; Pestka, J. J. Toxicology of Deoxynivalenol (Vomitoxin). *J Toxicol Environ Health* **1996**, *48* (1), 1–34. https://doi.org/10.1080/009841096161447.

(118) Audenaert, K.; Vanheule, A.; Höfte, M.; Haesaert, G. Deoxynivalenol: A Major Player in the Multifaceted Response of Fusarium to Its Environment. *Toxins (Basel)* **2013**, *6* (1), 1–19. https://doi.org/10.3390/toxins6010001.

(119) Pestka, J. J.; Smolinski, A. T. Deoxynivalenol: Toxicology and Potential Effects on Humans. *J Toxicol Environ Health B Crit Rev* **2005**, *8* (1), 39–69. https://doi.org/10.1080/10937400590889458.

(120) Hoogenboom, L. A. P.; Bokhorst, J. G.; Northolt, M. D.; van de Vijver, L. P. L.; Broex, N. J. G.; Mevius, D. J.; Meijs, J. A. C.; Van der Roest, J. Contaminants and Microorganisms in Dutch Organic Food Products: A Comparison with Conventional Products. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **2008**, *25* (10), 1195–1207. https://doi.org/10.1080/02652030802014930.

(121) Giraud, F.; Pasquali, M.; Jarroudi, M. E.; Vrancken, C.; Brochot, C.; Cocco, E.; Hoffmann, L.; Delfosse, P.; Bohn, T. Fusarium Head Blight and Associated Mycotoxin Occurrence on Winter Wheat in Luxembourg in 2007/2008. *Food Addit Contam. Part A* **2010**, *27* (6), 825–835. https://doi.org/10.1080/19440040903567232.

(122) van der Fels-Klerx, H. J.; de Rijk, T. C.; Booij, C. J. H.; Goedhart, P. W.; Boers, E. a. M.; Zhao, C.; Waalwijk, C.; Mol, H. G. J.; van der Lee, T. a. J. Occurrence of Fusarium Head Blight Species and Fusarium Mycotoxins in Winter Wheat in the Netherlands in 2009. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **2012**, *29* (11), 1716–1726. https://doi.org/10.1080/19440049.2012.685891.

(123) González, H. H. L.; Moltó, G. A.; Pacin, A.; Resnik, S. L.; Zelaya, M. J.; Masana, M.; Martínez, E. J. Trichothecenes and Mycoflora in Wheat Harvested in Nine Locations in Buenos Aires Province, Argentina. *Mycopathologia* **2008**, *165* (2), 105–114. https://doi.org/10.1007/s11046-007-9084-x.

(124) Ji, F.; Xu, J.; Liu, X.; Yin, X.; Shi, J. Natural Occurrence of Deoxynivalenol and Zearalenone in Wheat from Jiangsu Province, China. *Food Chem* **2014**, *157*, 393–397. https://doi.org/10.1016/j.foodchem.2014.02.058.

(125) Shephard, G. S.; van der Westhuizen, L.; Katerere, D. R.; Herbst, M.; Pineiro, M. Preliminary Exposure Assessment of Deoxynivalenol and Patulin in South Africa. *Mycotoxin Res* **2010**, *26* (3), 181–185. https://doi.org/10.1007/s12550-010-0052-9.

(126) Calori-Domingues, M. A.; Bernardi, C. M. G.; Nardin, M. S.; de Souza, G. V.; dos Santos, F. G. R.; Stein, M. de A.; Gloria, E. M. da; Dias, C. T. dos S.; de Camargo, A. C. Co-Occurrence and Distribution of Deoxynivalenol, Nivalenol and Zearalenone in Wheat from Brazil. *Food Addit Contam Part B Surveill* **2016**, *9* (2), 142–151. https://doi.org/10.1080/19393210.2016.1152598.

(127) Marques, M. F.; Martins, H. M.; Costa, J. M.; Bernardo, F. Co-Occurrence of Deoxynivalenol and Zearalenone in Crops Marketed in Portugal. *Food Addit Contam Part B Surveill* **2008**, *1* (2), 130–133. https://doi.org/10.1080/02652030802253983.

(128) Janaviciene, S.; Mankeviciene, A.; Suproniene, S.; Kochiieru, Y.; Keriene, I. The Prevalence of Deoxynivalenol and Its Derivatives in the Spring Wheat Grain from

Different Agricultural Production Systems in Lithuania. *Food Additives & Contaminants: Part A* **2018**, *35* (6), 1179–1188. https://doi.org/10.1080/19440049.2018.1427893.

(129) Xu, W.; Han, X.; Li, F. Co-Occurrence of Multi-Mycotoxins in Wheat Grains Harvested in Anhui Province, China. *Food Control* **2019**, *96*, 180–185. https://doi.org/10.1016/j.foodcont.2018.09.006.

(130) Waché, Y. J.; Valat, C.; Postollec, G.; Bougeard, S.; Burel, C.; Oswald, I. P.; Fravalo, P. Impact of Deoxynivalenol on the Intestinal Microflora of Pigs. *Int J Mol Sci* **2008**, *10* (1), 1–17. https://doi.org/10.3390/ijms10010001.

(131) Kolf-Clauw, M.; Castellote, J.; Joly, B.; Bourges-Abella, N.; Raymond-Letron, I.; Pinton, P.; Oswald, I. P. Development of a Pig Jejunal Explant Culture for Studying the Gastrointestinal Toxicity of the Mycotoxin Deoxynivalenol: Histopathological Analysis. *Toxicology in Vitro* **2009**, *23* (8), 1580–1584. https://doi.org/10.1016/j.tiv.2009.07.015.

(132) Awad, W. A.; Aschenbach, J. R.; Zentek, J. Cytotoxicity and Metabolic Stress Induced by Deoxynivalenol in the Porcine Intestinal IPEC-J2 Cell Line: Deoxynivalenol Effects on Intestinal Epithelial Cells. *J Anim Physiol Anim Nutr (Berl)* **2012**, *96* (4), 709–716. https://doi.org/10.1111/j.1439-0396.2011.01199.x.

(133) Zhang, X.; Jiang, L.; Geng, C.; Cao, J.; Zhong, L. The Role of Oxidative Stress in Deoxynivalenol-Induced DNA Damage in HepG2 Cells. *Toxicon* **2009**, *54* (4), 513–518. https://doi.org/10.1016/j.toxicon.2009.05.021.

(134) Bony, S.; Carcelen, M.; Olivier, L.; Devaux, A. Genotoxicity Assessment of Deoxynivalenol in the Caco-2 Cell Line Model Using the Comet Assay. *Toxicol Lett* **2006**, *166* (1), 67–76. https://doi.org/10.1016/j.toxlet.2006.04.010.

(135) Hégarat, L. L.; Takakura, N.; Simar, S.; Nesslany, F.; Fessard, V. The in Vivo Genotoxicity Studies on Nivalenol and Deoxynivalenol. *EFSA Supporting Publications* **2014**, 33 pp. https://doi.org/10.2903/sp.efsa.2014.EN-697.

(136) Groschwitz, K. R.; Hogan, S. P. Intestinal Barrier Function: Molecular Regulation and Disease Pathogenesis. *J Allergy Clin Immunol* **2009**, *124* (1), 3–22. https://doi.org/10.1016/j.jaci.2009.05.038.

(137) Payros, D.; Alassane-Kpembi, I.; Pierron, A.; Loiseau, N.; Pinton, P.; Oswald, I. P. Toxicology of Deoxynivalenol and Its Acetylated and Modified Forms. *Arch Toxicol* **2016**, *90* (12), 2931–2957. https://doi.org/10.1007/s00204-016-1826-4.

(138) Srinivasan, B.; Kolli, A. R.; Esch, M. B.; Abaci, H. E.; Shuler, M. L.; Hickman, J. J. TEER Measurement Techniques for in Vitro Barrier Model Systems. *J Lab Autom* **2015**, *20* (2), 107–126. https://doi.org/10.1177/2211068214561025.

(139) Pinton, P.; Nougayrède, J.-P.; Del Rio, J.-C.; Moreno, C.; Marin, D. E.; Ferrier, L.; Bracarense, A.-P.; Kolf-Clauw, M.; Oswald, I. P. The Food Contaminant Deoxynivalenol, Decreases Intestinal Barrier Permeability and Reduces Claudin Expression. *Toxicol Appl Pharmacol* **2009**, *237* (1), 41–48. https://doi.org/10.1016/j.taap.2009.03.003.

(140) Zhang, Z.-Q.; Wang, S.-B.; Wang, R.-G.; Zhang, W.; Wang, P.-L.; Su, X.-O. Phosphoproteome Analysis Reveals the Molecular Mechanisms Underlying Deoxynivalenol-Induced Intestinal Toxicity in IPEC-J2 Cells. *Toxins (Basel)* **2016**, *8* (10), 270. https://doi.org/10.3390/toxins8100270.

(141) Smith, M.-C.; Hymery, N.; Troadec, S.; Pawtowski, A.; Coton, E.; Madec, S. Hepatotoxicity of Fusariotoxins, Alone and in Combination, towards the HepaRG Human Hepatocyte Cell Line. *Food Chem Toxicol* **2017**, *109* (Pt 1), 439–451. https://doi.org/10.1016/j.fct.2017.09.022.

(142) Smith, M.-C.; Timmins-Schiffman, E.; Coton, M.; Coton, E.; Hymery, N.; Nunn, B. L.; Madec, S. Differential Impacts of Individual and Combined Exposures of Deoxynivalenol and Zearalenone on the HepaRG Human Hepatic Cell Proteome. *J Proteomics* **2018**, *173*, 89–98. https://doi.org/10.1016/j.jprot.2017.11.025.

(143) Smith, M.-C.; Madec, S.; Pawtowski, A.; Coton, E.; Hymery, N. Individual and Combined Toxicological Effects of Deoxynivalenol and Zearalenone on Human

Hepatocytes in in Vitro Chronic Exposure Conditions. *Toxicol Lett* **2017**, *280*, 238–246. https://doi.org/10.1016/j.toxlet.2017.08.080.

(144) Gu, W.; Zhu, P.; Jiang, D.; He, X.; Li, Y.; Ji, J.; Zhang, L.; Sun, Y.; Sun, X. A Novel and Simple Cell-Based Electrochemical Impedance Biosensor for Evaluating the Combined Toxicity of DON and ZEN. *Biosens Bioelectron* **2015**, *70*, 447–454. https://doi.org/10.1016/j.bios.2015.03.074.

(145) Xia, S.; Zhu, P.; Pi, F.; Zhang, Y.; Li, Y.; Wang, J.; Sun, X. Development of a Simple and Convenient Cell-Based Electrochemical Biosensor for Evaluating the Individual and Combined Toxicity of DON, ZEN, and AFB1. *Biosens Bioelectron* **2017**, *97*, 345–351. https://doi.org/10.1016/j.bios.2017.06.002.

(146) Zhou, H.; George, S.; Hay, C.; Lee, J.; Qian, H.; Sun, X. Individual and Combined Effects of Aflatoxin B1, Deoxynivalenol and Zearalenone on HepG2 and RAW 264.7 Cell Lines. *Food Chem Toxicol* **2017**, *103*, 18–27. https://doi.org/10.1016/j.fct.2017.02.017.

(147) Sun, L.-H.; Lei, M.; Zhang, N.-Y.; Zhao, L.; Krumm, C. S.; Qi, D.-S. Hepatotoxic Effects of Mycotoxin Combinations in Mice. *Food Chem Toxicol* **2014**, *74*, 289–293. https://doi.org/10.1016/j.fct.2014.10.020.

(148) Peng, Z.; Chen, L.; Nüssler, A. K.; Liu, L.; Yang, W. Current Sights for Mechanisms of Deoxynivalenol-Induced Hepatotoxicity and Prospective Views for Future Scientific Research: A Mini Review: DON-Induced Hepatotoxicity and Prospective Views. *J Appl Toxicol* **2017**, *37* (5), 518–529. https://doi.org/10.1002/jat.3428.

(149) Marin, D. E.; Motiu, M.; Taranu, I. Food Contaminant Zearalenone and Its Metabolites Affect Cytokine Synthesis and Intestinal Epithelial Integrity of Porcine Cells. *Toxins (Basel)* **2015**, *7* (6), 1979–1988. https://doi.org/10.3390/toxins7061979.

(150) Pinton, P.; Oswald, I. P. Effect of Deoxynivalenol and Other Type B Trichothecenes on the Intestine: A Review. *Toxins (Basel)* **2014**, *6* (5), 1615–1643. https://doi.org/10.3390/toxins6051615.

(151) Bouhet, S.; Oswald, I. P. The Effects of Mycotoxins, Fungal Food Contaminants, on the Intestinal Epithelial Cell-Derived Innate Immune Response. *Vet Immunol and Immunopathol* **2005**, *108* (1), 199–209. https://doi.org/10.1016/j.vetimm.2005.08.010.

(152) Ji, J.; Wang, Q.; Wu, H.; Xia, S.; Guo, H.; Blaženović, I.; Zhang, Y.; Sun, X. Insights into Cellular Metabolic Pathways of the Combined Toxicity Responses of Caco-2 Cells Exposed to Deoxynivalenol, Zearalenone and Aflatoxin B1. *Food and Chemical Toxicology* **2019**, *126*, 106–112. https://doi.org/10.1016/j.fct.2018.12.052.

(153) Bensassi, F.; Gallerne, C.; Sharaf el dein, O.; Hajlaoui, M. R.; Lemaire, C.; Bacha, H. In Vitro Investigation of Toxicological Interactions between the Fusariotoxins Deoxynivalenol and Zearalenone. *Toxicon* **2014**, *84*, 1–6. https://doi.org/10.1016/j.toxicon.2014.03.005.

(154) Zhou, H.; George, S.; Li, C.; Gurusamy, S.; Sun, X.; Gong, Z.; Qian, H. Combined Toxicity of Prevalent Mycotoxins Studied in Fish Cell Line and Zebrafish Larvae Revealed That Type of Interactions Is Dose-Dependent. *Aquat Toxicol* **2017**, *193*, 60–71. https://doi.org/10.1016/j.aquatox.2017.09.030.

(155) Ren, Z. H.; Deng, H. D.; Deng, Y. T.; Deng, J. L.; Zuo, Z. C.; Yu, S. M.; Shen, L. H.; Cui, H. M.; Xu, Z. W.; Hu, Y. C. Effect of the Fusarium Toxins, Zearalenone and Deoxynivalenol, on the Mouse Brain. *Environ Toxicol Pharmacol* **2016**, *46*, 62–70. https://doi.org/10.1016/j.etap.2016.06.028.

(156) Cai, G.; Sun, K.; Xia, S.; Feng, Z.; Zou, H.; Gu, J.; Yuan, Y.; Zhu, J.; Liu, Z.; Bian, J. Decrease in Immune Function and the Role of Mitogen-Activated Protein Kinase (MAPK) Overactivation in Apoptosis during T Lymphocytes Activation Induced by Zearalenone, Deoxynivalenol, and Their Combinations. *Chemosphere* **2020**, *255*, 126999. https://doi.org/10.1016/j.chemosphere.2020.126999.

(157) Li, Y.; Wang, T. Q.; Wu, J.; Zhang, X. L.; Xu, Y. Y.; Qian, Y. Z. Multi-Parameter Analysis of Combined Hepatotoxicity Induced by Mycotoxin Mixtures in HepG2 Cells. *World Mycotoxin J.* **2018**, *11* (2), 225–235. https://doi.org/10.3920/WMJ2017.2201.

(158) Jia, R.; Liu, W.; Zhao, L.; Cao, L.; Shen, Z. Low Doses of Individual and Combined Deoxynivalenol and Zearalenone in Naturally Moldy Diets Impair Intestinal Functions via Inducing Inflammation and Disrupting Epithelial Barrier in the Intestine of Piglets. *Toxicol Lett* **2020**, *333*, 159–169. https://doi.org/10.1016/j.toxlet.2020.07.032.

(159) Kachlek, M.; Szabó-Fodor, J.; Blochné Bodnár, Z.; Horvatovich, K.; Kovács, M. Preliminary Results on the Interactive Effects of Deoxynivalenol, Zearalenone and Fumonisin B1 on Porcine Lymphocytes. *Acta Vet Hung.* **2017**, *65* (3), 340–353. https://doi.org/10.1556/004.2017.033.

(160) Zhang, W.; Zhang, S.; Zhang, M.; Yang, L.; Cheng, B.; Li, J.; Shan, A. Individual and Combined Effects of Fusarium Toxins on Apoptosis in PK15 Cells and the Protective Role of N-Acetylcysteine. *Food Chem Toxicol* **2018**, *111*, 27–43. https://doi.org/10.1016/j.fct.2017.10.057.

(161) Liang, Z.; Ren, Z.; Gao, S.; Chen, Y.; Yang, Y.; Yang, D.; Deng, J.; Zuo, Z.; Wang, Y.; Shen, L. Individual and Combined Effects of Deoxynivalenol and Zearalenone on Mouse Kidney. *Environ Toxicol Pharmacol.* **2015**, *40* (3), 686–691. https://doi.org/10.1016/j.etap.2015.08.029.

(162) Ren, Z.; Deng, H.; Deng, Y.; Liang, Z.; Deng, J.; Zuo, Z.; Hu, Y.; Shen, L.; Yu, S.; Cao, S. Combined Effects of Deoxynivalenol and Zearalenone on Oxidative Injury and Apoptosis in Porcine Splenic Lymphocytes in Vitro. *Exp Toxicol Pathol* **2017**, *69* (8), 612–617. https://doi.org/10.1016/j.etp.2017.05.008.

(163) Smith, M.-C.; Madec, S.; Troadec, S.; Coton, E.; Hymery, N. Effects of Fusariotoxin Co-Exposure on THP-1 Human Immune Cells. *Cell Biol Toxicol* **2018**, *34* (3), 191–205. https://doi.org/10.1007/s10565-017-9408-7.

(164) Ji, J.; Zhu, P.; Pi, F.; Sun, C.; Jiang, H.; Sun, J.; Wang, X.; Zhang, Y.; Sun, X. GC-TOF/MS-Based Metabolomic Strategy for Combined Toxicity Effects of Deoxynivalenol and Zearalenone on Murine Macrophage ANA-1 Cells. *Toxicon* **2016**, *120*, 175–184. https://doi.org/10.1016/j.toxicon.2016.08.003.

(165) Smith, M.-C.; Gheux, A.; Coton, M.; Madec, S.; Hymery, N.; Coton, E. In Vitro Co-Culture Models to Evaluate Acute Cytotoxicity of Individual and Combined Mycotoxin Exposures on Caco-2, THP-1 and HepaRG Human Cell Lines. *Chem Biol Interact* **2018**, *281*, 51–59. https://doi.org/10.1016/j.cbi.2017.12.004.

(166) Szabó, A.; Szabó-Fodor, J.; Fébel, H.; Mézes, M.; Balogh, K.; Bázár, G.; Kocsó, D.; Ali, O.; Kovács, M. Individual and Combined Effects of Fumonisin B1, Deoxynivalenol and Zearalenone on the Hepatic and Renal Membrane Lipid Integrity of Rats. *Toxins (Basel)* **2017**, *10* (1), 4. https://doi.org/10.3390/toxins10010004.

(167) Szabó-Fodor, J.; Szabó, A.; Kócsó, D.; Marosi, K.; Bóta, B.; Kachlek, M.; Mézes, M.; Balogh, K.; Kövér, G.; Nagy, I.; Glávits, R.; Kovács, M. Interaction between the Three Frequently Co-Occurring Fusarium Mycotoxins in Rats. *J Anim Physiol Anim Nutr (Berl)* **2019**, *103* (1), 370–382. https://doi.org/10.1111/jpn.13013.

(168) Ji, J.; Zhu, P.; Cui, F.; Pi, F.; Zhang, Y.; Li, Y.; Wang, J.; Sun, X. The Antagonistic Effect of Mycotoxins Deoxynivalenol and Zearalenone on Metabolic Profiling in Serum and Liver of Mice. *Toxins (Basel)* **2017**, *9* (1), 28. https://doi.org/10.3390/toxins9010028.

(169) Ji, J.; Zhu, P.; Blaženović, I.; Cui, F.; Gholami, M.; Sun, J.; Habimana, J.; Zhang, Y.; Sun, X. Explaining Combinatorial Effects of Mycotoxins Deoxynivalenol and Zearalenone in Mice with Urinary Metabolomic Profiling. *Sci Rep* **2018**, *8*, 3762. https://doi.org/10.1038/s41598-018-21555-y.

(170) Vanhoutte, I.; Audenaert, K.; De Gelder, L. Biodegradation of Mycotoxins: Tales from Known and Unexplored Worlds. *Front. Microbiol.* **2016**, *7*, 561. https://doi.org/10.3389/fmicb.2016.00561.

(171) Cheli, F.; Pinotti, L.; Rossi, L.; Dell'Orto, V. Effect of Milling Procedures on Mycotoxin Distribution in Wheat Fractions: A Review. *LWT - Food Sci and Technol* **2013**, *54* (2), 307–314. https://doi.org/10.1016/j.lwt.2013.05.040.

(172) Lancova, K.; Hajslova, J.; Kostelanska, M.; Kohoutkova, J.; Nedelnik, J.; Moravcova, H.; Vanova, M. Fate of Trichothecene Mycotoxins during the Processing:

Milling and Baking. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **2008**, *25* (5), 650–659. https://doi.org/10.1080/02652030701660536.

(173) Yener, S.; Köksel, H. Effects of Washing and Drying Applications on Deoxynivalenol and Zearalenone Levels in Wheat. *World Mycotoxin J* **2013**, *6* (3), 335–341. https://doi.org/10.3920/WMJ2013.1544.

(174) Tibola, C. S.; Fernandes, J. M. C.; Guarienti, E. M. Effect of Cleaning, Sorting and Milling Processes in Wheat Mycotoxin Content. *Food Control* **2016**, *60*, 174–179. https://doi.org/10.1016/j.foodcont.2015.07.031.

(175) Seitz, L. M.; Eustace, W. D.; Mohr, H. E.; Shogren, M. D.; Yamazaki, W. T. Cleaning, Milling, and Baking Tests with Hard Red Winter Wheat Containing Deoxynivalenol. *Cereal chem* **1986**, *63* (2), 146–150.

(176) Kushiro, M. Effects of Milling and Cooking Processes on the Deoxynivalenol Content in Wheat. *Int J Mol Sci* **2008**, *9* (11), 2127–2145. https://doi.org/10.3390/ijms9112127.

(177) Trigo-Stockli, D. M. Effect of Processing on Deoxynivalenol and Other Trichothecenes. In *Mycotoxins and Food Safety*; DeVries, J. W., Trucksess, M. W., Jackson, L. S., Eds.; Advances in Experimental Medicine and Biology; Springer US: Boston, MA, 2002; pp 181–188. https://doi.org/10.1007/978-1-4615-0629-4_18.

(178) Brodal, G.; Aamot, H. U.; Almvik, M.; Hofgaard, I. S. Removal of Small Kernels Reduces the Content of Fusarium Mycotoxins in Oat Grain. *Toxins (Basel)* **2020**, *12* (5). https://doi.org/10.3390/toxins12050346.

(179) Simionato, E.; Sylos, C. Effect of Cooking on the Levels of Aflatoxin B 1 and Ochratoxin A in Rice. *Braz J Food Technol* **2004**, *7*, 173.

(180) Yazdanpanah, H.; Mohammadi, T.; Abouhossain, G.; Cheraghali, A. M. Effect of Roasting on Degradation of Aflatoxins in Contaminated Pistachio Nuts. *Food Chem Toxicol* **2005**, *43* (7), 1135–1139. https://doi.org/10.1016/j.fct.2005.03.004.

(181) Ryu, D.; Hanna, M. A.; Eskridge, K. M.; Bullerman, L. B. Heat Stability of Zearalenone in an Aqueous Buffered Model System. *J. Agric. Food Chem.* **2003**, *51* (6), 1746–1748. https://doi.org/10.1021/jf0210021.

(182) Kabak, B. The Fate of Mycotoxins during Thermal Food Processing. *J Sci Food Agric* **2009**, *89* (4), 549–554. https://doi.org/10.1002/jsfa.3491.

(183) Wolf, C. E.; Bullerman, L. B. Heat and PH Alter the Concentration of Deoxynivalenol in an Aqueous Enviroment. *J Food Prot* **1998**, *61* (3), 365–367. https://doi.org/10.4315/0362-028X-61.3.365.

(184) El-Banna, A. A.; Lau, P.-Y.; Scott, P. M. Fate of Mycotoxins During Processing of Foodstuffs II - Deoxynivalenol (Vomitoxin) During Making of Egyptian Bread. *J Food Prot* **1983**, *46* (6), 484–486. https://doi.org/10.4315/0362-028X-46.6.484.

(185) Scott, P.; Kanhere, S. R.; Lau, P. Y.; Dexter, J.; Greenhalgh, R. Effects of Experimental Flour Milling and Breadbaking on Retention of Deoxynivalenol (Vomitoxin) in Hard Red Spring Wheat. *Cereal chem* **1983**, *60* (6), 421–424.

(186) Fuchs, E.; Binder, E. M.; Heidler, D.; Krska, R. Structural Characterization of Metabolites after the Microbial Degradation of Type A Trichothecenes by the Bacterial Strain BBSH 797. *Food Addit Contam.* **2002**, *19* (4), 379–386. https://doi.org/10.1080/02652030110091154.

(187) Boudergue, C.; Burel, C.; Dragacci, S.; Favrot, M.; Fremy, J.; Massimi, C.; Prigent, P.; Debongnie, P.; Pussemier, L.; Boudra, H.; Morgavi, D.; Oswald, I.; Perez, A.; Avantaggiato, G. Review of Mycotoxin-detoxifying Agents Used as Feed Additives: Mode of Action, Efficacy and Feed/Food Safety. *EFSA Supporting Publications* **2009**, *6* (9). https://doi.org/10.2903/sp.efsa.2009.EN-22.

(188) Bordini, J. g.; Borsato, D.; Oliveira, A. s.; Ono, M. a.; Zaninelli, T. h.; Hirooka, E. y.; Ono, E. y. s. In Vitro Zearalenone Adsorption by a Mixture of Organic and Inorganic Adsorbents: Application of the Box Behnken Approach. *World Mycotoxin J* **2015**, *8* (3), 291–299. https://doi.org/10.3920/WMJ2013.1675.

(189) Solís-Cruz, B.; Hernández-Patlán, D.; Beyssac, E.; Latorre, J. D.; Hernandez-Velasco, X.; Merino-Guzman, R.; Tellez, G.; López-Arellano, R. Evaluation of Chitosan and Cellulosic Polymers as Binding Adsorbent Materials to Prevent Aflatoxin B1, Fumonisin B1, Ochratoxin, Trichothecene, Deoxynivalenol, and Zearalenone Mycotoxicoses Through an In Vitro Gastrointestinal Model for Poultry. *Polymers (basel)* **2017**, *9* (10), 529. https://doi.org/10.3390/polym9100529.

(190) Armando, M. R.; Pizzolitto, R. P.; Dogi, C. A.; Cristofolini, A.; Merkis, C.; Poloni, V.; Dalcero, A. M.; Cavaglieri, L. R. Adsorption of Ochratoxin A and Zearalenone by Potential Probiotic Saccharomyces Cerevisiae Strains and Its Relation with Cell Wall Thickness. *J Appl Microbiol* **2012**, *113* (2), 256–264. https://doi.org/10.1111/j.1365-2672.2012.05331.x.

(191) Sabater-Vilar, M.; Malekinejad, H.; Selman, M. H. J.; van der Doelen, M. A. M.; Fink-Gremmels, J. In Vitro Assessment of Adsorbents Aiming to Prevent Deoxynivalenol and Zearalenone Mycotoxicoses. *Mycopathologia* **2007**, *163* (2), 81–90. https://doi.org/10.1007/s11046-007-0093-6.

(192) Avantaggiato, G.; Solfrizzo, M.; Visconti, A. Recent Advances on the Use of Adsorbent Materials for Detoxification of *Fusarium* Mycotoxins. *Food Addit Contam* **2005**, *22* (4), 379–388. https://doi.org/10.1080/02652030500058312.

(193) Guan, S.; He, J.; Young, J. C.; Zhu, H.; Li, X.-Z.; Ji, C.; Zhou, T. Transformation of Trichothecene Mycotoxins by Microorganisms from Fish Digesta. *Aquaculture* **2009**, *290* (3), 290–295. https://doi.org/10.1016/j.aquaculture.2009.02.037.

(194) BIOMIN BBSH® 797 - Anitmycotoxin Additive | Proultry.com, avicultura para profesionales http://poultry.proultry.com/products/biomin-gmbh/biomin-bbsh-797-antimycotoxin-additive (accessed 2019 -07 -25).

(195) Young, J. C.; Zhou, T.; Yu, H.; Zhu, H.; Gong, J. Degradation of Trichothecene Mycotoxins by Chicken Intestinal Microbes. *Food Chem Toxicol* **2007**, *45* (1), 136–143. https://doi.org/10.1016/j.fct.2006.07.028.

(196) Böswald, C.; Engelhardt, G.; Vogel, H.; Wallnöfer, P. R. Metabolism of the Fusarium Mycotoxins Zearalenone and Deoxynivalenol by Yeast Strains of Technological Relevance. *Nat Toxins* **1995**, *3* (3), 138–144. https://doi.org/10.1002/nt.2620030304.

(197) Brodehl, A.; Möller, A.; Kunte, H.-J.; Koch, M.; Maul, R. Biotransformation of the Mycotoxin Zearalenone by Fungi of the Genera Rhizopus and Aspergillus. *FEMS Microbiol Lett* **2014**, *359* (1), 124–130. https://doi.org/10.1111/1574-6968.12586.

Chapter 2: Investigating the extent to which ZEN can induce damage on IPEC-J2 cells and feed supplements can protect against damage observed

Abstract

Zearalenone (ZEN) is an oestrogenic mycotoxin that has been shown to have a negative effect on the reproductive function of animals. The structure of ZEN resembles that of naturally occurring oestrogens, whereby it binds to oestrogenic receptors, resulting in hormonal disturbances. The European Food Safety Authority (EFSA) has suggested that the maximum level ZEN in feed for sows and fattening pigs should not exceed 0.25 ppm. The aim of this study was to investigate the effect of ZEN on a porcine intestinal epithelial IPEC-J2 cell model. ZEN was shown to induce cytotoxicity in IPEC-J2 cells in a time and dose dependent manner at concentrations of 3 ppm and above. The comet assay and the TUNEL assay showed that 25 ppm ZEN induced DNA damage and apoptosis, respectively. A potential protective effect of the feed supplement Sel-Plex (Se-Y) and the polyunsaturated fatty acids component of the mycotoxin binder Mycosorb A+ (My-A+), individually and combined, against ZEN induced toxicity was also explored. Cells were treated with the feed supplements for 48 h prior to 24 h exposure to ZEN. A reduction in ZEN associated toxicity was observed in cells that had been pre-treated with Se-Y, My-A+ and Se-Y + My-A+ when compared to cells that were not pre-treated. Overall, it was shown that ZEN induced cytotoxicity, DNA damage and apoptosis in IPEC-J2 cells and these effects were mitigated by pre-incubation with the feed supplement.

2.1 Introduction

Ingestion of ZEN contaminated feed can result in negative health effects in animals, including disturbances in the hormonal balance and the reproductive system [1]. At a cellular level, exposure to ZEN has been shown to induce apoptosis, inhibit protein synthesis and inhibit cell proliferation [2–4]. Additionally, as discussed in greater detail in Chapter 1, ZEN has also been shown to induce genotoxicity in some cell lines [5,6]. The comet assay and terminal deoxynucleotidyl transferase nick-end labelling (TUNEL) assay are two commonly used method for the detection of DNA fragmentation [7,8]. These two assays are used here to determine the apoptotic and genotoxic effects of ZEN on IPEC-J2 cells.

The comet assay, also known as single-cell gel electrophoresis (SCGE), is a method for the analysis of DNA damage. It can be used to measure DNA strand breaks in eukaryotic cells [7,9]. In an alkaline comet assay, cells are embedded in low melting agarose and are electrophoresed at a high pH. During the unwinding of DNA, broken or damaged fragments migrate away from the nucleus, forming comet shaped structures, which can be observed by fluorescence microscopy. DNA damage can therefore be determined by analysing the comet tail, towards which the damaged DNA migrates, with the intact DNA staying in the nucleoid at the head of the comet [10,11]. The alkaline comet assay can detect both single and double strand breaks whereas a neutral comet assay detects only double strand breaks [12].

Apoptosis, also known as programmed cell death, is a naturally occurring process, which plays an important role in the development of adult tissues and the removal of damaged cells in eukaryotes [13,14]. The TUNEL assay is used to detect apoptotic DNA fragmentation [15]. This method consists of fixing and permeabilizing cells after they have been harvested in order to allow the TUNEL reagents to penetrate the nucleus. The cells are fixed with formaldehyde and then permeabilised with ethanol. The terminal deoxynucleotidyl transferase (TdT) catalyses the addition of 2'-deoxyuridine 5'-triphosphate dUTPs to the exposed 3'-hydroxyl ends generated by the DNA fragmentation that occurs during apoptosis [15,16]. However, the TUNEL assay is non-specific as TUNEL staining can label 3'-hydroxyl termini exposed due to necrotic cell death as well as apoptosis [14–16]. The comet and TUNEL assays are often used together to detect and quantify DNA fragmentation.

After the consumption of contaminated food or feed the intestinal epithelium can be exposed to mycotoxins as it is the first barrier against foreign matter in food or feed [17–19]. As well as filtering harmful toxins and pathogens, the epithelium is also important for the absorption of nutrients [20]. Ingestion of mycotoxins can result in harmful effects due to the alteration of intestinal barrier functions as well as nutrient absorption [20]. Thus, intestinal epithelial cells (IECs) can rapidly absorb a high concentration of mycotoxins after ingestion of contaminated food or feed [21].

The IPEC-J2 cell line was derived from intestinal porcine epithelial cells isolated from the jejunum of neonatal unsuckled piglets. It is a non-transformed, primary cell line that is being increasingly used in many studies as an *in vitro* model, including those involving mycotoxins, as it closely mimics the physiology of the intestinal epithelium [22,23]. One study demonstrated the induction of cell toxicity and apoptosis by OTA on IPEC-J2 cells [24]. Exposure to AFB1 and ZEN together had a synergistic effect on the cytotoxicity of IPEC-J2 cells [25]. Elsewhere, the effects of DON and various *Fusarium* metabolites, including enniatins, beauvericin, apicidin and aurofusarin, on the intestinal barrier function of IPEC-J2 cells was measured over 72 h [26]. IPEC-J2 cells were used in another study to investigate the apoptotic and necrotic effects of the *fusarium* mycotoxins DON, T-2 toxin, ZEN and fumonisin B_1 [27].

2.1.3 Chapter aims

The chapter presented here has two main aims. The first was to investigate the effects of ZEN on IPEC-J2 cells at levels currently considered safe and at elevated levels. The effect of ZEN on cell viability was observed following 24 and 48 h exposure. The genotoxic and apoptotic effect of ZEN was investigated following 24 h exposure. As the small intestine of an adult pig is approximately 18 metres long, the time required for the ingestion of feed and the passage of digeta along the gastrointestinal tract has been estimated at approximately 24 h [91]. For this reason, exposure time of less than 24 h was not investigated. The second aim of this chapter was to investigate the extent to which selenium yeast (Se-Y) and Mycosorb A+ (My-A+) can protect against ZEN toxicity, both individually and in combination. The role of Se-Y and My-A+ as feed additives will be discussed in greater detail in Chapter 3.

2.2 Materials and methods

2.2.1 Cell culture

IPEC-J2 cells were grown in Dulbecco's Modified Eagle's Medium/ Nutrient Mixture F-12 Ham (Sigma-Aldrich) supplemented with 10% heat inactivated porcine serum (Sigma-Aldrich) and 1% penicillin streptomycin (Sigma-Aldrich) at 37°C in a humidified 5% CO_2 atmosphere (Galaxy S CO_2 Incubator, Model No: 170-200, RS Biotech Laboratory Equipment Ltd., Irvine, United Kingdom). The adherent cells were passaged by dislodging the cells using trypsin/EDTA (Sigma-Aldrich). They passaged every 3-4 days when they had reached near confluence. All cell culture work was carried out in a Class II biological safety cabinet (BioAire Aura 2000 BS; Bioair Instruments, Pavia, Italy).

2.2.2 Zearalenone preparation

ZEN (Sigma Aldrich) was bought in powder form and dissolved in ethanol (EtOH) to prepare a 7000 ppm stock solution. The stock solution was stored at -20°C and was diluted as necessary in serum free medium. The final concentration of EtOH was kept below 1% during cell culture work.

2.2.3 Preparation of selenised yeast powder and Mycosorb A+

Selenised yeast powder (0.5g; Sel-Plex, Alltech Inc.) and Mycosorb A+ (0.5g; Alltech Inc.) were placed in separate 50 mL sterilin tubes. Five millilitres of 0.1 M sodium phosphate buffer (pH 6.0) was added to each of the tubes, followed by 2 mL of 0.2 M HCl. The pH of the solution was then adjusted to 2.0 using 1 M HCl/NaOH as necessary. One millilitre of 50 units per mL pepsin and 0.5 mL 5 mg/mL chloramphenicol was then added. The solutions were incubated at 39°C for 2 h with gentle shaking. Following this, 2 mL of 0.2 M sodium phosphate buffer (pH 6.8) was added, followed by 1 mL of 0.6 M NaOH. The pH of the solution was adjusted to 6.8. Pancreatin solution (1 mL of 10 mg/mL) was added and the digestion was incubated for 4 h at 39°C with gentle shaking. The tubes were then centrifuged at 8000 x g for 15 min and the supernatants were collected and transferred to ultrafiltration tubes with 10,000 Daltons molecular cut off (Vivaspin 20, Sartorious). These were centrifuged at 8000 x g for 3 h at 4°C. The filtrate was then aliquoted and stored at -20°C until use.

2.2.4 Cell viability assay

Alamar Blue (AB) (Invitrogen, Bioscience Ltd.) and CFDA-AM (5-carboxyfluorescein diacetate acetoxymethyl ester; Invitrogen, Bioscience Ltd.) were used for the analysis of cell toxicity. Cells were seeded in a black flat-bottomed 96 well plate at 2 x 10^4 cells/mL in DMEM with 2% porcine serum. For analysis involving Se-Y and/or My-A+, the cells were incubated with digested Se-Y (0.4 ppm Se), My-A+ (2g/kg) or both for 48 h. Cells were insulted with various concentrations of ZEN and incubated for 24 or 48 h, as indicated in the text. For the CFDA-AM/AB combined solution, a 4 µM stock solution of CFDA-AM was made up in DMSO. The working reagent was made up with 1/1000 dilution of the CFDA-AM stock solution and a 1/10 dilution of AB, made up with serum free medium up to the total volume required. After incubation, the medium was removed from the well plate and a 100 µL of the combined reagent was added. This was then incubated for 30 min. Fluorescence was measured at 530 nm excitation/590 nm emission for AB and 485 nm excitation/535 nm emission for CFDA-AM using a multiwell scanning spectrophotometer (Safire II; Tecan Group Ltd., Männedorf, Switzerland).

2.2.5 TUNEL Assay

The TUNEL assay was carried out according to the manufacturer's instructions (Phoenix Flow Systems, San Diego, CA). After pre-incubation of the cells with Se-Y (0.4 ppm Se) and My A+ (2 g/kg), followed by incubation with ZEN for 24 h, the cells were trypsinised, fixed with 4% paraformaldehyde in PBS, and then stored in 70% EtOH at -20 °C for at least 18 h. The cells were then incubated in the DNA labelling mix, which consisted of TDT enzyme and Brd-UTP antibody for 1 h at 37 °C. Following the incubation, the cells were rinsed with the rinsing buffer and then incubated in the Anti-BrdU-FITC staining mixture for 30 min in the dark. After the incubation, 150 µL rinsing buffer was added to each sample. The samples were transferred to a round-bottomed 96 well plate and data was acquired on a Guava benchtop Flow Cytometer (Guava easyCyte 8Ht; Merck Millipore, Cork, Ireland) and analysed using FlowJo software (FlowJo LLC, Ashland, USA).

2.2.6 Alkaline Comet Assay

IPEC-J2 cells were incubated with ZEN for 24 h at a cell concentration of 1 x 10^5 cells/mL. For analysis involving Se-Y and/or My-A+, the cells were incubated with digested Se-Y (0.4 ppm Se), My-A+ (2g/kg) or both for 48 h prior to ZEN exposure. The

cells were combined with low melting agarose and placed on a comet slide (Trevigen, Gaithersburg, USA). The slides were incubated in the dark at 4 °C for 10 min and then immersed in 4°C lysis solution overnight. Once the excess solution was drained, the slides were immersed in Alkaline Unwinding Solution for 20 min at room temperature. Alkaline electrophoresis solution (850 mL) at 4°C was then added to the Comet Assay ES unit (Bio-Techne, Minnesote, USA). The slides were placed in the electrophoresis slide tray and covered with the overlay. Gel electrophoresis was carried out at 21 V for 30 min. The slides were then immersed in water for 5 min twice and once in 70% EtOH and then dried for 15-20 min at 37°C. 100 µL of diluted SYBR Gold staining solution (Invitrogen, Bioscience Ltd.) was placed on the slides and incubated for 30 min in the dark. The slides were then rinsed in water, dried and viewed at 10X magnification by fluorescent microscopy (NIKON Ti-E Epifluorescence Microscope) at excitation/emission wavelength of 496/522 nm. Percentage tail intensity was scored using the Comet Score Software (TriTek Corp, Summerduck, VA). The median of 50 comets per slide was calculated and the mean of the median of 3 slides per sample was measured.

2.3 Results

2.3.1 A concentration of 2% ethanol is not toxic to IPEC-J2 cells

As a 'vehicle control' the AB/CFDA-AM assay was first used to determine if there was any cytotoxicity due to 1% EtOH on IPEC-J2 cells. AB measures the metabolic activity of the cells and CFDA-AM measures cell membrane integrity. Figure 2.1 shows that there was no cytotoxic effect on IPEC-J2 cells due to 1% ethanol after 48 h. This is in agreement with the results obtained by Goossens *et al.* where it was determined that 1% ethanol was non-cytotoxic for IPEC-J2 cells after 72 h [27]. The final concentration of ethanol in cell culture treatments with ZEN was kept below 1%.

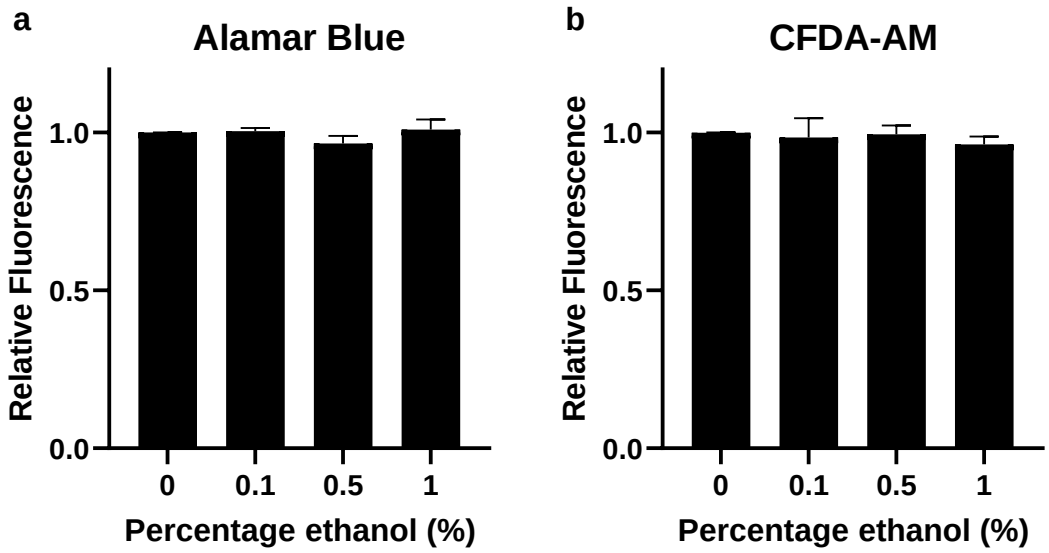

Figure 2.1: *The effect of 0 - 1% ethanol on IPEC-J2 cells over 48 h; (a) Alamar Blue assay and (b) CFDA-AM assay. Results are expressed as the mean of triplicates ± SD and are presented as relative to the control (0% ethanol).*

2.3.2 ZEN is not toxic to IPEC-J2 cells at maximum EFSA recommended levels

The EFSA upper limit for ZEN in pig feed is set at 0.25 ppm [28]. The combined AB and CFDA-AM cell viability assay was used to measure the cytotoxicity of ZEN on IPEC-J2 cells. As seen in Figure 2.2 a and 2.2 b, the results showed that ZEN was not cytotoxic to the cells at the EFSA limits after 24 h exposure. At 48 h, however, there was a significant decrease in cell viability at 0.5 ppm. ZEN was found to be significantly cytotoxic at 3 ppm at 24 h.

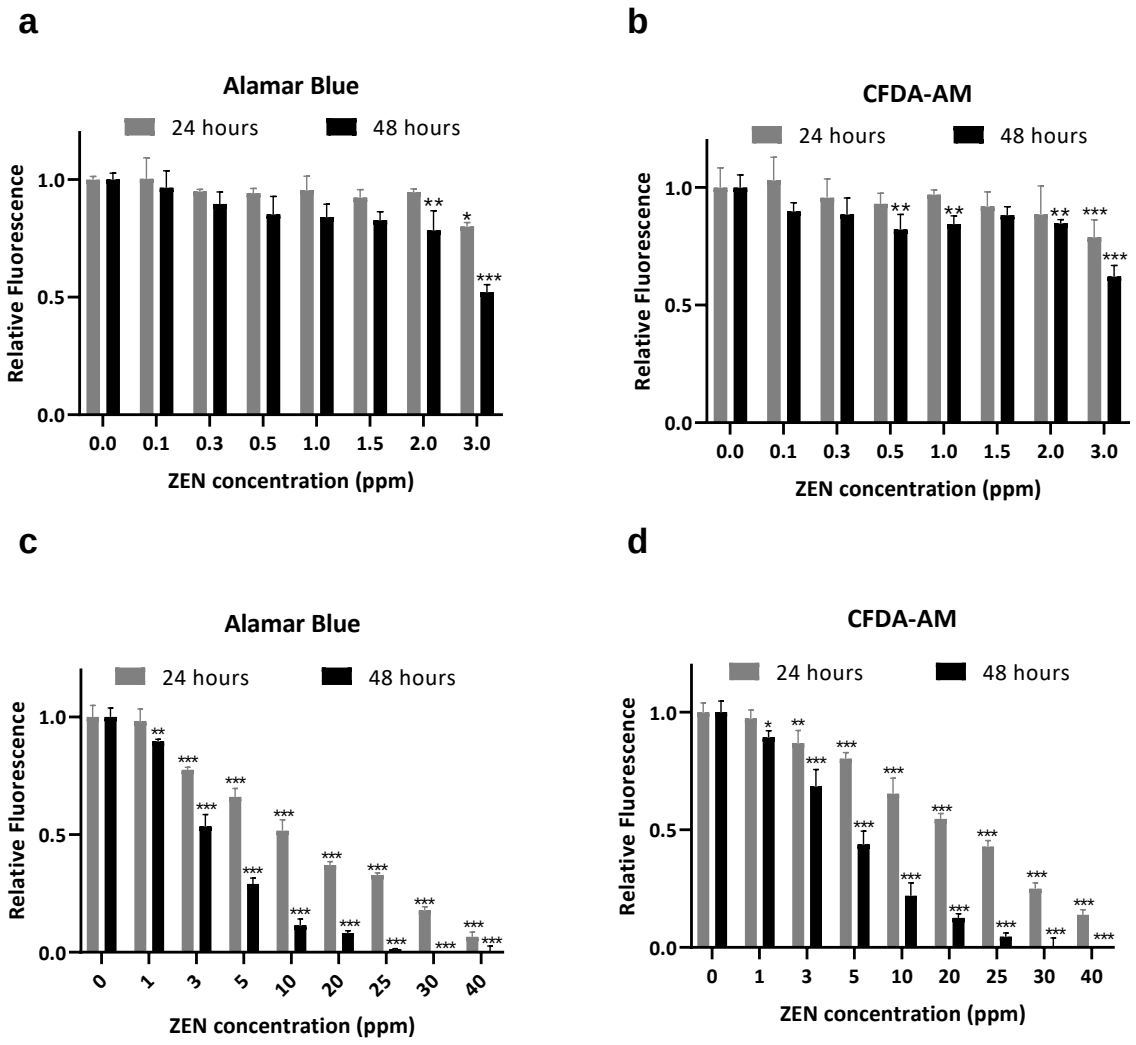

Figure 2.2: *The effect on IPEC-J2 viability following a 24 and 48 h exposure to ZEN. The graphs above represent IPEC-J2 cells treated with a) lower concentrations of ZEN as analysed by Alamar blue, b) lower concentrations of ZEN as analysed by CFDA-AM, c) higher concentrations of ZEN as analysed by Alamar blue, d) higher concentrations of ZEN as analysed by CFDA-AM. Results are expressed as the mean of triplicates ± SD and are presented as relative to the control (0 ppm ZEN).*

The viability of the IPEC-J2 cells were measured at a higher ZEN concentration range of 0 – 40 ppm. The results in Figure 2.2 c and 2.2 d showed significant decreases in cell viability with increasing exposure time and concentrations illustrating a time and dose dependent response.

2.3.3 ZEN induces genotoxicity at concentrations above EFSA guidelines

The alkaline comet assay was used to analyse DNA damage in IPEC-J2 cells induced by ZEN. Cells were incubated with increasing concentrations of ZEN for 24 h and analysed by fluorescent microscopy. As with cell viability, there was no DNA damage detected by the comet assay at the EFSA limits. However, when higher concentrations of ZEN were used, it was shown to induce genotoxicity in IPEC-J2. A significant increase in tail DNA was observed at 25 ppm, indicating DNA damage by strand breaks. No significant DNA damage was observed at concentrations lower than 25 ppm. Representative images of untreated cells (left) and cells treated with 25 ppm ZEN (right) are shown in Figure 2.3 a. The bar chart in figure 2.3 b shows the increase in DNA tail percentage with increasing concentration at 25 ppm and higher.

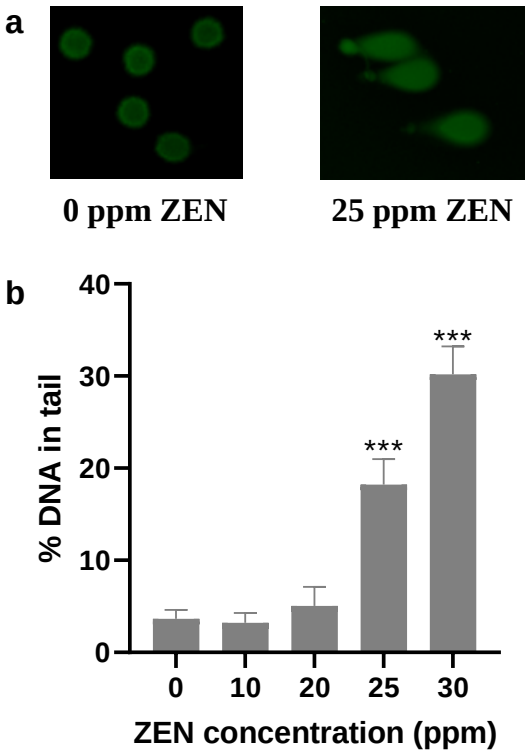

Figure 2.3: *ZEN induces DNA damage in IPEC-J2 cells. A) Representative images of cells treated with 0 ppm ZEN (left) and 25 ppm ZEN (right) at 24 h. B) Comet assay showing the percentage tail DNA as determined by Comet Score software for IPEC-J2 cells incubated with ZEN for 24 h. Data in the bar chart presented is the mean of the median ± SD of triplicate samples. Significant differences between negative control cells and cells insulted with ZEN are highlighted with asterisks. This was calculated by one way ANOVA followed by Dunnett's test (*P<0.05, **P<0.01,***P<0.001).*

2.3.4 ZEN induces apoptosis at concentrations above EFSA guidelines

The TUNEL assay was used to determine ZEN induced apoptosis in IPEC-J2 cells. Cells were treated with increasing concentrations of ZEN for 24 h. As with the comet assay, the TUNEL assay showed a significant decrease in TUNEL negative values, indicating apoptosis, at 25 ppm. Representative histograms for the apoptosis of cells treated with 0, 10, 25 and 30 ppm ZEN, respectively, are shown in figure 2.4 a. Figure 2.4 b shows the decrease in the TUNEL negative population with increasing concentrations over 25 ppm. As seen in figure 2.4 b, there was no significant apoptosis observed below 25 ppm.

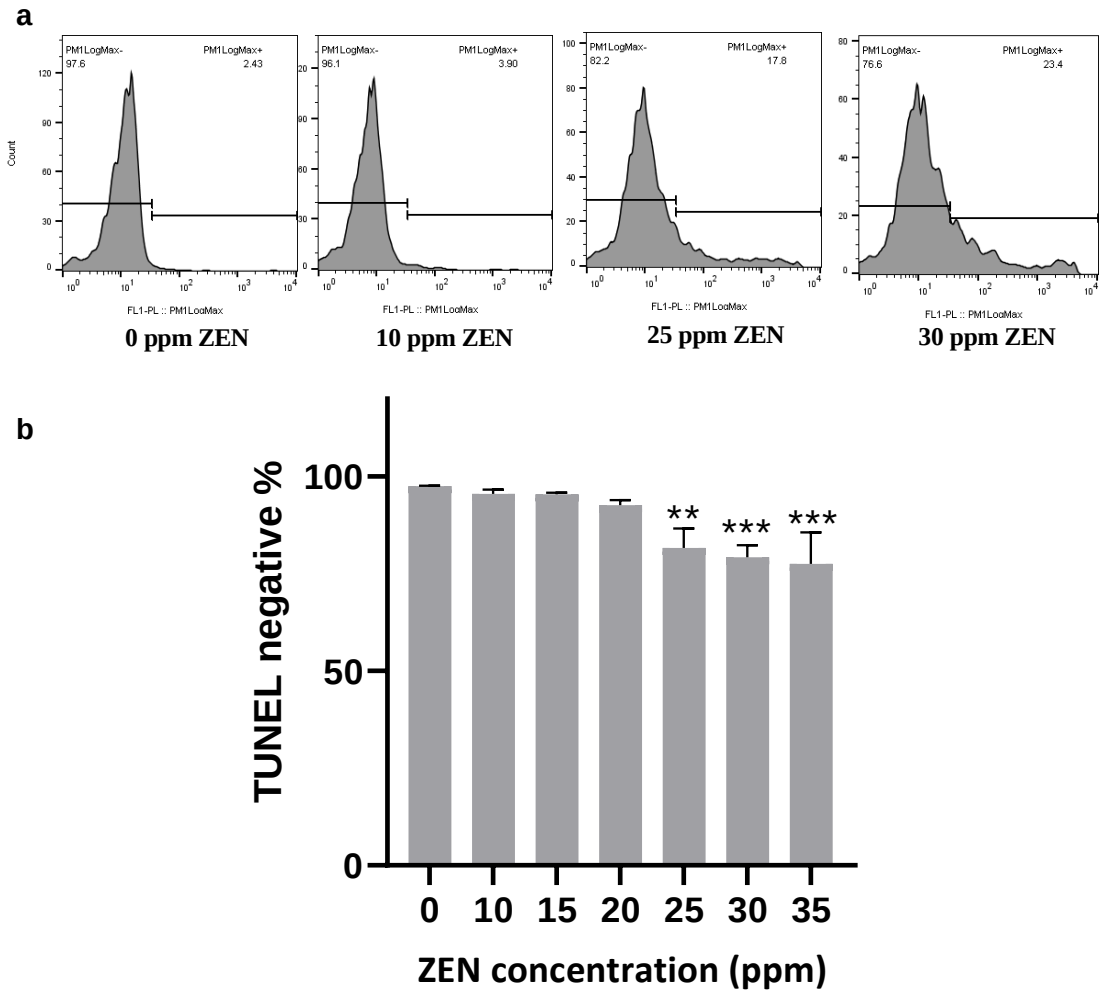

Figure 2.4: *TUNEL assay on IPEC-J2 cells exposed to increasing concentrations of ZEN for 24 h. A) Representative histogram plots of cells treated with 0, 10, 25 and 30 ppm ZEN. B) TUNEL negative values for cells incubated with increasing concentrations of ZEN. Data in the bar chart is presented as the mean ± SD of triplicate samples. Significant difference between control cells ((0 ppm ZEN) and cells insulted with ZEN are highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

2.3.5 Assessment of Se-Y and My-A+ for toxicity on IPEC-J2 cells

The effects of the supplements Se-Y and My A+ on IPEC-J2 cells were first investigated using the AB/CFDA-AM dual viability assay in control experiments prior to inclusion of ZEN. The cells were incubated with Se-Y and My-A+ for 48 h. The concentration of Se (in the case of Se-Y) used for the experiment was 0.4 ppm. This concentration was chosen as it was shown by another study that 0.4 ppm Se from Se-Y was not toxic to IPEC-J2 cells [51]. My-A+ is typically added to animal feed at 2 g/kg and the level used in this work was chosen in an effort to reflect that level following a simulated digestion of the product as outlined in section 2.2.3. No significant cytotoxicity was observed when either Se-Y or My-A+ were used either individually or in combination (Figure 2.5 a and b). The individual and combined effects of Se-Y and My-A+ on IPEC-J2 cells were also investigated using the TUNEL and comet assay. The results (Figure 2.5 c and d) showed that incubation of the cells with the feed supplements for 48 h did not result in a genotoxic or apoptotic effect.

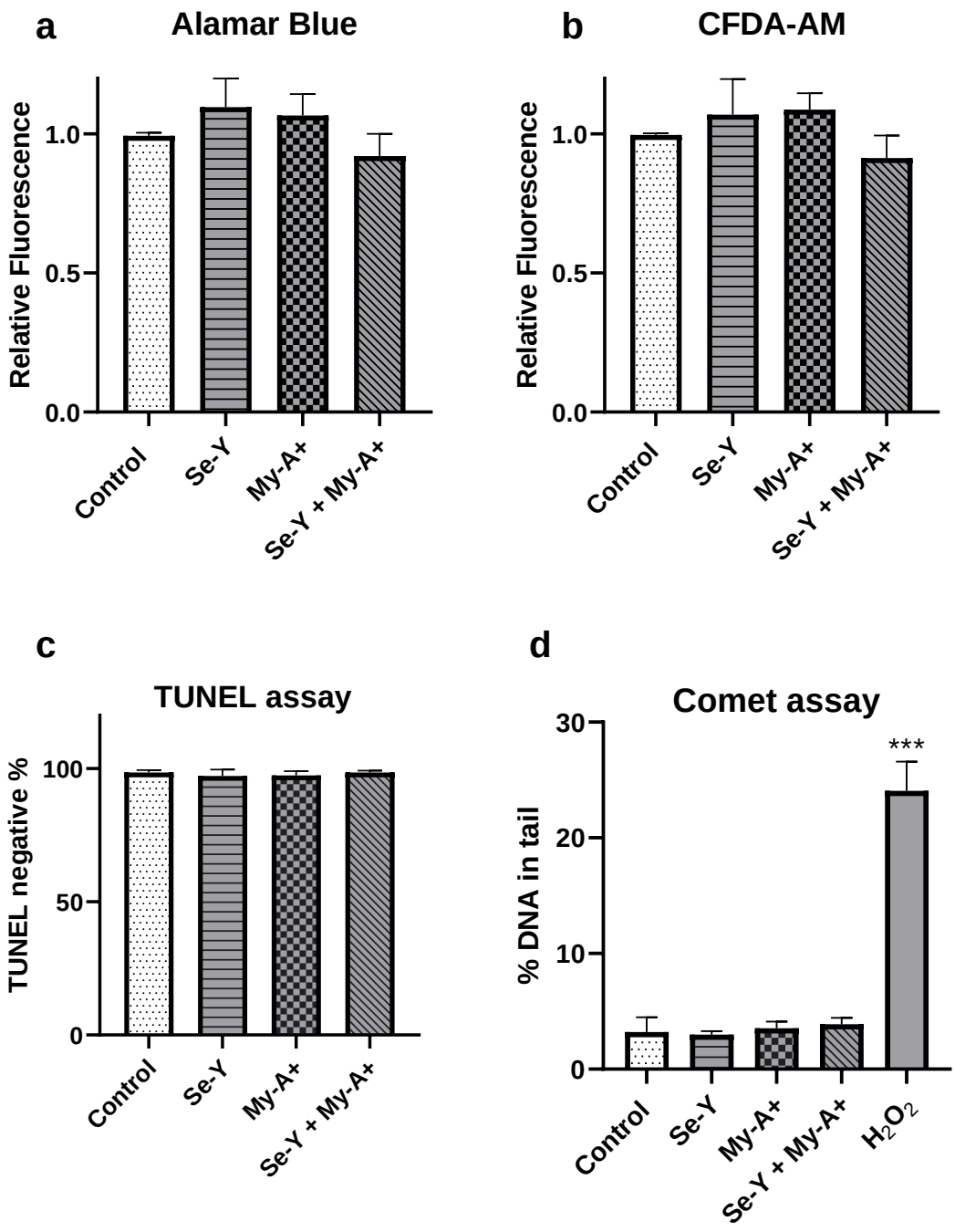

Figure 2.5: *Se-Y and My-A+ do not significantly affect IPEC-J2 viability, genotoxicity and apoptosis as determined by AB/CFDA-AM dual assay (graph a and b), TUNEL assay (graph c) and comet assay (graph d). Cells were incubated in Se-Y, My-A+ and a combination of Se-Y and My-A+ for 48 h. Results are expressed as the mean of triplicates ± SD and are presented as relative to the negative control. Control: no supplementation.*

2.3.6 Sel-Plex and Mycosorb A+ exhibit protective effects against ZEN induced cytotoxicity

In order to investigate the potential of the supplements to mitigate against ZEN-induced cell damage IPEC-J2 cells were pre-incubated with Se-Y and My-A+ for 48 h, followed by a 24 h incubation with 3 ppm or 10 ppm ZEN. Figure 2.6 shows that significant protective effects against cytotoxicity were observed in cells pre-treated with the feed additives for 48 h when compared to cells that were not pre-treated. Higher cell viability was observed in cells treated with Se-Y, My-A+ and Se-Y and My-A+ than cells only treated with 3 ppm or 10 ppm ZEN. However, there was no significant difference observed between the three pre-treatments.

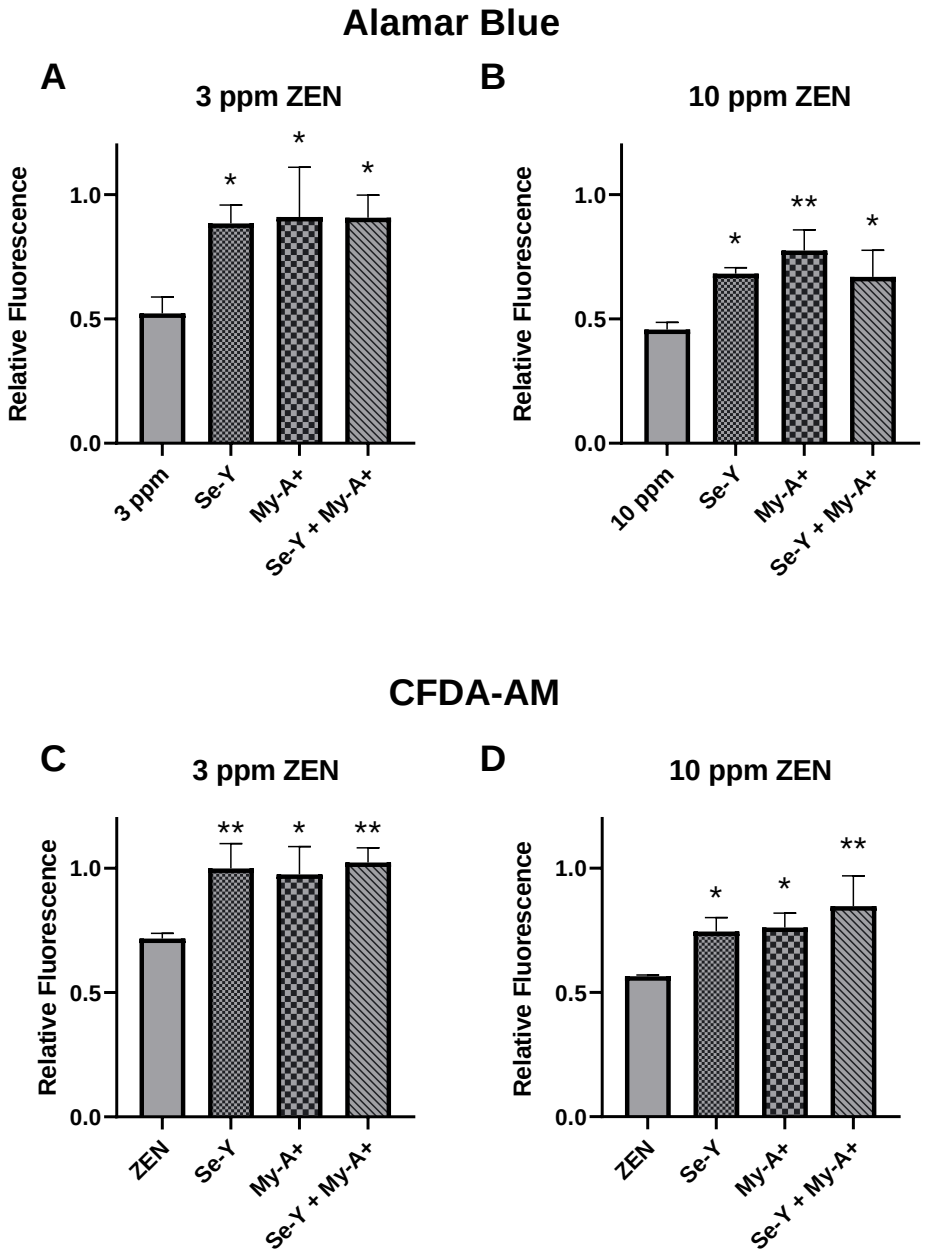

Figure 2.6: *Se-Y and My-A+ mitigate against ZEN induced cytotoxicity in IPEC-J2 cells. The graphs above represent a) cells treated with 3 ppm ZEN and analysed by CFDA-AM b) cells treated with 10 ppm ZEN and analysed by CFDA-AM c) cells treated with 3 ppm ZEN analysed by alamar blue and d) cells treated with 10 ppm ZEN analysed by alamar blue. In all cases cells were pre-incubated for 48h with supplement (as indicated underneath) followed by insult with ZEN for 24 h. Results are expressed as the mean of triplicates ± SD and are presented as relative to the negative control (0 ppm ZEN, no supplements). Significant difference between the ZEN treated cells with no pre-treatments and pre-treated samples were calculated by one-way ANOVA (*P<0.05, **P<0.01, ***P<0.001).*

2.3.7 Sel-Plex and Mycosorb A+ exhibit protective effects against ZEN induced DNA damage

In order to investigate the potential of the supplements to mitigate against ZEN-induced DNA damage IPEC-J2 cells were pre-incubated with Se-Y and My-A+ for 48 h, followed by a 24 h incubation with 25 ppm ZEN. The comet assay was then used to investigate any protective effects of the feed supplements against DNA damage. A significant decrease in DNA damage was observed only in cells that were pre-treated with the feed supplements (figure 2.7). It can be seen that ZEN induced a DNA tail value of 15.19% whereas this value was reduced to 5.72%, 6.17% and 5.5% in cells pre-treated with Se-Y, My-A+ and Se-Y + My-A+ respectively. There was no significant difference between the protective effects exhibited by the different pre-treatments.

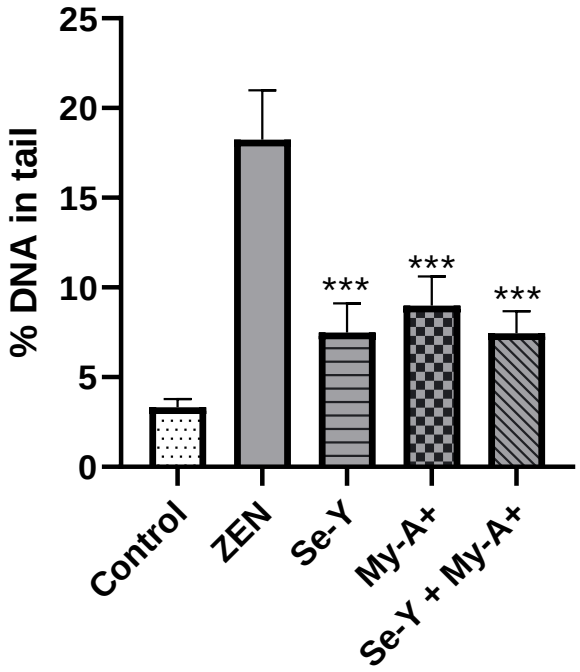

Figure 2.7: *Se-Y and My-A+ mitigate against ZEN induced DNA damage in IPEC-J2 cells IPEC-J2 cells were pre-treated with Se-Y, My-A+ or a combination of both for 48 h followed by a 24 h exposure to 25 ppm ZEN and analysed for DNA damage by comet assay. Data in the bar chart is presented as the mean of the median ± SD of triplicate samples. Significant differences between the ZEN treated cells with no pre-treatments and pre-treated cells are highlighted by one way ANOVA followed by Dunnett's test(*P<0.05, **P<0.01,***P<0.001). Control = No ZEN, no supplementation, ZEN = ZEN, no supplements, Se-Y/My-A+/Se-Y+My-A+ = ZEN + supplementation.*

2.3.8 Sel-Plex and Mycosorb A+ mitigate ZEN induced apoptosis in IPEC-J2 cells

Cells were pre-incubated with Se-Y and My-A+ for 48 h, followed by a 24 h incubation with 25 ppm ZEN. The TUNEL assay was then used to measure the protective effect of the feed supplements when used individually and combined against apoptosis induced by ZEN. It can be seen from Figure 2.8 that ZEN induced apoptosis in IPEC-J2 cells and that there was a significant increase in the TUNEL negative population in cells that were pre-treated with the feed supplements when compared to the cells with no pre-treatment.

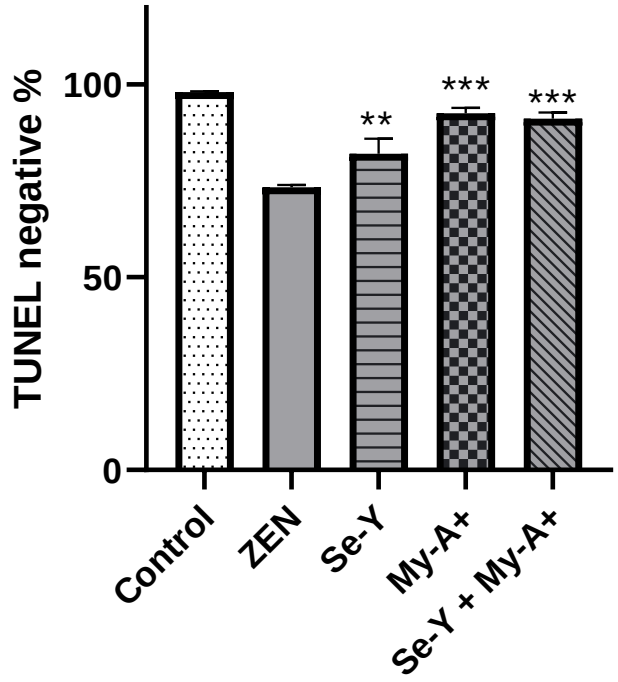

Figure 2.8: *Se-Y and My-A+ mitigate against ZEN induced apoptosis in IPEC-J2 cells. IPEC-J2 cells were pre-treated with Se-Y, My-A+ or a combination of both for 48 h followed by a 24-h exposure to 25 ppm ZEN and analysed by TUNEL assay. Data in the bar chart is presented as the mean ± SD of triplicate samples. Significant differences between ZEN treated cells with no pre-treatments and pre-treated cells are highlighted by one way ANOVA followed by Dunnett's test (*P<0.05, **P<0.01,***P<0.001). Control = No ZEN, no supplementation, ZEN = ZEN, no supplements, Se-Y/My-A+/Se-Y+My-A+ = ZEN + supplementation.*

2.4 Discussion

ZEN is a naturally occurring *Fusarium* mycotoxin, often found in maize, barley, wheat and oats [30,31]. The intestine is the first physiological barrier for mycotoxin contaminated feed and thus is the first organ targeted by the contaminants [32]. Therefore, this study used a porcine intestinal epithelial cell model (IPEC-J2) to study the effects of ZEN.

The guideline set by EFSA for the level of ZEN in feeding stuffs for piglets and gilts is 0.1 ppm and for feeding stuffs for sows and fattening pigs is 0.25 ppm [28]. The dual AB/CFDA-AM cell viability assay was used to measure the cytotoxic effects of ZEN. The cellular metabolic activity was measured by AB and the membrane integrity of the cells was measured by CFDA-AM. The results showed that a 24 h exposure to 0.25 ppm ZEN was not significantly cytotoxic to IPEC-J2 cells. After 24 h exposure, cytotoxicity was only observed at concentrations of 3 ppm and higher. With the higher doses tested, ZEN was found to be cytotoxic to the cells in a dose dependent manner. An increase in cytotoxicity was also observed when the exposure time was increased to 48 h. The results of this study are in agreement with that of another study using IPEC-J2 cells, where a decrease in cell viability was reported in cells treated with 3.18 – 31.84 ppm ZEN for 24 h, a concentration that is significantly above the EFSA guideline [2]. Similar to our results, a time and dose dependent response to ZEN was also observed in another study in which rat liver cells were used [33]. A dose dependent response was also reported in human hepatoma HepG2 and murine leukaemia virus-induced tumour RAW 264.7 cells [33,34]. Therefore, the cytotoxic effect of ZEN observed on IPEC-J2 appears to be comparable to that seen in other cell types.

In this study, the alkaline comet assay was used to measure the extent to which DNA strand breaks were induced by ZEN. At the EFSA limits for ZEN in pig feed, ZEN was not found to induce genotoxicity to IPEC-J2. However, DNA damage was induced at 24 h when the concentration was increased to 25 ppm. The measure of DNA damage was to be seen from the increase in the amount of DNA in the tails of the comets. There was 4.73% DNA in tail measured in the control cells (0 ppm ZEN). This value then increased to 15.19% when cells were incubated for 24 h with 25 ppm ZEN. When the concentration was increased to 30 ppm, a further increase in the percentage of DNA in tail was observed at 28.44 %. This correlates with findings from studies with different cell lines, e.g. DNA damage induced by ZEN was also observed in Chang liver cells treated with 15.9 ppm

and above for 24 h [35]. Similarly, DNA damage was observed by the comet assay, when human embryonic kidney (HEK293) cells were treated with 3.18 ppm and 6.37 ppm ZEN for 2 h [3]. With embryonic zebrafish, ZEN was shown to induce DNA strand breaks at the much lower concentrations of 0.75 ppm and 0.95 ppm [36]. The comet assay was also used to measure the induction of DNA damage by ZEN in HepG2 cells. Here the effect of increasing concentration of ZEN (1.6 – 31.84 μM) was measured after 3 h and 24 h exposure and it was found that ZEN induced DNA damage in a dose dependent manner. Additionally, higher levels of damage were observed after 24 h exposure when compared to the shorter exposure time of 3 h [6]. Another study also using HepG2 cells also found that ZEN induced DNA damage in a dose dependent manner after 24 h exposure. That study also reported that metabolites of ZEN, namely α-ZOL and β-ZOL, were more toxic to the cells than ZEN, with α-ZOL inducing then most DNA damage [37]. However, it was found that ZEN did not induce DNA damage in Chinese hamster ovary cells following a 24 h incubation at 8 ppm [38]. These studies show that the induction of ZEN can vary with the cell type.

As with the cell viability and comet assay, the TUNEL assay results showed that ZEN at the EFSA limit did not induce apoptosis. It was found that 24 h exposure to ZEN at concentrations of 25 ppm and above resulted in apoptosis in IPEC-J2 cells as detected by the TUNEL assay. Significant decreases in the percentages of TUNEL negative populations were observed with concentrations higher than 25 ppm, thus indicating a dose dependent response. These results agree with another study demonstrating the apoptotic effect of ZEN on porcine granulosa cells, where results from the TUNEL and annexin/PI assay showed a dose dependent increase in apoptosis following a 24 h exposure to ZEN [39]. Similarly, Leydig cells treated with increasing concentrations of ZEN resulted in increasing percentage of apoptotic cells [40], correlating with another study showing that a 24 h exposure to 9.55 ppm ZEN induced apoptosis in bovine mammary epithelial cells [4]. The TUNEL and annexin/PI assays were also used to measure the level of apoptosis induced by ZEN on mouse endometrial stromal cells. Again, a dose dependent response was observed after a 24 h incubation with ZEN. ZEN treatment resulted in the activation of caspase-9, caspase-3 and regulation of Bcl-2 family proteins, thus triggering apoptosis in the cells [41]. The induction of apoptosis by ZEN via the activation of caspase-3 has also been reported in HepG2 cells, where a significant dose-dependent increase in caspase-3 activity was observed [42]. Overall, it has been shown that

ZEN is not toxic to IPEC-J2 cells at the concentrations set by EFSA for the level of ZEN in feeding stuffs for piglets and gilts. At higher concentrations, ZEN was found to reduce cell viability and induce DNA damage and apoptosis in a time and dose dependent manner.

Se-Y and My-A+ are both feed supplements produced by Alltech Ltd. and protective effects of these two supplements, when used both individually and in combination, against ZEN toxicity was evident in this study. Se is an essential dietary trace element that is naturally present in food and is known to have anti-oxidant properties [43]. Se has been used previously as a protective agent against mycotoxin induced toxicity. Selenomethionine was shown to be protective against OTA induced nephrotoxicity in porcine kidney PK15 cells [44]. In another study, rats were fed a naturally mycotoxin contaminated diet, with or without the presence of Se enriched yeast. The results showed that the hepatotoxicity induced by ochratoxin was reduced in the group that was fed organic Se [45]. The protective effects of Se against ZEN induced apoptosis have also been demonstrated in chicken spleen lymphocytes [46]. Se has also been shown to have ameliorative effects against ZEN induced reproductive damage in male mice [47].

Mycosorb A+ is a yeast cell wall based feed additive derived from *Saccharomyces cerevisiae* and algae [48]. YCW have been used as a mycotoxin adsorbent to reduce the toxic effects of mycotoxins in animals. The protective effect of another YCW product (Mycosorb) against mycotoxin-induced toxicity has been demonstrated, where a protective effect against T-2 toxin induced toxicity was observed. Enhanced mitigation was observed when Mycosorb was combined with organic Se (Sel-Plex) [49]. In the study presented here however, enhanced mitigation of damage due to ZEN was not observed when My-A+ was used as a co-supplement with Sel-Plex. This may be due to the difference in the concentration and content of the supplements used. The Mycosorb used in the study is not enriched with PUFAs as My-A+ is. Additionally, the study used 1g/kg Mycosorb and 0.3 ppm Se-Y, which is lower than the concentrations used in this project. Supplementation with Mycosorb was also shown to result in protection against feed naturally contaminated with a mixture of mycotoxins (AFs (B1, B2, G1, G2), DON, ZEN and OTA) in broiler chicks [50]. Similarly, protective effects were also observed in another study using broiler chicks fed with a diet contaminated with AFB1 [51].

The algae in My-A+ is rich in PUFAs, which are known to have antioxidant properties. The use of PUFAs in the mitigation of mycotoxins has not been studied widely. However, there are some published reports of a protective effect against toxicity induced by various other sources. Female rats were used in one such study, where the rats were divided in groups and were treated with n-3 FAs and lead acetate. n-3 was shown to have a protective effect against the DNA damage, induced by lead acetate. Additionally, a protective effect against the hepatoxicity and renal toxicity was also observed [52]. Elsewhere, DHA and ARA were shown to have a protective effect against DNA fragmentation and increased caspase-3 levels due to serum starvation in neuronal cells [53]. In another study, pre-treatment with the n-3 FA DHA mitigated hydroxynoneal induced apoptosis in human umbilical vein endothelial cells [54]. These studies provide evidence of the protective effects of PUFA against toxicity induced by various sources, thus indicating that the presence of PUFA in My-A+ could contribute to the observed mitigation against ZEN induced toxicity.

In this study, the cells were pre-treated with the feed supplements for 48 h prior to exposure with ZEN. This pre-incubation time was chosen as it allowed the cells enough time to metabolise Se. Additionally, a study by Lynch *et al*. showed that 48 h pre-incubation with Se-Y resulted in a protective effect against cadmium-induced toxicity in IPEC-J2 cells [29]. The cell viability assay showed that after 24 h exposure to ZEN, a decrease in cell viability was observed at 3 ppm. Therefore, this concentration was used to investigate the protective effect of the feed supplements against the cytotoxicity of ZEN against IPEC-J2. A protective effect of feed supplementation was demonstrated here, with cells that were pre-treated with the feed supplements for 48 h prior to 24 h ZEN exposure having higher cell viability when compared to the cells were not pre-treated. This was shown for supplementation with all three dosing regimens, i.e. Se-Y, My-A+, Se-Y and My-A+. This clearly demonstrates that the incubation of the cells with the supplements resulted in a protective effect against ZEN induced cytotoxicity. Similar effects were observed with the comet assay and the TUNEL assay. For these assays, 25 ppm ZEN was used to investigate the protective effects, as this was the lowest concentration of ZEN where induced DNA strand breaks and apoptosis were observed. The results of the assays showed that cells that underwent a 48 h pre-treatment with the feed supplements had reduced ZEN induced toxicity. The concentration of Se used in the study was chosen as it has been specified by EFSA that 0.4 ppm Se in animal feed is safe

and this amount has previously shown to be non-toxic to IPEC-J2 cells [29,55]. For My-A+, the recommended dose for addition in animal feed is 2g/kg and the dilution was carried out to reflect this. Therefore, these were the concentrations chosen for the combination of the feed additives also. However, there was no significant difference observed between the ameliorative effects of Se-Y, My-A+ and Se-Y + My-A+. It is possible that a maximum level of mitigation has been achieved by the feed supplements individually. However, this theory could be investigated by examining the individual and combined effects of Se-Y and My-A+ at lower concentrations.

2.5 Conclusion

From the results presented, it was found that ZEN did not reduce cell viability at a concentration of 0.25 ppm, which is the maximum EU allowable ZEN in feed for sows and fattening pigs. When higher concentrations of ZEN was used, it was found that ZEN had a dose dependent negative effect on cell viability, genotoxicity and apoptosis of IPEC-J2 cells. The protective effect of the feed supplements against ZEN induced damage was also analysed. Cells treated with Se-Y and My-A+, individually and in combination, for 48 h prior to ZEN resulted in the reduction of the toxicity induced by ZEN. Thus, it was shown that Se and PUFAs could mitigate against ZEN toxicity in IPEC-J2 cells. However, the protective effects observed with the feed supplements individually were shown to be similar to that observed with the supplements combined.

2.6 References

(1) Minervini, F.; Dell'Aquila, M. E. Zearalenone and Reproductive Function in Farm Animals. *Int J Mol Sci* **2008**, *9* (12), 2570–2584. https://doi.org/10.3390/ijms9122570.

(2) Wang, X.; Yu, H.; Fang, H.; Zhao, Y.; Jin, Y.; Shen, J.; Zhou, C.; Zhou, Y.; Fu, Y.; Wang, J.; Zhang, J. Transcriptional Profiling of Zearalenone-Induced Inhibition of IPEC-J2 Cell Proliferation. *Toxicon* **2019**, *172*, 8–14. https://doi.org/10.1016/j.toxicon.2019.10.004.

(3) Gao, F.; Jiang, L.; Chen, M.; Geng, C.; Yang, G.; Ji, F.; Zhong, L.; Liu, X. Genotoxic Effects Induced by Zearalenone in a Human Embryonic Kidney Cell Line. *Mutat Res.* **2013**, *755* (1), 6–10. https://doi.org/10.1016/j.mrgentox.2013.04.009.

(4) Fu, Y.; Jin, Y.; Zhao, Y.; Shan, A.; Fang, H.; Shen, J.; Zhou, C.; Yu, H.; Zhou, Y. F.; Wang, X.; Wang, J.; Li, R.; Wang, R.; Zhang, J. Zearalenone Induces Apoptosis in Bovine Mammary Epithelial Cells by Activating Endoplasmic Reticulum Stress. *J Dairy Sci.* **2019**, *102* (11), 10543–10553. https://doi.org/10.3168/jds.2018-16216.

(5) Venkataramana, M.; Chandra Nayaka, S.; Anand, T.; Rajesh, R.; Aiyaz, M.; Divakara, S. T.; Murali, H. S.; Prakash, H. S.; Lakshmana Rao, P. V. Zearalenone Induced Toxicity in SHSY-5Y Cells: The Role of Oxidative Stress Evidenced by N-Acetyl Cysteine. *Food Chem Toxicol* **2014**, *65*, 335–342. https://doi.org/10.1016/j.fct.2013.12.042.

(6) Hassen, W.; Ayed-Boussema, I.; Oscoz, A. A.; De Cerain Lopez, A.; Bacha, H. The Role of Oxidative Stress in Zearalenone-Mediated Toxicity in Hep G2 Cells: Oxidative DNA Damage, Gluthatione Depletion and Stress Proteins Induction. *Toxicology* **2007**, *232* (3), 294–302. https://doi.org/10.1016/j.tox.2007.01.015.

(7) Langie, S. A. S.; Azqueta, A.; Collins, A. R. The Comet Assay: Past, Present, and Future. *Frontiers in Genetics* **2015**, *6* (Aug), 1–3. https://doi.org/10.3389/fgene.2015.00266.

(8) Mirzayans, R.; Murray, D. Do TUNEL and Other Apoptosis Assays Detect Cell Death in Preclinical Studies? *Int J Mol Sci* **2020**, *21* (23), 9090. https://doi.org/10.3390/ijms21239090.

(9) Collins, a R. The Comet Assay for DNA Damage and Repair: Principles, Applications, and Limitations. *Molecular Biotechnology* **2004**, *26* (3), 249–261. https://doi.org/10.1385/MB:26:3:249.

(10) Møller, P.; Loft, S.; Ersson, C.; Koppen, G.; Dusinska, M.; Collins, A. On the Search for an Intelligible Comet Assay Descriptor. *Frontiers in Genetics* **2014**, *5* (JUL), 3–6. https://doi.org/10.3389/fgene.2014.00217.

(11) Braafladt, S.; Reipa, V.; Atha, D. H. The Comet Assay: Automated Imaging Methods for Improved Analysis and Reproducibility. *Scientific Reports* **2016**, *6* (July), 1–9. https://doi.org/10.1038/srep32162.

(12) Peycheva, E.; Georgieva, M.; Miloshev, G. Comparison Between Alkaline and Neutral Variants of Yeast Comet Assay. *Biotechnol Biotechnol Equip* **2009**, *23* (1), 1090–1092. https://doi.org/10.1080/13102818.2009.10817618.

(13) Kerr, J. F. R.; Wyllie, A. H.; Currie, A. R. Apoptosis: A Basic Biological Phenomenon with Wide-Ranging Implications in Tissue Kinetics. *Br J Cancer* **1972**, *26* (4), 239–257. https://doi.org/10.1038/bjc.1972.33.

(14) Loo, D. T. In Situ Detection of Apoptosis by the TUNEL Assay: An Overview of Techniques. In *DNA Damage Detection In Situ, Ex Vivo, and In Vivo: Methods and Protocols*; Didenko, V. V., Ed.; Humana Press: Totowa, NJ, 2011; Vol. 682, pp 3–13. https://doi.org/10.1007/978-1-60327-409-8_1.

(15) Majtnerová, P.; Roušar, T. An Overview of Apoptosis Assays Detecting DNA Fragmentation. *Mol Biol Rep.* **2018**, *45* (5), 1469–1478. https://doi.org/10.1007/s11033-018-4258-9.

(16) Darzynkiewicz, Z.; Galkowski, D.; Zhao, H. Analysis of Apoptosis by Cytometry Using TUNEL Assay. *Methods* **2008**, *44* (3), 250–254. https://doi.org/10.1016/j.ymeth.2007.11.008.

(17) Marin, D. E.; Motiu, M.; Taranu, I. Food Contaminant Zearalenone and Its Metabolites Affect Cytokine Synthesis and Intestinal Epithelial Integrity of Porcine Cells. *Toxins (Basel)* **2015**, *7* (6), 1979–1988. https://doi.org/10.3390/toxins7061979.

(18) Pinton, P.; Oswald, I. P. Effect of Deoxynivalenol and Other Type B Trichothecenes on the Intestine: A Review. *Toxins (Basel)* **2014**, *6* (5), 1615–1643. https://doi.org/10.3390/toxins6051615.

(19) Bouhet, S.; Oswald, I. P. The Effects of Mycotoxins, Fungal Food Contaminants, on the Intestinal Epithelial Cell-Derived Innate Immune Response. *Vet Immunol and Immunopathol* **2005**, *108* (1), 199–209. https://doi.org/10.1016/j.vetimm.2005.08.010.

(20) Liew, W.-P.-P.; Mohd-Redzwan, S. Mycotoxin: Its Impact on Gut Health and Microbiota. *Front Cell Infect Microbiol.* **2018**, *8*, 60. https://doi.org/10.3389/fcimb.2018.00060.

(21) Wang, J.-S.; Groopman, J. D. DNA Damage by Mycotoxins. *Mutat Res* **1999**, *424* (1–2), 167–181. https://doi.org/10.1016/S0027-5107(99)00017-2.

(22) Vergauwen, H. The IPEC-J2 Cell Line. In *The Impact of Food Bioactives on Health*; Verhoeckx, K., Cotter, P., López-Expósito, I., Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., Wichers, H., Eds.; Springer International Publishing: Cham, 2015; pp 125–134. https://doi.org/10.1007/978-3-319-16104-4_12.

(23) Zakrzewski, S. S.; Richter, J. F.; Krug, S. M.; Jebautzke, B.; Lee, I.-F. M.; Rieger, J.; Sachtleben, M.; Bondzio, A.; Schulzke, J. D.; Fromm, M.; Günzel, D. Improved Cell Line IPEC-J2, Characterized as a Model for Porcine Jejunal Epithelium. *PLoS One* **2013**, *8* (11), e79643. https://doi.org/10.1371/journal.pone.0079643.

(24) Wang, H.; Chen, Y.; Zhai, N.; Chen, X.; Gan, F.; Li, H.; Huang, K. Ochratoxin A-Induced Apoptosis of IPEC-J2 Cells through ROS-Mediated Mitochondrial

Permeability Transition Pore Opening Pathway. *J Agric Food Chem* **2017**, *65* (48), 10630–10637. https://doi.org/10.1021/acs.jafc.7b04434.

(25) Huang, W.; Chang, J.; Wang, P.; Liu, C.; Yin, Q.; Song, A.; Gao, T.; Dang, X.; Lu, F. Effect of Compound Probiotics and Mycotoxin Degradation Enzymes on Alleviating Cytotoxicity of Swine Jejunal Epithelial Cells Induced by Aflatoxin B1 and Zearalenone. *Toxins* **2019**, *11* (1), 12. https://doi.org/10.3390/toxins11010012.

(26) Springler, A.; Vrubel, G.-J.; Mayer, E.; Schatzmayr, G.; Novak, B. Effect of Fusarium-Derived Metabolites on the Barrier Integrity of Differentiated Intestinal Porcine Epithelial Cells (IPEC-J2). *Toxins* **2016**, *8* (11), 345. https://doi.org/10.3390/toxins8110345.

(27) Goossens, J.; Pasmans, F.; Verbrugghe, E.; Vandenbroucke, V.; De Baere, S.; Meyer, E.; Haesebrouck, F.; De Backer, P.; Croubels, S. Porcine Intestinal Epithelial Barrier Disruption by the Fusarium Mycotoxins Deoxynivalenol and T-2 Toxin Promotes Transepithelial Passage of Doxycycline and Paromomycin. *BMC Vet Res.* **2012**, *8* (1), 245. https://doi.org/10.1186/1746-6148-8-245.

(28) European Commission. Commission Recommendation of 17 August 2006 on the Presence of Deoxynivalenol, Zearalenone, Ochratoxin A, T-2 and HT-2 and Fumonisins in Products Intended for Animal Feeding. *Official J. Eur. Union* **2006**.

(29) Lynch, S. J.; Horgan, K. A.; White, B.; Walls, D. Selenium Source Impacts Protection of Porcine Jejunal Epithelial Cells from Cadmium-Induced DNA Damage, with Maximum Protection Exhibited with Yeast-Derived Selenium Compounds. *Biol Trace Elem Res* **2017**, *176* (2), 311–320. https://doi.org/10.1007/s12011-016-0828-7.

(30) Zhang, G.-L.; Feng, Y.-L.; Song, J.-L.; Zhou, X.-S. Zearalenone: A Mycotoxin With Different Toxic Effect in Domestic and Laboratory Animals' Granulosa Cells. *Front Genet.* **2018**, *9*, 667. https://doi.org/10.3389/fgene.2018.00667.

(31) Poór, M.; Kunsági-Máté, S.; Sali, N.; Kőszegi, T.; Szente, L.; Peles-Lemli, B. Interactions of Zearalenone with Native and Chemically Modified Cyclodextrins and Their Potential Utilization. *J Photochem Photobiol B* **2015**, *151*, 63–68. https://doi.org/10.1016/j.jphotobiol.2015.07.009.

(32) Wang, Y.-L.; Zhou, X.-Q.; Jiang, W.-D.; Wu, P.; Liu, Y.; Jiang, J.; Wang, S.-W.; Kuang, S.-Y.; Tang, L.; Feng, L. Effects of Dietary Zearalenone on Oxidative Stress, Cell Apoptosis, and Tight Junction in the Intestine of Juvenile Grass Carp (Ctenopharyngodon Idella). *Toxins (Basel)* **2019**, *11* (6), 333. https://doi.org/10.3390/toxins11060333.

(33) Sun, L.-H.; Lei, M.; Zhang, N.-Y.; Gao, X.; Li, C.; Krumm, C. S.; Qi, D.-S. Individual and Combined Cytotoxic Effects of Aflatoxin B1, Zearalenone, Deoxynivalenol and Fumonisin B1 on BRL 3A Rat Liver Cells. *Toxicon* **2015**, *95*, 6–12. https://doi.org/10.1016/j.toxicon.2014.12.010.

(34) Zhou, H.; George, S.; Hay, C.; Lee, J.; Qian, H.; Sun, X. Individual and Combined Effects of Aflatoxin B1, Deoxynivalenol and Zearalenone on HepG2 and RAW 264.7 Cell Lines. *Food Chem Toxicol* **2017**, *103*, 18–27. https://doi.org/10.1016/j.fct.2017.02.017.

(35) Kang, C.; Lee, H.; Yoo, Y.-S.; Hah, D.-Y.; Kim, C. H.; Kim, E.; Kim, J. S. Evaluation of Oxidative DNA Damage Using an Alkaline Single Cell Gel Electrophoresis (SCGE) Comet Assay, and the Protective Effects of N-Acetylcysteine Amide on Zearalenone-Induced Cytotoxicity in Chang Liver Cells. *Toxicol Res* **2013**, *29* (1), 43–52. https://doi.org/10.5487/TR.2013.29.1.043.

(36) Muthulakshmi, S.; Maharajan, K.; Habibi, H. R.; Kadirvelu, K.; Venkataramana, M. Zearalenone Induced Embryo and Neurotoxicity in Zebrafish Model (Danio Rerio): Role of Oxidative Stress Revealed by a Multi Biomarker Study. *Chemosphere* **2018**, *198*, 111–121. https://doi.org/10.1016/j.chemosphere.2018.01.141.

(37) Tatay, E.; Espín, S.; García-Fernández, A.-J.; Ruiz, M.-J. Oxidative Damage and Disturbance of Antioxidant Capacity by Zearalenone and Its Metabolites in Human Cells. *Toxicol In Vitro* **2017**, *45* (Pt 3), 334–339. https://doi.org/10.1016/j.tiv.2017.04.026.

(38) Tatay, E.; Font, G.; Ruiz, M.-J. Cytotoxic Effects of Zearalenone and Its Metabolites and Antioxidant Cell Defense in CHO-K1 Cells. *Food Chem Toxicol* **2016**, *96*, 43–49. https://doi.org/10.1016/j.fct.2016.07.027.

(39) Zhu, L.; Yuan, H.; Guo, C.; Lu, Y.; Deng, S.; Yang, Y.; Wei, Q.; Wen, L.; He, Z. Zearalenone Induces Apoptosis and Necrosis in Porcine Granulosa Cells via a Caspase-3- and Caspase-9-Dependent Mitochondrial Signaling Pathway. *J Cell Physiol* **2012**, *227* (5), 1814–1820. https://doi.org/10.1002/jcp.22906.

(40) Wang, Y.; Zheng, W.; Bian, X.; Yuan, Y.; Gu, J.; Liu, X.; Liu, Z.; Bian, J. Zearalenone Induces Apoptosis and Cytoprotective Autophagy in Primary Leydig Cells. *Toxicol Lett* **2014**, *226* (2), 182–191. https://doi.org/10.1016/j.toxlet.2014.02.003.

(41) Hu, J.; Xu, M.; Dai, Y.; Ding, X.; Xiao, C.; Ji, H.; Xu, Y. Exploration of Bcl-2 Family and Caspases-Dependent Apoptotic Signaling Pathway in Zearalenone-Treated Mouse Endometrial Stromal Cells. *Biochem Biophys Res Commun*. **2016**, *476* (4), 553–559. https://doi.org/10.1016/j.bbrc.2016.05.161.

(42) Ayed-Boussema, I.; Bouaziz, C.; Rjiba, K.; Valenti, K.; Laporte, F.; Bacha, H.; Hassen, W. The Mycotoxin Zearalenone Induces Apoptosis in Human Hepatocytes (HepG2) via P53-Dependent Mitochondrial Signaling Pathway. *Toxicol in Vitro* **2008**, *22* (7), 1671–1680. https://doi.org/10.1016/j.tiv.2008.06.016.

(43) Reich, H. J.; Hondal, R. J. Why Nature Chose Selenium. *ACS Chem Biol* **2016**, *11* (4), 821–841. https://doi.org/10.1021/acschembio.6b00031.

(44) Gan, F.; Xue, H.; Huang, Y.; Pan, C.; Huang, K. Selenium Alleviates Porcine Nephrotoxicity of Ochratoxin A by Improving Selenoenzyme Expression In Vitro. *PLoS One* **2015**, *10* (3), e0119808. https://doi.org/10.1371/journal.pone.0119808.

(45) Yu, J.; Chen, D.; Yu, B. Protective Effects of Selenium and Vitamin E on Rats Consuming Maize Naturally Contaminated with Mycotoxins. *Front Agric China* **2009**, *3* (1), 95–99. https://doi.org/10.1007/s11703-009-0015-0.

(46) Xiao, Y.; Xu, S.; Zhao, S.; Liu, K.; Lu, Z.; Hou, Z. Protective Effects of Selenium against Zearalenone-Induced Apoptosis in Chicken Spleen Lymphocyte via an Endoplasmic Reticulum Stress Signaling Pathway. *Cell Stress and Chaperones* **2019**, *24* (1), 77–89. https://doi.org/10.1007/s12192-018-0943-9.

(47) Long, M.; Yang, S.; Wang, Y.; Li, P.; Zhang, Y.; Dong, S.; Chen, X.; Guo, J.; He, J.; Gao, Z.; Wang, J. The Protective Effect of Selenium on Chronic Zearalenone-Induced Reproductive System Damage in Male Mice. *Molecules* **2016**, *21* (12), 1687. https://doi.org/10.3390/molecules21121687.

(48) Alltech. Mycosorb A+® https://www.knowmycotoxins.com/mycotoxin-mitigation/mycosorb-a/ (accessed 2019 -02 -14).

(49) Dvorska, J. E.; Pappas, A. C.; Karadas, F.; Speake, B. K.; Surai, P. F. Protective Effect of Modified Glucomannans and Organic Selenium against Antioxidant Depletion in the Chicken Liver Due to T-2 Toxin-Contaminated Feed Consumption. *Comp Biochem Physiol C Toxicol Pharmacol* **2007**, *145* (4), 582–587. https://doi.org/10.1016/j.cbpc.2007.02.005.

(50) Mohaghegh, A.; Chamani, M.; Shivazad, M.; Sadeghi, A. A.; Afzali, N. Effect of Esterified Glucomannan on Broilers Exposed to Natural Mycotoxin-Contaminated Diets. *J Appl Anim Res* **2017**, *45* (1), 285–291. https://doi.org/10.1080/09712119.2016.1174122.

(51) Wade, M. R.; Sapcota, D. Effect of Dietary Esterified Glucomannan on the Performance of Broiler Chickens During Experimental Aflatoxicosis. *Anim Nutr Technology* **2017**, *17* (1), 107. https://doi.org/10.5958/0974-181X.2017.00011.7.

(52) Abdou, H. M.; Hassan, M. A. Protective Role of Omega-3 Polyunsaturated Fatty Acid against Lead Acetate-Induced Toxicity in Liver and Kidney of Female Rats. *Biomed Res. Int.* **2014**, *2014*, e435857. https://doi.org/10.1155/2014/435857.

(53) Kim, H.-Y.; Akbar, M.; Kim, K.-Y. Inhibition of Neuronal Apoptosis by Polyunsaturated Fatty Acids. *J Mol Neurosci* **2001**, *16* (2–3), 223–227. https://doi.org/10.1385/JMN:16:2-3:223.

(54) Pfrommer, C. A.; Erl, W.; Weber, P. C. Docosahexaenoic Acid Induces Ciap1 MRNA and Protects Human Endothelial Cells from Stress-Induced Apoptosis. *Am J Physiol Heart Circ Physiol* **2006**, *290* (6), H2178–H2186. https://doi.org/10.1152/ajpheart.00933.2005.

(55) European Food Safety Authority. Scientific Opinion on Safety and Efficacy of Sel-Plex® (Organic Form of Selenium Produced by Saccharomyces Cerevisiae CNCM I-3060) for All Species. *EFSA Journal* **2011**, *9* (4), 2110. https://doi.org/10.2903/j.efsa.2011.2110.

Chapter 3: Investigating the extent to which DON can induce damage on IPEC-J2 cells and feed supplements can protect against damage observed

Abstract

Deoxynivalenol (DON) is a *Fusarium* mycotoxin and a common contaminant of cereal grains, which are often used as raw materials in animal feed. Pigs are particularly susceptible to DON toxicity and ingestion of the toxin can result in detrimental health effects in animals, including vomiting, diarrhoea, reduced growth and loss of appetite. The European Food Safety Authority (EFSA) has recommended that the level of DON in pig feed should not exceed 0.9 ppm. The first aim of this study was to investigate the effect of DON on a porcine intestinal epithelial IPEC-J2 cell model. Exposure to DON led to the reduction of cell viability when this toxin was present at concentrations lower than that recommended by EFSA. Additionally, a time and dose dependent cytotoxic effect was observed. DON was also shown to induce apoptosis following 24 h exposure at 0.9 ppm. The comet assay showed that DON did not induce DNA damage at levels up to 5 ppm following 24 h exposure. The second aim of this chapter was to investigate the potential protective effects of the feed supplements Se-Y and My-A+. The results showed that pre-incubating the cells with the feed supplements resulted in reduced cytotoxicity and apoptosis induced by DON. Overall, it was found that DON induced cytotoxicity and apoptosis in IPEC-J2 cells but DNA damage was not detected by the comet assay.

3.1 Introduction

Mycotoxins are found to contaminate various staple food used as raw materials for animal feed. They can have adverse effects on the health of farm animals including pigs, poultry and ruminants [1,2]. Due to the negative health effects of mycotoxins, it has become increasingly important to study mitigation methods for the reduction of toxins in animal feed. Many mycotoxin decontamination strategies have been studied to reduce the amount of toxin that is ultimately consumed and this includes methods such as cleaning, sorting, heating and adsorption [3–7]. In Chapter 2, the protective effects of the feed additives against ZEN toxicity was investigated. However, in order to understand the mechanism of their protective effects, the mode of interaction of these feed additive should be explored. Feed additives and supplementations are often combined with animal feed in order to reduce the toxic effects of mycotoxins in animals [7]. Selenium (Se) supplementation has been used to mitigate against toxicity of the contaminated feed [8,9]. Additionally, yeast cell wall (YCW) based feed additives are increasingly being used as a means of adsorbing and detoxifying mycotoxins in the digestive tract of the animal [7,10].

3.1.1 Selenium

Se is an essential dietary trace element that is naturally present in food, and was discovered in 1817 by Swedish chemist Jons Jacob Brezelius [11]. It is a member of Group VIA (16) in the periodic table of elements, also known as the chalcogens or the oxygen group and is a metalloid. It is found naturally in ore minerals and in soil and can exist in different molecular forms [12]. Se can exist in four oxidation states in nature: selenide (Se(-2)), elemental Se (Se(0)), selenite (Se(+4)) and selenate (Se(+6)) [13,14].

The main Se compound found in the body is selenocysteine (Se-C) which is the 21st amino acid and proteins made up of or containing SeCys are known as selenoproteins. Glutathione peroxidase (GSH-Px) was the first selenoprotein to be discovered [15,16]. GSH-Px is an enzymatic antioxidant that inhibits cellular damage from oxidative stress by the reduction of hydrogen peroxides to water [17,18]. However, the activity of this selenoprotein is related to the Se concentration available in the body. Therefore, a lower intake of Se can result in the reduction of antioxidant activity of GSH-Px which has the potential to result in increased oxidative stress [19,20].

Se is essential for humans and animals as deficiency of the element can result in negative health effects such as liver necrosis, immune deficiencies, fertility issues, cystic ovaries etc. [14,21]. Keshan's disease, which is a cardiomyopathy of young women and children and Kashin-Beck disease, which is an endemic osteoarthropathy, are both also associated with Se deficiency [19,21]. The dietary reference value given by the WHO and Food and Agriculture Organisation (FAO) in 2004 was 34 and 26 µg/day for men and women, respectively, within the age group of 19-65 years. For men and women older than 65, this value was 33 and 25 µg/day, respectively [22].

Se deficiency can also result in negative health effects in animals such as lower milk and wool production as well as lower weight gain and reduced fertility in animals [23,24]. Ruminants are more susceptible to Se deficiency as they absorb Se less efficiently than other animal groups, with 77% retention of orally ingested Se observed in swine, compared with only 29% retention in sheep [23,25,26]. Although Se deficiency can result in negative health impacts, excess Se has also been found to be toxic. Acute Se toxicity can result in vomiting, discolouration of nails, hair loss and foul breath odour [27,28].

Dietary Se is the most practical method of Se intake, with plants being the main source for both animals and humans [29]. However, the concentration of Se in plants is highly dependent on the Se concentration in the soil, which can vary geographically [19,29,30]. Se occurs as elemental Se, selenates, selenites and organic Se in soil [31,32]. The chemical properties of Se are similar to those of sulphur, therefore the uptake of Se in plants occurs via sulphate transporters in the plasma membranes of the root cells [33,34]. Se competes with sulphate in the soil for uptake by plants, and thus the presence of sulfate ions can inhibit the uptake of selenate [34,35]. Selenate is often favoured over selenite and organic Se for plant uptake by sulphate transporters [34]. Alkaline soils in oxic conditions often favour selenate and neutral to acidic soils under less oxic conditions favour selenite [33]. After the uptake of selenate or selenite by plants, selenates are reduced to selenite, which is then reduced to selenide and then converted into organic forms, especially selenomethionine (Se-M) and (Se-C) [33,34,36].

Bioavailability is defined as the fraction of ingested and absorbed nutrient that is utilised for normal physiological functions or storage [19,37,38]. Thus, the bioavailability of Se is dependent on how well it is absorbed and Se uptake in the body is dependent on the

source of Se. The majority of orally ingested Se is absorbed, first, in the duodenum, followed by the jeunum and ileum [14].

The inorganic form selenite is passively absorbed by simple diffusion and selenate is actively absorbed with the sodium ions through the co-transport pathway [39,40]. Once selenite is absorbed, it is immediately non-enzymatically reduced to dihydrogen selenide (H_2Se) by glutathione (GSH) [14,28,39]. The selenide is then transported to the liver for selenoprotein synthesis [38]. In the case of selenate, it requires several passes of blood via the liver before it is reduced to selenite then selenide and a considerable amount of the absorbed selenate is excreted in the urine before it is reduced as it is not as readily taken up by red blood cells as selenite [39,41].

The absorption of organic Se occurs in the small intestine by active transport via amino acid transport mechanisms [39,40]. The Se from Se-M can be metabolised to Se-C, which is then converted into hydrogen selenide by β-lyase. It can also be used for the synthesis of proteins as it can freely substitute for methionine [39,41–43]. Excess Se is excreted in breath or urine after methylation of H_2Se [43].

Due to the varying concentration of Se in soil and the deleterious health effects caused by Se deficiency, Se supplementation is often added to animal feed. Se supplements are commercially available in organic form including Se-M and Se-enriched yeast (Se-Y) and inorganic forms including sodium selenite (Na_2SeO_3) and sodium selenate (Na_2SeO_4) [43]. Inorganic forms of Se supplementation is often used for animal feed, as they are less expensive [43].

Several studies have shown important differences in bioavailability between organic and inorganic forms of Se supplementations, consistently indicating that organic forms have higher bioavailability [44–46]. In one such study, the bioavailability of three Se based food supplements, namely Se-Y, selenate based supplement and selenite based supplement was analysed. This was carried out by investigating the fraction of soluble Se that was transported through a monolayer of Caco-2 cells and the fraction that was actually retained in the cells during the process of absorption. The results showed that there was a significant difference between the bioavailability of the three supplements. It was found that selenite showed the lowest fraction of bioavailable Se. The selenate based supplement showed the highest bioavailable fraction, followed by Se-Y [44]. This result was comparable with another study where the bioavailability of orally ingested Se-Y was

compared with that of Se-Ni in rats. The study also concluded that Se-Y had a higher bioavailability than selenite [45]. The effect of selenite, selenate and Se-Y on dairy cows was investigated, where the cows were split into three groups and each group was supplemented with 3 mg of one of the supplements. There was also a control group of cows, which were not given any supplements. After 12 weeks, the blood Se concentration was determined to be approximately 35% higher in the selenite and selenate groups and approximately 60% higher in the Se yeast group than in the control. There was no significant difference between the selenite and selenate groups. A similar trend was observed in the average Se concentration in milk. The average Se concentration in milk was 16.4, 16.4, 31.2 and 14 µg/L for selenite, selenate, yeast and control group respectively. A plateau was reached within one week of supplementation. The results of this study shows that Se from Se-Y was better absorbed than Se from inorganic sources [47].

Reactive oxygen species (ROS) such as superoxide and peroxide radicals can result in DNA damage by oxidation of nitrogenous DNA bases. Aerobic organisms have enzymatic and non-enzymatic antioxidants that are effective in inhibiting the harmful effects of ROS [18]. Selenoenzymes such as GSH-Px and thioredoxin reductase (TrxR) have been shown to have antioxidant properties [19,48]. GSH-Px is an enzymatic antioxidant, and its main role is to maintain low levels of hydrogen peroxide in the cell, which in turn reduces free radical damage [17,18,48]. The main function of the TrxR is to maintain thioredoxin in a reduced state for removal of harmful hydrogen peroxide [19,21].

Due to its antioxidant properties, the protective effect of Se has been an area of interest. A study carried out by Tran *et al*. investigated the protective effect of Se against DNA damage caused by mercury in haemocytes from *Mytilus edulis* (blue mussel) [49]. Using the comet assay, it was found that when the haemocytes were exposed to 20 µg/L mercury chloride ($HgCl_2$), there were high levels of single strand DNA breaks. The results showed that pre-exposure to 4 µg/L sodium selenite reduced DNA damaged induced by the mercury. The same study also showed that GPx activity doubled after three days in the presence of Se [49]. In another study, Se-Y was found to have a protective effect against lead induced DNA damage in liver cells. HepG2 cells were treated with 1 µg/mL Se-Y and 40 µg/mL lead nitrate ($Pb(NO_3)_2$) for 24 h. The comet assay was then used to show that the percentage DNA damage decreased from 70% to 50% when compared to cells treated with 40 µg/mL $Pb(NO_3)_2$ alone. Furthermore, a 50% reduction in DNA damage

was found when the cells were treated with 1 μg/mL Se-Y and 80 μg/mL $Pb(NO_3)_2$ in comparison to the 80 μg/mL $Pb(NO_3)_2$ control [50]. Similar results were found in another study that investigated the effect of Se against cadmium (Cd) induced damage. Pre-incubation of Se-Y for 48 h prior to 24 h incubation with Cd resulted in remedial effects against Cd-induced DNA damage [51].

3.1.2 Mycotoxin binder

One of the methods of post-harvest mycotoxin elimination is adsorption by using a feed supplement. Mycotoxin binders are adsorbents that are added to animal feed and that can eliminate mycotoxins from the digestive tract of the animal [52]. There are various adsorption agents available but their effectiveness in the removal of mycotoxins can vary [53]. Zeolites are hydrated aluminosilicate minerals that have been investigated for their mycotoxin binding abilities [54]. Natural zeolites with high clintoptilolite resulted in effective adsorption of AFB1 [55]. Organically modified zeolites were found to adsorb AFB1, OTA and Zearalenone [56]. Activated charcoal is a non-toxic adsorbent powder that is formed by the pyrolysis of various organic compounds [57]. It has been shown to be effective in the removal of mycotoxins including AFB1, OTA and ZEN [58–60]. Bentonite clay is another material used in the adsorption of mycotoxins. One study has showed that bentonite clay had a higher binding affinity for AFB1 than for DON [58]. Elsewhere, it was shown that bentonite clay had a protective effect against ZEN induced toxicity in Caco-2 and THP-1 cells [61].

In the recent years, yeast cell walls have been used as a mycotoxin binder product. This type of organic binder has been known to bind to and remove mycotoxins in the digestive tract of animals [62–64]. Yeast cell wall (YCW) was found to be effective in the adsorption of AFB1 (92.7% adsorption) and DON (22.9% adsorption) [58]. Another study showed that a YCW product from baker's yeast could adsorb 68% of ZEN, 29% of AFB1 and 62% of OTA during challenge with these toxins [65].

Mycosorb A+ (My-A+) is a mycotoxin binder, produced by Alltech Ltd., which works by reducing mycotoxin absorption within the animal gut. It is an YCW based additive derived from the yeast *Saccharomyces cerevisiae*. As well as yeast, My-A+ also contains heterotrophically grown algae. The carbohydrate components of both the yeast and algal cell walls binds to mycotoxins and removes them from the digestive tract of the animal

[63]. The algal component in the binder is rich in polyunsaturated fatty acids (PUFAs) which provide it with antioxidant properties [66,67].

PUFAs are long hydrocarbon chains with two or more *cis* double bonds present in their structure [68]. There are two groups of PUFAs, namely omega-3 (n-3) and omega-6 (n-6). The structural difference between n-3 and n-6 fatty acids (FAs) is the position of the first double bond. n-3 FAs have their first double bond between the third and fourth carbon from the methyl terminal and the first double bond in n-6 FAs is between the sixth and seventh carbon atoms [69–71].

Humans and other mammals are unable to synthesise PUFAs and therefore must obtain them through dietary means [72,73]. The two essential PUFAs are α-linolenic acid (ALA), which is the parent n-3 FA and linoleic acid (LA), which is the parent n-6 FA [74,75]. Long chain FAs are synthesised within the body from ALA and LA. Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are both long chain n-3 FAs that are synthesised from ALA. These long chain FAs can also be obtained via dietary means. Similarly, dihomo-y-linolenic acid (DGLA) and arachidonic acid (ARA) are n-6 FAs synthesised in the body from LA [75–77]. However, DGLA and ARA are scarcely available via dietary means [78]. n-3 PUFAs are mainly found in fish, meat and eggs, rapeseed, walnuts, chia seeds and flaxseeds. n-6 PUFAs are found in various oils including sunflower, safflower and corn oil as well as nuts and seeds including walnuts and sunflower seeds [79,80].

n-3 PUFAs have been shown to have anti-inflammatory properties and there is published evidence that they can reduce the risk of cardiovascular diseases and depression [81–85]. DHA is important in the regulation of brain function and neuroinflammation and is also required in maintaining normal retinal functions [76,86,87]. PUFAs have also been shown to mitigate against DNA damage. One study showed the mitigation of H_2O_2-induced oxidative stress-induced DNA damage by n-3 PUFAs EPA and DHA in human aortic endothelial cells [88]. In another study, rats were split into two groups and fed different diets. One group was fed fish oil, which was rich in n-3 PUFAs and the other group was fed safflower oil, which was rich in n-6 PUFAs. The rats were then injected with ferric nitrilotriacetate to induce oxidative stress. DNA damage was induced in the livers of both groups but significantly less damage was observed in the group that was fed the fish oil diet indicating that the fish oil supplement rich in n-3 PUFAs had a protective effect against DNA damage induced by oxidative stress [89]. Elsewhere, DHA was shown to have

a protective effect against apoptosis in retinal neurons induced by oxidative damage [90]. These studies show the protective effect of PUFAs against DNA damage and apoptosis induced by various sources.

3.1.3 Chapter aims

The aim of this chapter was to study the effect DON has on IPEC-J2 cells. Here, DON was shown to induce cytotoxicity and apoptosis following exposure for 24 h. However, DNA damage was not detected using the comet assay. The protective effect of the feed supplements Sel-Plex (Se-Y) and Mycosorb A+ (My-A+), both individually and combined, against DON induced damage was also investigated. Both feed supplements, used separately and in combination, were seen to have a protective effect against DON induced damage.

3.2 Materials and Methods

3.2.1 Cell culture

Cell culture methods were carried out as outlined in Chapter 2 (Section 2.2.1)

3.2.2 Deoxynivalenol preparation

Deoxynivalenol (Sigma Aldrich) was bought in powder form and dissolved in ethanol to prepare a 2500 ppm stock solution. The stock solution was stored at -20°C and was diluted as necessary in serum free medium. The final concentration of ethanol was kept below 1% during cell culture. IPEC-J2 cells were incubated with 0-1% ethanol for 24 and 48 h.

3.2.3 Preparation of selenised yeast powder and mycosorb A+

Se-Y and My-A+ (obtained directly from Alltech Ltd.) were prepared as outlined in section 2.2.3.

3.2.4 Cell viability assay

Alamar Blue and CFDA-AM were used for the analysis of cell toxicity. Cells were seeded in a black flat-bottomed 96 well plate at 2 x 10^4 cells/mL in DMEM (see 2.2.1) with 2% porcine serum. Cells were incubated with DON for 24 h. The cell viability assay was then carried out as outlined in Chapter 2 (Section 2.2.4). For analyses involving Se-Y and/or My-A+, the cells were incubated with digested Se-Y (0.4 ppm Se), My-A+ (2g/kg) or both for 48 h, prior to incubation with mycotoxins.

3.2.5 TUNEL Assay

Cells were pre-incubated with the feed supplements for 48 h, when required, followed by a 24 h incubation with DON. The TUNEL assay was then carried out according to the manufacturer's instructions (Phoenix Flow Systems, San Diego, CA) as outlined in the Chapter 2 (Sections 2.2.5).

3.2.6 Alkaline Comet Assay

Cells were pre-incubated with the feed supplements for 48 h, when required, followed by a 24 h incubation with DON. The comet assay was then carried out as outlined in the Chapter 2 (Sections 2.2.6).

3.3 Results

3.3.1 DON is toxic to IPEC-J2 cells at levels below the EFSA limit

The cytotoxic effect of DON at concentrations between 0 - 0.9 ppm was investigated on IPEC-J2 cells. The cells were incubated with DON for 24 and 48 h. The highest concentration of DON was chosen as 0.9 ppm for the cytotoxicity assay as it is the current maximum concentration of DON for feeding stuffs for pigs as set by the EU [2]. The cell viability was measured by a combined cell viability assay made up of AB and CFDA-AM. The results in Figure 3.1 show that DON was toxic to IPEC-J2 cells in a time and dose dependent manner.

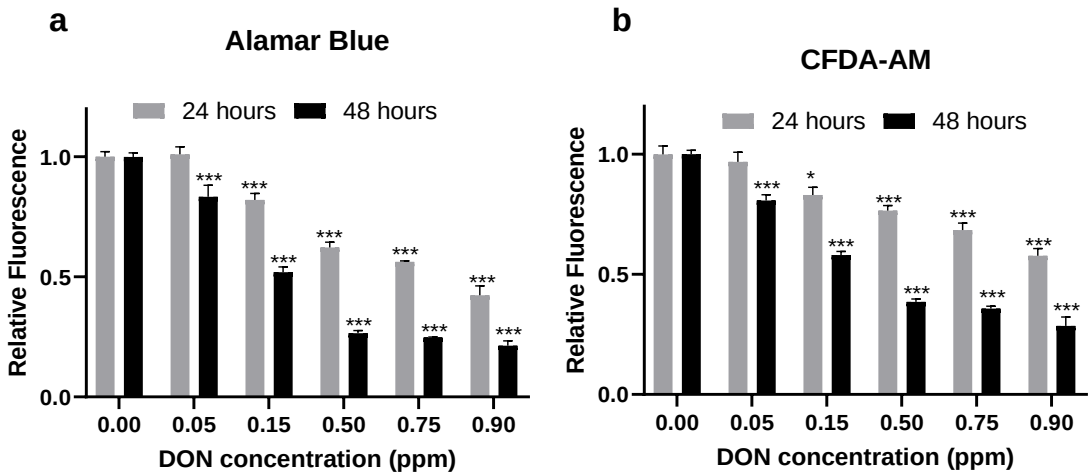

Figure 3.1: *The effect on IPEC-J2 viability following 24 and 48 h exposure to DON. Results were obtained using the combined AB/CFDA-AM dual assay (a: alamar blue, b: CFDA-AM). Significant differences between control cells (0 ppm DON) and cells insulted with DON were determined by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

The results show that DON was found to be significantly cytotoxic to the cells at 0.15 ppm following 24 h exposure. A decrease in cell viability was observed with increasing DON concentration. Additionally DON was found to be more toxic to the cells following the longer exposure time of 48 h.

3.3.2 DON induces apoptosis in IPEC-J2 cells

The TUNEL assay was used to determine apoptosis-induced DNA fragmentation in IPEC-J2 cells after incubation with DON for 24 h. DON induced apoptosis was analysed after DON exposure for 24 h, at concentrations of 0 – 5 ppm. The sizes of TUNEL positive cell populations indicated the extent of apoptosis and TUNEL negative values

represented healthy cells. The bar chart in Figure 3.2 represents the TUNEL negative percentage of IPEC-J2 cells. The TUNEL negative value of healthy control cells was 97.1%. Increasing DON concentrations resulted in decreased TUNEL negative populations.

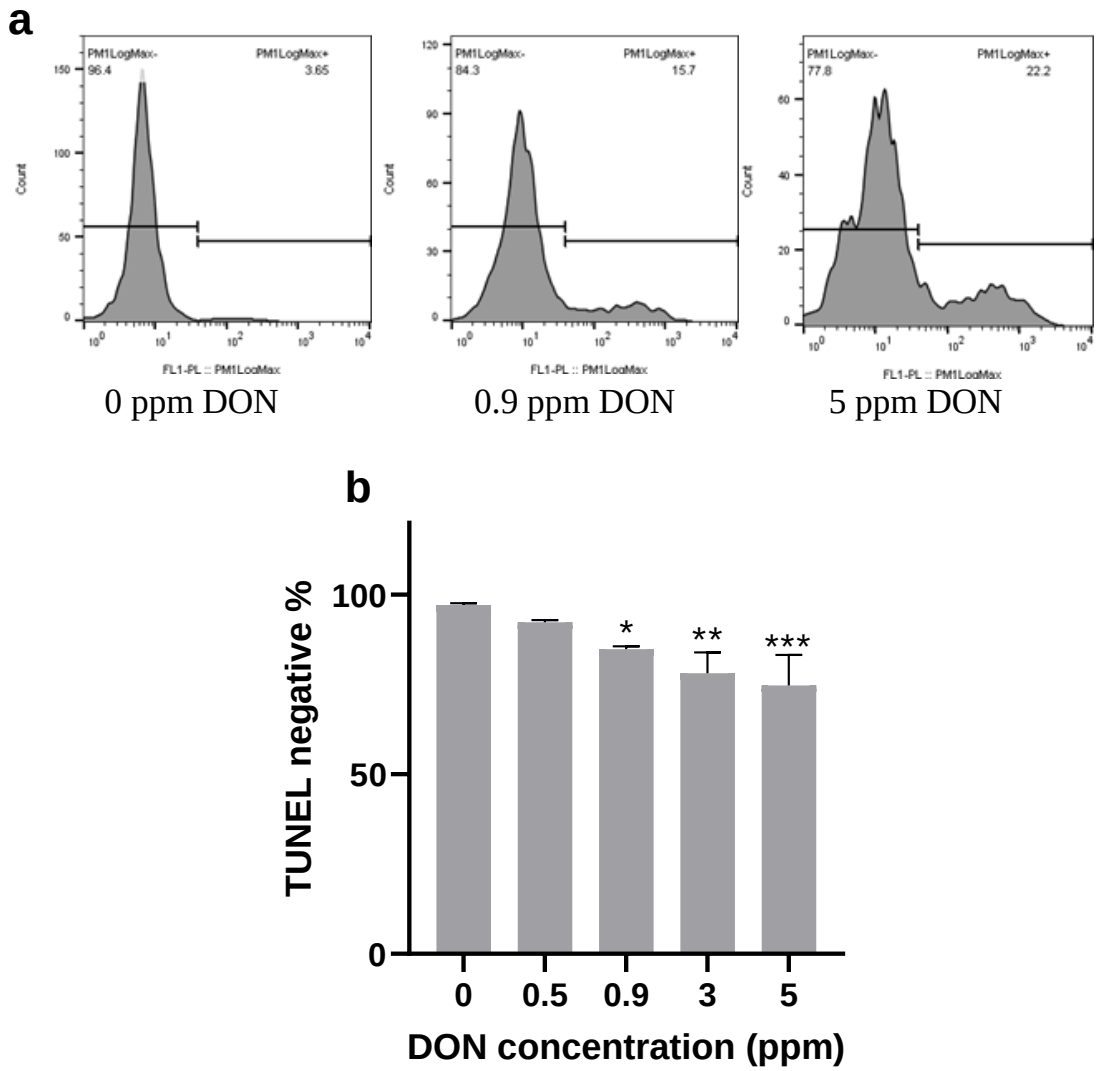

Figure 3.2: *DON induces apoptosis in IPEC-J2 cells. Cells were exposed to increasing concentrations of DON for 24 h and analysed by the TUNEL assay. A) Representative histogram plots of cells treated with 0 ppm, 0.9 ppm and 5 ppm DON. B) TUNEL negative values for cells incubated increasing concentrations of DON. Data in the bar chart is presented as the mean ± SD of triplicate samples. Significant differences between control cells (0 ppm DON) and cells insulted with DON were calculated one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

Exposure to 0.5 ppm DON did not induce apoptosis even though significant cytotoxic effects were observed at this concentration by AB and CFDA-AM. DON was shown to induce apoptosis to IPEC-J2 cells at a concentration of 0.9 ppm. The TUNEL negative population decreased with increasing DON concentration.

3.3.3 DON-induced DNA damage was not detected by comet assay

The alkaline comet assay was used to analyse DNA damage in IPEC-J2 cells caused by various concentrations of DON. This assay is used to detect both single and double stranded DNA damage. Cells were incubated with various concentration of DON, in the range of 0 – 5 ppm, for 24 h and the slides were viewed at 10X magnification by fluorescent microscopy. No comets were observed for the cells that had been incubated with DON (Figure 3.3). In contrast, comets can be seen on the slide with cells insulted with hydrogen peroxide (H_2O_2) for 30 min, which was the positive control. No DNA damage by DON was detected by the comet assay at concentrations up to 5 ppm.

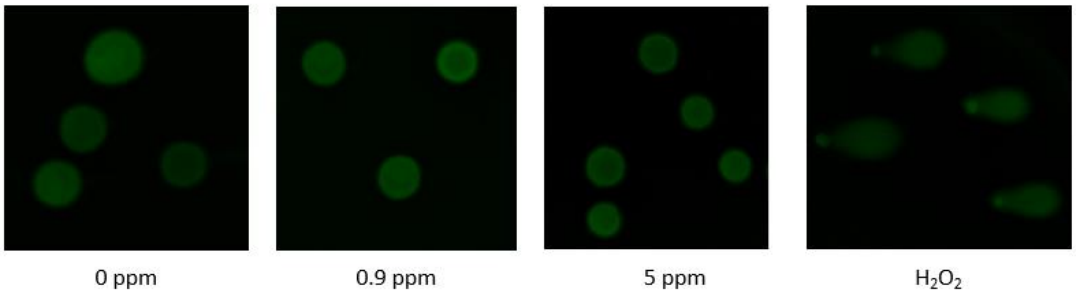

Figure 3.3: *DON does not induce DNA damage in IPEC-J2 at concentrations up to 5 ppm. Comet analysis of IPEC-J2 cells incubated with various concentration of DON for 24 hours.*

The Comet Score software was used to analyse the data collected from the microscope. The bar chart in Figure 3.4 shows that there was no significant difference in the percentage tail DNA between the healthy cells (0 ppm DON) and cells insulted with DON.

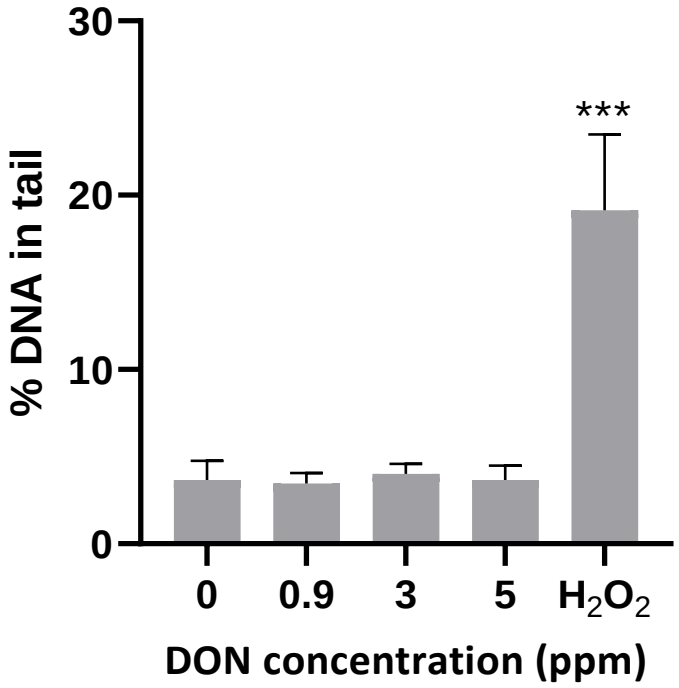

Figure 3.4: *Percentage tail DNA as determined by Comet Score software for IPEC-J2 cells incubated with DON for 24 hours. Data in the bar chart is presented as the mean of the median ± SD of triplicate samples. Significant difference between negative control cells and cells insulted with DON was highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

3.3.4 Sel-Plex and Mycosorb A+ exhibited protective effects against DON induced cytotoxicity

The AB/CFDA-AM dual cell viability assays were carried out on DON treated cells with and without pre-treatment with Se-Y and My-A+. Cells were pre-incubated with Se-Y and My-A+ for 48 h, followed by a 24 h incubation with 0.15 ppm or 0.9 ppm DON. An increase in cell viability was observed in the cells that were pre-incubated with the feed supplements when compared to the cells that were exposed to DON alone.

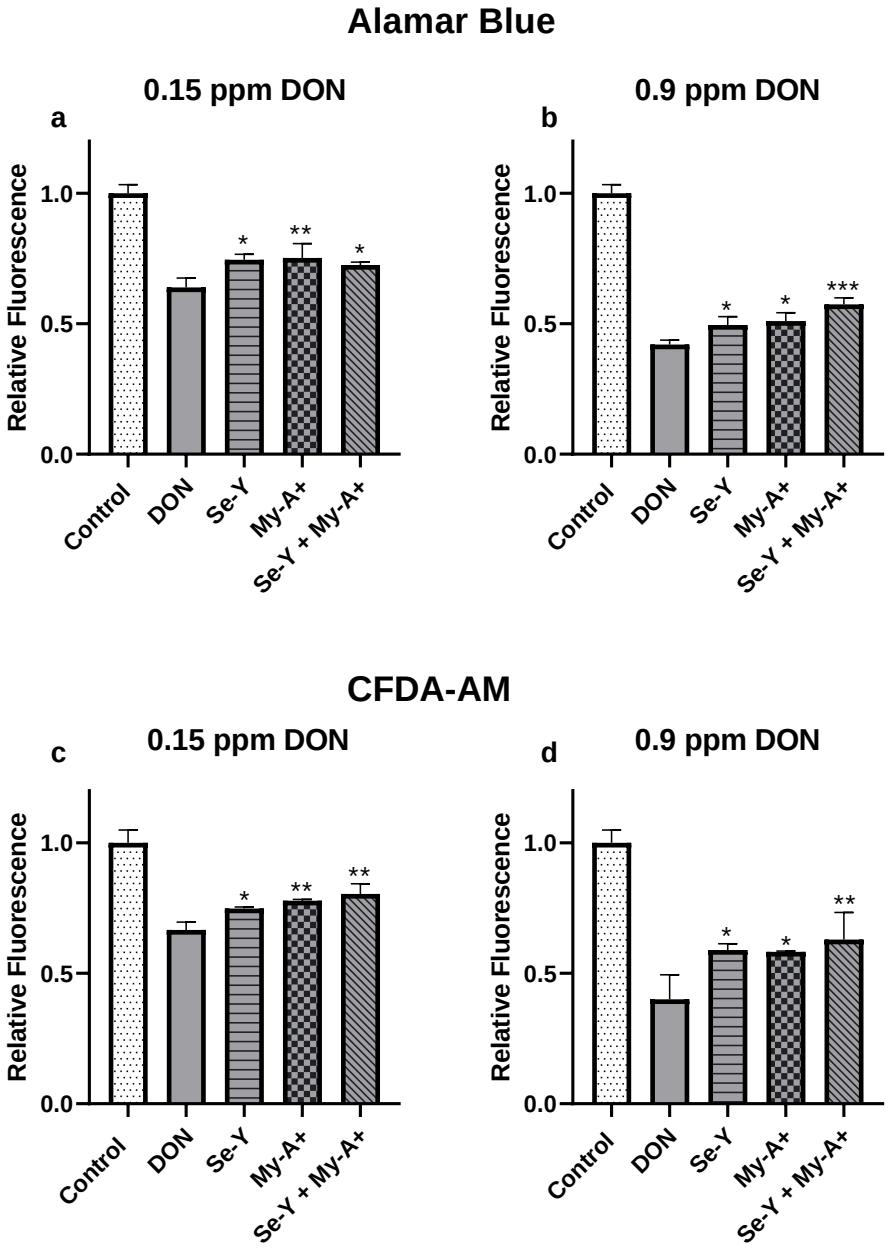

Figure 3.5: *Se-Y and My-A+ mitigate against DON induced cytotoxicity in IPEC-J2 cells. The graphs above represent a) cells treated with 0.15 ppm DON and analysed by alamar blue b) cells treated with 0.9 ppm DON and analysed by alamar blue c) cells treated with 0.15 ppm DON analysed by CFDA-AM and d) cells treated with 0.9 ppm DON analysed by CFDA-AM. In all cases cells were pre-incubated for 48 h with supplement (as indicated underneath) followed by insult with DON for 24 h. Results are expressed as the mean of triplicates ± SD and are presented as relative to the negative control (0 ppm DON, no supplements). Significant difference between the untreated and pre-treated samples were calculated by one-way ANOVA (*P<0.05, **P<0.01, ***P<0.001).*

3.3.5 Se-Y and My-A+ mitigate against DON induced apoptosis in IPEC-J2 cells

TUNEL assay was then used to investigate the protective effect of the feed supplements against DON damage. Cells were pre-incubated with Se-Y, My-A+ and Se-Y + My-A+ for 48 hours followed by a 24 hour incubation with 0.9 ppm DON. The results of the assay are summarised in Figure 3.6 as a histogram and a bar chart. Pre-incubation of the cells with the supplements resulted in an increase in the population of TUNEL negative cells. This shows that supplementation correlated with a reduction in the extent of DON-associated apoptosis.

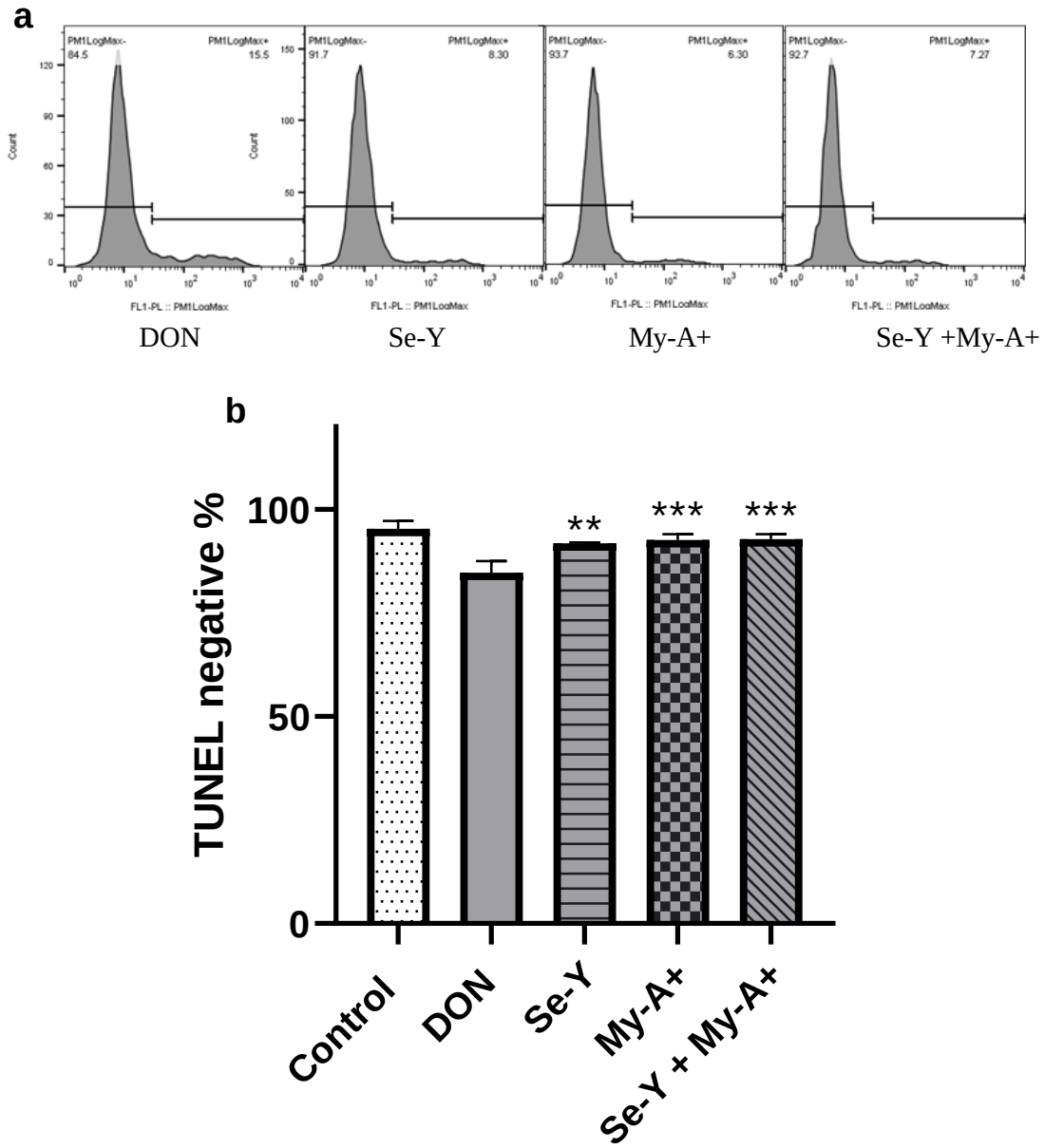

Figure 3.6: *Se-Y and My-A+ mitigate against DON induced apoptosis in IPEC-J2 cells. IPEC-J2 cells were pre-treated with Se-Y, My-A+ or a combination of both for 48 h followed by a 24-h exposure to 0.9 ppm DON and analysed by TUNEL assay. The upper panels show a representative histogram plots of cells with no-pre-treatment, cell pre-treated with Se-Y, My-A+ and Se-Y + My-A+. Data in the bar chart was obtained by represents the percentage TUNEL negative values presented as the mean ± SD of triplicate samples. Significant differences between cells with no pre-treatment (0.9 ppm DON, no supplements) and pre-treated cells are highlighted by one way ANOVA followed by Dunnett's test (*P<0.05, **P<0.01,***P<0.001).*

3.4 Discussion

The common worldwide occurrence of DON in grains is concerning as grains are used as raw materials for animal feed, including wheat, barley, corn and maize [92]. Cereal grains and their by-products are an important source of energy and protein for farm livestock [93]. Thus, the presence of DON in grains, even at low levels, can result in detrimental health effects in animals, especially in pigs. The cytotoxic, apoptotic and genotoxic effects DON on IPEC-J2 cells were investigated in this chapter.

The AB/CFDA-AM dual assay was employed to measure the cytotoxicity of the mycotoxin at various concentrations. Both the cellular metabolic activity and the membrane activity were measured with this assay. The recommended maximum level of DON in pig feed as directed by EFSA is 0.9 ppm. From the results of the cell viability assay, DON-induced cytotoxicity was observed at a concentration of 0.15 ppm, significantly lower than the EFSA recommendation, following 24 h exposure. Following a longer exposure of 48 h, it was found that an even lower concentration of 0.05 ppm DON exhibited cytotoxic effects on the cells. Thus, the results of the study showed that DON had a time and dose dependent detrimental effect on IPEC-J2. These results agree with the results of the study carried out by Awad *et al.*, where the effect of DON on IPEC-J2 cells was investigated by cell count and lactase dehydrogenase (LDH) release assay. The results of that study showed that with increasing DON concentration there was a decrease in total cell count and an increase in LDH release, which reflected the cytotoxic effects of DON [94]. DON also had a time and dose dependent effect on the viability of HepG2 cells [95]. A similar dose dependent response was also observed on the cell viability of Caco-2 cells, measured with the MTS assay, with toxicity noted at a DON concentration as low as 0.3 ppm [96]. These results are consistent with this study, which shows that DON induces cytotoxicity in a time and dose dependent manner.

The TUNEL assay was used in this study to measure the level of apoptosis induced by DON in IPEC-J2 cells. A decrease in the percentage of TUNEL negative cells was observed with increasing DON concentration, indicating increasing numbers of apoptotic cells. A concentration 0.5 ppm DON did not induce apoptosis, even though it was shown to induce significant cytotoxicity in the cells. Significant apoptosis was observed with 24 h exposure to 0.9 ppm DON. Higher concentrations of DON were shown to decrease the TUNEL negative population indicating the increase in apoptotic cells. Similar results

were obtained in a study by Kang *et al.*, where it was found that 0.5 ppm did not induce significant apoptosis on IPEC-J2 cells. The study showed that exposure to 1 ppm and 2 ppm DON for 24 h resulted in increased percentage of both early and late apoptotic cells [97]. Other studies using the IPEC-J2 cells also observed the induction of apoptosis by DON [98,99]. In another study, an increase in apoptosis of mouse endometrial stromal cells (ESCs) was also observed with increasing DON concentration [100]. Elsewhere, DON was also shown to induce apoptosis in bovine mammary epithelial cells [101]. These studies show that DON can induce apoptosis in various cell types.

The alkaline comet assay can detect both single and double stranded DNA breaks. Here, the assay was used to detect DNA damage induced by DON in IPEC-J2 cells. However, no comets were seen when the cells were incubated with concentrations of DON up to 0.9 ppm, which was the maximum concentration of DON permissible in swine feed. The cells were then incubated in higher DON concentrations up to 5 ppm but there was still no DNA damage detected. Each experiment was carried out with a positive control, where cells were incubated with H_2O_2 for 30 minutes and comets were detected for this control as seen in Figure 3.3 and 3.4.

Other studies have detected DNA damage by DON using the comet assay on different cell types. A study by Zhang *et al.* used the comet assay to investigate the effect of DON on HepG2 liver cells. The study showed that there was a dose-dependent increase in the percentage tail DNA when cells were treated with DON at concentrations in the range of 1.1 – 8.9 ppm [102]. Elsewhere, dose-dependent DNA damage was observed by the comet assay in Caco-2 cells [103]. In another study, the genotoxicity of DON was investigated in TK6 (human lymphoblastoid) cells. The cells were exposed to 0 – 25 ppm DON for 3 h or 24 h. The results showed no significant increase in DNA migration at the concentration and times tested, similar to what was observed with in this work with the IPEC-J2 cells [104]. Another study also showed that DON did not induce genotoxicity in human hepatoma HepaRG cells using the comet assay [105]. These studies show that the genotoxic effects of DON may be cell dependent, as it has been shown to induce DNA damage in some cells but not others.

There are various methods of detecting DNA damage, with comet assay and TUNEL assay being amongst them and in general, the TUNEL assay is found to be more sensitive for detection of DNA fragmentation [106], which could explain why DNA damage by DON

was detected by TUNEL but not by comet in this study. It may be necessary to increase the concentration range of DON being used in the experiment here, in order to detect DNA damage by comet.

The protective effects of the feed supplements Se-Y and My-A+ was analysed also using the cell viability and the TUNEL assay. It was found that 24 h exposure to DON resulted in lower cytotoxic effects in cells that were pre-treated with the supplements. The supplemented cells also displayed lower levels apoptosis. It was found there was a significant increase in the TUNEL negative population in the cells that were pre-incubated with the supplements in comparison to those that were not. This shows that the supplements showed protective effects against DON induced apoptosis and cytotoxicity.

Se is an essential dietary trace mineral that has been previously shown to have protective effect against DON toxicity. One study showed the ameliorative effects of sodium selenite against DON induced oxidative damage to porcine splenic lymphocytes [107]. Supplementation with Se-enriched yeast was shown to counteract most of the plasma indicator alterations induced by DON in broiler chickens [108]. DON induced cytotoxic injury and metabolism imbalance in human chondrocytes was also reversed by Se [109]. A glucomannan based mycotoxin adsorbent, Mycosorb, was used in one study to mitigate the toxic effects on DON in broiler chickens. Mycosorb counteracted many of the effects that were induced by DON such as depletion of plasma levels of magnesium, triglycerides and total protein, but did not prevent the toxic effect on calcium metabolism [110]. Mycosorb was also shown to reduce the toxic effects of *Fusarium* mycotoxins (including DON, ZEN and 15-acetyl DON) on the reproductive performance of gilts [111]. An *in vitro* study also used IPEC-J2 cells to show the protective effects of Mycosorb against DON damage. DON was shown to decrease TEER and reduce cell viability and this effect was counteracted by Mycosorb [112]. One of the components of My-A+ that lends its protective effects is the PUFAs present in the algae. PUFAs have shown to alleviate toxicity induced by various sources. One study showed that long chain n-3 PUFAs, EPA and DHA could mitigate DON induced toxicity in IPEC-1 cells. EPA and DHA were shown to prevent cell injury induced by DON and improve intestinal barrier integrity. Exposure to DON also increased ROS production, which was subsequently decreased by EPA and DHA [113]. In another study, in the spleen of male mice, DON was shown to induce the expression of various genes that could be responsible for its immune dysregulation and inflammatory effect. In mice that were fed n-3 PUFA diet, the effects of DON were mitigated [114].

Elsewhere, n-3 PUFAs DHA and EPA were shown to attenuate the early stages of IgA nephropathy induced in mice following DON exposure. However, the parent n-3 FA ALA did not have an effect on DON induced IgA nephropathy [115]. A follow up study by the same authors showed that DHA mitigated DON induced IgA dysregulation and nephropathy in a dose dependent manner [116]. These results corroborate with the results of this chapter where it was shown that both Se from Se-Y supplement and PUFAs from My-A+ had a protective effect against DON induced damage on IPEC-J2 cells.

From the results of this chapter and Chapter 2, it can be concluded that both ZEN and DON have toxic effects on IPEC-J2 cells. Comparing the data, DON was more toxic to the cells than ZEN. It was found that 24 h exposure to 3 ppm ZEN was required to significantly reduce the cell viability, however, with DON a significant difference was observed with only 0.15 ppm exposure. Similarly, 0.9 ppm DON was shown to induce apoptosis, but with ZEN, a much higher concentration of 25 ppm was used. However, although ZEN was shown to induce DNA damage in the cells, DON did not. A study by Wan *et al.* also showed that DON was more toxic to IPEC-J2 cells than ZEN, following a 48 h exposure [117]. Similarly, DON was shown to be more cytotoxic than ZEN to HepG2 cells and Raw 26.7 cells [118]. Elsewhere, similar results were also observed with PK15 cells [119]. These results highlight the higher toxic effects of DON compared to ZEN.

3.5 Conclusion

From the results presented, it can be seen from the cell viability and the TUNEL assay that the cytotoxic and apoptotic effects of DON on IPEC-J2 cells are dose dependent. The results of the comet assay showed that DON did not induce DNA damage on the epithelial at the conditions tested. Elsewhere, DON was found to induce DNA damage on Caco-2 and HepG2 cell lines but not on TK6 and HepaRg cells, indicating that its potential toxicity may be cell dependent. This could explain the absence of observed genotoxicity by DON in this study. The protective effects of Se-Y, My-A+ and Se-Y + My-A+ against DON toxicity was also investigated in this chapter. It was found that pre-incubating the cells for 48 h prior to 24 h exposure to DON resulted in reduced cytotoxic and apoptotic effects, when compared to the cells that were not pre-incubated.

3.6 References

(1) Iheshiulor, O.; Esonu, B. O.; Chuwuka, O. K.; Omede, A. A.; Ifeanyi Charles, O.; Ifeanyi PhD, O. Effects of Mycotoxins in Animal Nutrition: A Review. *Asian J Anim Sci* **2011**, *5* (1), 19–33. https://doi.org/10.3923/ajas.2011.19.33.

(2) Ren, Z.; Guo, C.; Yu, S.; Zhu, L.; Wang, Y.; Hu, H.; Deng, J. Progress in Mycotoxins Affecting Intestinal Mucosal Barrier Function. *Int J Mol Sci* **2019**, *20* (11), 2777. https://doi.org/10.3390/ijms20112777.

(3) Cheli, F.; Pinotti, L.; Rossi, L.; Dell'Orto, V. Effect of Milling Procedures on Mycotoxin Distribution in Wheat Fractions: A Review. *LWT - Food Sci and Technol* **2013**, *54* (2), 307–314. https://doi.org/10.1016/j.lwt.2013.05.040.

(4) Lancova, K.; Hajslova, J.; Kostelanska, M.; Kohoutkova, J.; Nedelnik, J.; Moravcova, H.; Vanova, M. Fate of Trichothecene Mycotoxins during the Processing: Milling and Baking. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* **2008**, *25* (5), 650–659. https://doi.org/10.1080/02652030701660536.

(5) Bretz, M.; Beyer, M.; Cramer, B.; Knecht, A.; Humpf, H.-U. Thermal Degradation of the *Fusarium* Mycotoxin Deoxynivalenol. *J Agric Food Chem* **2006**, *54* (17), 6445–6451. https://doi.org/10.1021/jf061008g.

(6) Wolf, C. E.; Bullerman, L. B. Heat and PH Alter the Concentration of Deoxynivalenol in an Aqueous Enviroment. *J Food Prot* **1998**, *61* (3), 365–367. https://doi.org/10.4315/0362-028X-61.3.365.

(7) Čolović, R.; Puvača, N.; Cheli, F.; Avantaggiato, G.; Greco, D.; Đuragić, O.; Kos, J.; Pinotti, L. Decontamination of Mycotoxin-Contaminated Feedstuffs and Compound Feed. *Toxins* **2019**, *11* (11), 617. https://doi.org/10.3390/toxins11110617.

(8) Limaye, A.; Yu, R.-C.; Chou, C.-C.; Liu, J.-R.; Cheng, K.-C. Protective and Detoxifying Effects Conferred by Dietary Selenium and Curcumin against AFB1-Mediated Toxicity in Livestock: A Review. *Toxins* **2018**, *10* (1), 25. https://doi.org/10.3390/toxins10010025.

(9) Placha, I.; Borutova, R.; Gresakova, L.; Petrovic, V.; Faix, S.; Leng, L. Effects of Excessive Selenium Supplementation to Diet Contaminated with Deoxynivalenol on Blood Phagocytic Activity and Antioxidative Status of Broilers. *J Anim Physiol Anim Nutr (Berl)* **2009**, *93* (6), 695–702. https://doi.org/10.1111/j.1439-0396.2008.00857.x.

(10) Vila-Donat, P.; Marín, S.; Sanchis, V.; Ramos, A. J. A Review of the Mycotoxin Adsorbing Agents, with an Emphasis on Their Multi-Binding Capacity, for Animal Feed Decontamination. *Food Chem Toxicol* **2018**, *114*, 246–259. https://doi.org/10.1016/j.fct.2018.02.044.

(11) Reich, H. J.; Hondal, R. J. Why Nature Chose Selenium. *ACS Chem Biol* **2016**, *11* (4), 821–841. https://doi.org/10.1021/acschembio.6b00031.

(12) Perrone, D.; Monteiro, M.; Nunes, J. C. CHAPTER 1:The Chemistry of Selenium. In *Selenium: Chemistry, Analysis, Function and Effects*; 2015; pp 3–15. https://doi.org/10.1039/9781782622215-00003.

(13) National Research Council Staff; National Research Council (U.S.); Subcommittee on Selenium. *Selenium in Nutrition*; National Academy Press: Washington, D.C., 1983.

(14) Mahima; Verma, A. K.; Kumar, A.; Rahal, A.; Kumar, V.; Roy, D. Inorganic Versus Organic Selenium Supplementation: A Review. *Pak J Biol Sci* **2012**, *15* (9), 418–425. https://doi.org/10.3923/pjbs.2012.418.425.

(15) Bhabak, K. P.; Mugesh, G. Functional Mimics of Glutathione Peroxidase: Bioinspired Synthetic Antioxidants. *Acc Chem Res* **2010**, *43* (11), 1408–1419. https://doi.org/10.1021/ar100059g.

(16) Lu, J.; Holmgren, A. Selenoproteins. *J Biol Chem* **2009**, *284* (2), 723–727. https://doi.org/10.1074/jbc.R800045200.

(17) Nimse, S. B.; Pal, D. Free Radicals, Natural Antioxidants, and Their Reaction Mechanisms. *RSC Adv* **2015**, *5* (35), 27986–28006. https://doi.org/10.1039/C4RA13315C.

(18) Birben, E.; Sahiner, U. M.; Sackesen, C.; Erzurum, S.; Kalayci, O. Oxidative Stress and Antioxidant Defense: *World Allergy Organ J* **2012**, *5* (1), 9–19. https://doi.org/10.1097/WOX.0b013e3182439613.

(19) Tapiero, H.; Townsend, D. M.; Tew, K. D. The Antioxidant Role of Selenium and Seleno-Compounds. *Biomed Pharmacother* **2003**, *57* (3), 134–144. https://doi.org/10.1016/S0753-3322(03)00035-0.

(20) Chen, J. An Original Discovery: Selenium Deficiency and Keshan Disease (an Endemic Heart Disease). *Asia Pac J Clin Nutr* **2012**, *21* (3), 320–326.

(21) Brown, K.; Arthur, J. Selenium, Selenoproteins and Human Health: A Review. *Public Health Nutr* **2001**, *4* (2b), 593–599. https://doi.org/10.1079/PHN2001143.

(22) European Food Safety Authority (EFSA). Scientific Opinion on Dietary Reference Values for Selenium. *EFSA J* **2014**, *12* (10), 3846. https://doi.org/10.2903/j.efsa.2014.3846.

(23) Hefnawy, A. E. G.; Tórtora-Pérez, J. L. The Importance of Selenium and the Effects of Its Deficiency in Animal Health. *Small Rumin Res.* **2010**, *89* (2), 185–192. https://doi.org/10.1016/j.smallrumres.2009.12.042.

(24) Mehdi, Y.; Dufrasne, I. Selenium in Cattle: A Review. *Molecules* **2016**, *21* (4), 545. https://doi.org/10.3390/molecules21040545.

(25) Alfthan, G.; Eurola, M.; Ekholm, P.; Venäläinen, E.-R.; Root, T.; Korkalainen, K.; Hartikainen, H.; Salminen, P.; Hietaniemi, V.; Aspila, P.; Aro, A. Effects of Nationwide Addition of Selenium to Fertilizers on Foods, and Animal and Human Health in Finland: From Deficiency to Optimal Selenium Status of the Population. *J. Trace Elem. Med Biol.* **2015**, *31*, 142–147. https://doi.org/10.1016/j.jtemb.2014.04.009.

(26) Wright, P. L.; Bell, M. C. Comparative Metabolism of Selenium and Tellurium in Sheep and Swine. *Am J Physiol* **1966**, *211* (1), 6–10. https://doi.org/10.1152/ajplegacy.1966.211.1.6.

(27) MacFarquhar, J. K.; Broussard, D. L.; Melstrom, P.; Hutchinson, R.; Wolkin, A.; Martin, C.; Burk, R. F.; Dunn, J. R.; Green, A. L.; Hammond, R.; Schaffner, W.; Jones, T. F. Acute Selenium Toxicity Associated With a Dietary Supplement. *Arch Intern Med* **2010**, *170* (3), 256–261. https://doi.org/10.1001/archinternmed.2009.495.

(28) *Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids*; National Academies Press: Washington, D.C., 2000. https://doi.org/10.17226/9810.

(29) Gupta, M.; Gupta, S. An Overview of Selenium Uptake, Metabolism, and Toxicity in Plants. *Front. Plant Sci.* **2017**, *7*, 2074. https://doi.org/10.3389/fpls.2016.02074.

(30) White, P. J. Selenium Accumulation by Plants. *Ann Bot* **2016**, *117* (2), 217–235. https://doi.org/10.1093/aob/mcv180.

(31) Preda, C.; Vasiliu, I.; Bredetean, O.; Gabriela, C. D.; Ungureanu, M.-C.; Leustean, E. L.; Grigorovici, A.; Oprisa, C.; Vulpoi, C. Selenium in the Environment: Essential or Toxic to Human Health? *Environ Eng Manag J* **2015**, *15* (4), 913–921. https://doi.org/10.30638/eemj.2016.099.

(32) Mehdi, Y.; Hornick, J.-L.; Istasse, L.; Dufrasne, I. Selenium in the Environment, Metabolism and Involvement in Body Functions. *Molecules* **2013**, *18* (3), 3292–3311. https://doi.org/10.3390/molecules18033292.

(33) Bitterli, C.; Bañuelos, G. S.; Schulin, R. Use of Transfer Factors to Characterize Uptake of Selenium by Plants. *J Geochem Explor* **2010**, *107* (2), 206–216. https://doi.org/10.1016/j.gexplo.2010.09.009.

(34) Dumont, E.; Vanhaecke, F.; Cornelis, R. Selenium Speciation from Food Source to Metabolites: A Critical Review. *Anal Bioanal Chem* **2006**, *385* (7), 1304–1323. https://doi.org/10.1007/s00216-006-0529-8.

(35) Fordyce, F. M. Selenium Deficiency and Toxicity in the Environment. In *Essentials of Medical Geology*; Selinus, O., Ed.; Springer Netherlands: Dordrecht, 2013; pp 375–416. https://doi.org/10.1007/978-94-007-4375-5_16.

(36) Kieliszek, M.; Błażejak, S. Selenium: Significance, and Outlook for Supplementation. *Nutrition* **2013**, *29* (5), 713–718. https://doi.org/10.1016/j.nut.2012.11.012.

(37) Fox, T. E.; Heuvel, E. G. H. M. V. den; Atherton, C. A.; Dainty, J. R.; Lewis, D. J.; Langford, N. J.; Crews, H. M.; Luten, J. B.; Lorentzen, M.; Sieling, F. W.; Aken-Schneyder, P. van; Hoek, M.; Kotterman, M. J. J.; Dael, P. van; Fairweather-Tait, S. J. Bioavailability of Selenium from Fish, Yeast and Selenate: A Comparative Study in Humans Using Stable Isotopes. *Eur J Clin Nutr* **2004**, *58* (2), 343–349. https://doi.org/10.1038/sj.ejcn.1601787.

(38) Thiry, C.; Ruttens, A.; De Temmerman, L.; Schneider, Y.-J.; Pussemier, L. Current Knowledge in Species-Related Bioavailability of Selenium in Food. *Food Chem* **2012**, *130* (4), 767–784. https://doi.org/10.1016/j.foodchem.2011.07.102.

(39) Gresakova, L.; Cobanova, K.; Faix, S. Selenium Retention in Lambs Fed Diets Supplemented with Selenium from Inorganic or Organic Sources. *Small Rumin Res.* **2013**, *111* (1–3), 76–82. https://doi.org/10.1016/j.smallrumres.2012.10.009.

(40) Shini, S.; Sultan, A.; Bryden, W. Selenium Biochemistry and Bioavailability: Implications for Animal Agriculture. *Agriculture* **2015**, *5* (4), 1277–1288. https://doi.org/10.3390/agriculture5041277.

(41) Suzuki, K. T. Metabolomics of Selenium: Se Metabolites Based on Speciation Studies. *J Health Sci* **2005**, *51* (2), 107–114. https://doi.org/10.1248/jhs.51.107.

(42) Zeng, H. Selenium as an Essential Micronutrient: Roles in Cell Cycle and Apoptosis. *Molecules* **2009**, *14* (3), 1263–1278. https://doi.org/10.3390/molecules14031263.

(43) Rayman, M. P. The Use of High-Selenium Yeast to Raise Selenium Status: How Does It Measure Up? *Br J Nutr* **2004**, *92* (4), 557–573. https://doi.org/10.1079/BJN20041251.

(44) Thiry, C.; Schneider, Y.-J.; Pussemier, L.; De Temmerman, L.; Ruttens, A. Selenium Bioaccessibility and Bioavailability in Se-Enriched Food Supplements. *Biol*

Trace Elem Res **2013**, *152* (1), 152–160. https://doi.org/10.1007/s12011-013-9604-0.

(45) Zhang, S.-Q.; Shen, S.; Zhang, Y. Comparison of Bioavailability, Pharmacokinetics, and Biotransformation of Selenium-Enriched Yeast and Sodium Selenite in Rats Using Plasma Selenium and Selenomethionine. *Biol Trace Eleme Res* **2019**, *196* (2), 512–516. https://doi.org/10.1007/s12011-019-01935-9.

(46) Fairweather-Tait, S. J.; Collings, R.; Hurst, R. Selenium Bioavailability: Current Knowledge and Future Research Requirements. *AM J Clin Nutr* **2010**, *91* (5), 1484S-1491S. https://doi.org/10.3945/ajcn.2010.28674J.

(47) Ortman, K.; Pehrson, B. Effect of Selenate as a Feed Supplement to Dairy Cows in Comparison to Selenite and Selenium Yeast. *J Anim Sci* **1999**, *77* (12), 3365. https://doi.org/10.2527/1999.77123365x.

(48) Tinggi, U. Selenium: Its Role as Antioxidant in Human Health. *Environ Health Prev Med* **2008**, *13* (2), 102–108. https://doi.org/10.1007/s12199-007-0019-4.

(49) Tran, D.; Moody, A. J.; Fisher, A. S.; Foulkes, M. E.; Jha, A. N. Protective Effects of Selenium on Mercury-Induced DNA Damage in Mussel Haemocytes. *Aquat Toxicol* **2007**, *84* (1), 11–18. https://doi.org/10.1016/j.aquatox.2007.05.009.

(50) McKelvey, S. M.; Horgan, K. A.; Murphy, R. A. Chemical Form of Selenium Differentially Influences DNA Repair Pathways Following Exposure to Lead Nitrate. *J Trace Elem Med Biol* **2015**, *29*, 151–169. https://doi.org/10.1016/j.jtemb.2014.06.005.

(51) Lynch, S. J.; Horgan, K. A.; White, B.; Walls, D. Selenium Source Impacts Protection of Porcine Jejunal Epithelial Cells from Cadmium-Induced DNA Damage, with Maximum Protection Exhibited with Yeast-Derived Selenium Compounds. *Biol Trace Elem Res* **2017**, *176* (2), 311–320. https://doi.org/10.1007/s12011-016-0828-7.

(52) Awad, W. A.; Ghareeb, K.; Böhm, J.; Zentek, J. Decontamination and Detoxification Strategies for the *Fusarium* Mycotoxin Deoxynivalenol in Animal Feed and the Effectiveness of Microbial Biodegradation. *Food Addit Contam Part A*

Chem Anal Control Expo Risk Assess **2010**, *27* (4), 510–520. https://doi.org/10.1080/19440040903571747.

(53) Zain, M. E. Impact of Mycotoxins on Humans and Animals. *J Saudi Chem Soc* **2011**, *15* (2), 129–144. https://doi.org/10.1016/j.jscs.2010.06.006.

(54) Eroglu, N.; Emekci, M.; Athanassiou, C. G. Applications of Natural Zeolites on Agriculture and Food Production. *J Sci Food Agric* **2017**, *97* (11), 3487–3499. https://doi.org/10.1002/jsfa.8312.

(55) Tomasevic-Canovic, M.; Dakovic, A.; Markovic, V.; Stojsic, D. The Effect of Exchangeable Cations in Clinoptilolite and Montmorillonite on the Adsorption of Aflatoxin B1. *J Serb Chem Soc* **2001**, *66* (8), 555–561. https://doi.org/10.2298/JSC0108555T.

(56) Tomašević-Čanović, M.; Daković, A.; Rottinghaus, G.; Matijašević, S.; Đuričić, M. Surfactant Modified Zeolites—New Efficient Adsorbents for Mycotoxins. *Micropor and Mesopor Mat* **2003**, *61* (1), 173–180. https://doi.org/10.1016/S1387-1811(03)00365-2.

(57) Gregorio, M. C. D.; Neeff, D. V. de; Jager, A. V.; Corassin, C. H.; Carão, Á. C. de P.; Albuquerque, R. de; Azevedo, A. C. de; Oliveira, C. A. F. Mineral Adsorbents for Prevention of Mycotoxins in Animal Feeds. *Toxin Rev.* **2014**, *33* (3), 125–135. https://doi.org/10.3109/15569543.2014.905604.

(58) Kong, C.; Shin, S. Y.; Kim, B. G. Evaluation of Mycotoxin Sequestering Agents for Aflatoxin and Deoxynivalenol: An in Vitro Approach. *SpringerPlus* **2014**, *3* (1), 346. https://doi.org/10.1186/2193-1801-3-346.

(59) Kalagatur, N. K.; Karthick, K.; Allen, J. A.; Nirmal Ghosh, O. S.; Chandranayaka, S.; Gupta, V. K.; Krishna, K.; Mudili, V. Application of Activated Carbon Derived from Seed Shells of Jatropha Curcas for Decontamination of Zearalenone Mycotoxin. *Front. Pharmacol.* **2017**, *8*, 760. https://doi.org/10.3389/fphar.2017.00760.

(60) Var, I.; Kabak, B.; Erginkaya, Z. Reduction in Ochratoxin A Levels in White Wine, Following Treatment with Activated Carbon and Sodium Bentonite. *Food Control* **2008**, *19* (6), 592–598. https://doi.org/10.1016/j.foodcont.2007.06.013.

(61) Nones, J.; Solhaug, A.; Riella, H. G.; Eriksen, G. S.; Nones, J. Brazilian Bentonite and a New Modified Bentonite Material, BAC302, Reduce Zearalenone-Induced Cell Death. *World Mycotoxin J.* **2020**, *4*, 1–10. https://doi.org/10.3920/WMJ2019.2547.

(62) Oliveira, A.; Keller, K.; Deveza, M.; Keller, L.; Dias, E.; Martini-Santos, B.; Leitao, D.; Cavaglieri, L.; Rosa, C. Effect of Three Different Anti-Mycotoxin Additives on Broiler Chickens Exposed to Aflatoxin B1. *Arch. med. vet.* **2015**, *47* (2), 175–183. https://doi.org/10.4067/S0301-732X2015000200008.

(63) Alltech. Mycosorb A+® https://www.knowmycotoxins.com/mycotoxin-mitigation/mycosorb-a/ (accessed 2019 -02 -14).

(64) Çelýk, K.; Denlý, M.; Savas, T. Reduction of Toxic Effects of Aflatoxin B1 by Using Baker Yeast (Saccharomyces Cerevisiae) in Growing Broiler Chicks Diets. *Rev Bras de Zootec* **2003**, *32* (3), 615–619. https://doi.org/10.1590/S1516-35982003000300013.

(65) Joannis-Cassan, C.; Tozlovanu, M.; Hadjeba- Medjdoub, K.; Ballet, N.; Pfohl-Leszkowicz, A. Binding of Zearalenone, Aflatoxin B1, and Ochratoxin A by Yeast-Based Products: A Method for Quantification of Adsorption Performance. *J Food Prot* **2011**, *74* (7), 1175–1185. https://doi.org/10.4315/0362-028X.JFP-11-023.

(66) Lluís, L.; Taltavull, N.; Muñoz-Cortés, M.; Sánchez-Martos, V.; Romeu, M.; Giralt, M.; Molinar-Toribio, E.; Torres, J. L.; Pérez-Jiménez, J.; Pazos, M.; Méndez, L.; Gallardo, J. M.; Medina, I.; Nogués, M. R. Protective Effect of the Omega-3 Polyunsaturated Fatty Acids: Eicosapentaenoic Acid/Docosahexaenoic Acid 1:1 Ratio on Cardiovascular Disease Risk Markers in Rats. *Lipids Health Dis* **2013**, *12*, 140. https://doi.org/10.1186/1476-511X-12-140.

(67) Richard, D.; Kefi, K.; Barbe, U.; Bausero, P.; Visioli, F. Polyunsaturated Fatty Acids as Antioxidants. *Pharmacol Res* **2008**, *57* (6), 451–455. https://doi.org/10.1016/j.phrs.2008.05.002.

(68) Wiktorowska-Owczarek, A.; Berezińska, M.; Nowak, J. PUFAs: Structures, Metabolism and Functions. *Adv Clin Exp Med* **2015**, *24* (6), 931–941. https://doi.org/10.17219/acem/31243.

(69) Vrablík, M.; Prusikova, M.; Snejdrlová, M.; Zlatohlavek, L. Omega-3 Fatty Acids and Cardiovascular Disease Risk: Do We Understand the Relationship? *Physiol Res* **2009**, *58 Suppl 1*, S19–S26. https://doi.org/10.33549/physiolres.931860.

(70) Kaur, N.; Chugh, V.; Gupta, A. K. Essential Fatty Acids as Functional Components of Foods- a Review. *J Food Sci Technol* **2014**, *51* (10), 2289–2303. https://doi.org/10.1007/s13197-012-0677-0.

(71) Balić, A.; Vlašić, D.; Žužul, K.; Marinović, B.; Bukvić Mokos, Z. Omega-3 Versus Omega-6 Polyunsaturated Fatty Acids in the Prevention and Treatment of Inflammatory Skin Diseases. *Int J Mol Sci* **2020**, *21* (3). https://doi.org/10.3390/ijms21030741.

(72) Hulbert, A. J.; Kelly, M. A.; Abbott, S. K. Polyunsaturated Fats, Membrane Lipids and Animal Longevity. *J Comp Physiol B* **2014**, *184* (2), 149–166. https://doi.org/10.1007/s00360-013-0786-8.

(73) Malcicka, M.; Visser, B.; Ellers, J. An Evolutionary Perspective on Linoleic Acid Synthesis in Animals. *Evol Biol* **2018**, *45* (1), 15–26. https://doi.org/10.1007/s11692-017-9436-5.

(74) Sinclair, A. J.; Attar-Bashi, N. M.; Li, D. What Is the Role of α-Linolenic Acid for Mammals? *Lipids* **2002**, *37* (12), 1113–1123. https://doi.org/10.1007/s11745-002-1008-x.

(75) Higdon, J. Essential Fatty Acids https://lpi.oregonstate.edu/mic/other-nutrients/essential-fatty-acids (accessed 2021 -05 -06).

(76) Domenichiello, A. F.; Kitson, A. P.; Bazinet, R. P. Is Docosahexaenoic Acid Synthesis from α-Linolenic Acid Sufficient to Supply the Adult Brain? *Prog Lipid Res* **2015**, *59*, 54–66. https://doi.org/10.1016/j.plipres.2015.04.002.

(77) Saini, R. K.; Keum, Y.-S. Omega-3 and Omega-6 Polyunsaturated Fatty Acids: Dietary Sources, Metabolism, and Significance — A Review. *Life Sci* **2018**, *203*, 255–267. https://doi.org/10.1016/j.lfs.2018.04.049.

(78) Innes, J. K.; Calder, P. C. Omega-6 Fatty Acids and Inflammation. *Prostaglandins, Leukot and Essent Fatty Acids* **2018**, *132*, 41–48. https://doi.org/10.1016/j.plefa.2018.03.004.

(79) Meyer, B. J.; Mann, N. J.; Lewis, J. L.; Milligan, G. C.; Sinclair, A. J.; Howe, P. R. C. Dietary Intakes and Food Sources of Omega-6 and Omega-3 Polyunsaturated Fatty Acids. *Lipids* **2003**, *38* (4), 391–398. https://doi.org/10.1007/s11745-003-1074-0.

(80) Lemahieu, C.; Bruneel, C.; Ryckebosch, E.; Muylaert, K.; Buyse, J.; Foubert, I. Impact of Different Omega-3 Polyunsaturated Fatty Acid (n-3 PUFA) Sources (Flaxseed, Isochrysis Galbana, Fish Oil and DHA Gold) on n-3 LC-PUFA Enrichment (Efficiency) in the Egg Yolk. *J Funct Foods* **2015**, *19*, 821–827. https://doi.org/10.1016/j.jff.2015.04.021.

(81) Zhao, G.; Etherton, T. D.; Martin, K. R.; Vanden Heuvel, J. P.; Gillies, P. J.; West, S. G.; Kris-Etherton, P. M. Anti-Inflammatory Effects of Polyunsaturated Fatty Acids in THP-1 Cells. *Biochem Biophys Res Commun* **2005**, *336* (3), 909–917. https://doi.org/10.1016/j.bbrc.2005.08.204.

(82) Ruxton, C. H. S.; Reed, S. C.; Simpson, M. J. A.; Millington, K. J. The Health Benefits of Omega-3 Polyunsaturated Fatty Acids: A Review of the Evidence. *J Hum Nutr Diet* **2004**, *17* (5), 449–459. https://doi.org/10.1111/j.1365-277X.2004.00552.x.

(83) Von Schacky, C.; Harris, W. S. Cardiovascular Benefits of Omega-3 Fatty Acids. *Cardiovasc Res* **2007**, *73* (2), 310–315. https://doi.org/10.1016/j.cardiores.2006.08.019.

(84) Uauy, R.; Valenzuela, A. Marine Oils: The Health Benefits of n-3 Fatty Acids. *Nutrition* **2000**, *16* (7–8), 680–684. https://doi.org/10.1016/S0899-9007(00)00326-9.

(85) Shahidi, F.; Ambigaipalan, P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. *Annu. Rev. Food Sci. Technol.* **2018**, *9* (1), 345–381. https://doi.org/10.1146/annurev-food-111317-095850.

(86) Orr, S. K.; Palumbo, S.; Bosetti, F.; Mount, H. T.; Kang, J. X.; Greenwood, C. E.; Ma, D. W. L.; Serhan, C. N.; Bazinet, R. P. Unesterified Docosahexaenoic Acid Is Protective in Neuroinflammation. *J Neurochem* **2013**, *127* (3), 378–393. https://doi.org/10.1111/jnc.12392.

(87) Querques, G.; Forte, R.; Souied, E. H. Retina and Omega-3. *J Nutr Metab* **2011**, *2011*, 748361. https://doi.org/10.1155/2011/748361.

(88) Sakai, C.; Ishida, M.; Ohba, H.; Yamashita, H.; Uchida, H.; Yoshizumi, M.; Ishida, T. Fish Oil Omega-3 Polyunsaturated Fatty Acids Attenuate Oxidative Stress-Induced DNA Damage in Vascular Endothelial Cells. *PLoS One* **2017**, *12* (11), e0187934. https://doi.org/10.1371/journal.pone.0187934.

(89) Kikugawa, K.; Yasuhara, Y.; Ando, K.; Koyama, K.; Hiramoto, K.; Suzuki, M. Protective Effect of Supplementation of Fish Oil with High N−3 Polyunsaturated Fatty Acids against Oxidative Stress-Induced DNA Damage of Rat Liver in Vivo. *J. Agric. Food Chem.* **2003**, *51* (20), 6073–6079. https://doi.org/10.1021/jf030141v.

(90) Rotstein, N. P.; Politi, L. E.; German, O. L.; Girotti, R. Protective Effect of Docosahexaenoic Acid on Oxidative Stress-Induced Apoptosis of Retina Photoreceptors. *Invest Ophthalmol Vis Sci* **2003**, *44* (5), 2252–2259. https://doi.org/10.1167/iovs.02-0901.

(91) European Commission. Commission Recommendation of 17 August 2006 on the Presence of Deoxynivalenol, Zearalenone, Ochratoxin A, T-2 and HT-2 and Fumonisins in Products Intended for Animal Feeding. *Official J. Eur. Union* **2006**.

(92) Arvanitoyannis, I. S.; Tserkezou, P. Wheat, Barley and Oat Waste: A Comparative and Critical Presentation of Methods and Potential Uses of Treated Waste. *Int J Food Sci Technol* **2008**, *43* (4), 694–725. https://doi.org/10.1111/j.1365-2621.2006.01510.x.

(93) Placinta, C. M.; D'Mello, J. P. F.; Macdonald, A. M. C. A Review of Worldwide Contamination of Cereal Grains and Animal Feed with Fusarium Mycotoxins. *Animal Feed Science and Technology* **1999**, *78* (1), 21–37. https://doi.org/10.1016/S0377-8401(98)00278-8.

(94) Awad, W. A.; Aschenbach, J. R.; Zentek, J. Cytotoxicity and Metabolic Stress Induced by Deoxynivalenol in the Porcine Intestinal IPEC-J2 Cell Line: Deoxynivalenol Effects on Intestinal Epithelial Cells. *J Anim Physiol Anim Nutr (Berl)* **2012**, *96* (4), 709–716. https://doi.org/10.1111/j.1439-0396.2011.01199.x.

(95) Fernández-Blanco, C.; Elmo, L.; Waldner, T.; Ruiz, M.-J. Cytotoxic Effects Induced by Patulin, Deoxynivalenol and Toxin T2 Individually and in Combination in Hepatic Cells (HepG2). *Food Chem. Toxicol.* **2018**, *120*, 12–23. https://doi.org/10.1016/j.fct.2018.06.019.

(96) Cano-Sancho, G.; González-Arias, C. A.; Ramos, A. J.; Sanchis, V.; Fernández-Cruz, M. L. Cytotoxicity of the Mycotoxins Deoxynivalenol and Ochratoxin A on Caco-2 Cell Line in Presence of Resveratrol. *Toxicol In Vitro* **2015**, *29* (7), 1639–1646. https://doi.org/10.1016/j.tiv.2015.06.020.

(97) Kang, R.; Li, R.; Dai, P.; Li, Z.; Li, Y.; Li, C. Deoxynivalenol Induced Apoptosis and Inflammation of IPEC-J2 Cells by Promoting ROS Production. *Environmental Pollution* **2019**, *251*, 689–698. https://doi.org/10.1016/j.envpol.2019.05.026.

(98) Xu, X.; Yan, G.; Chang, J.; Wang, P.; Yin, Q.; Liu, C.; Zhu, Q.; Lu, F. Comparative Transcriptome Analysis Reveals the Protective Mechanism of Glycyrrhinic Acid for Deoxynivalenol-Induced Inflammation and Apoptosis in IPEC-J2 Cells. *Oxid Med Cell Longev* **2020**, *2020*, e5974157. https://doi.org/10.1155/2020/5974157.

(99) Gu, X.; Guo, W.; Zhao, Y.; Liu, G.; Wu, J.; Chang, C. Deoxynivalenol-Induced Cytotoxicity and Apoptosis in IPEC-J2 Cells Through the Activation of Autophagy by Inhibiting PI3K-AKT-MTOR Signaling Pathway. *ACS Omega* **2019**, *4* (19), 18478–18486. https://doi.org/10.1021/acsomega.9b03208.

(100) Dai, Y.; Xie, H.; Xu, Y. Evaluation of Deoxynivalenol-Induced Toxic Effects on Mouse Endometrial Stromal Cells: Cell Apoptosis and Cell Cycle. *Biochem Biophys Res Commun* . **2017**, *483* (1), 572–577. https://doi.org/10.1016/j.bbrc.2016.12.103.

(101) Wang, J.; Jin, Y.; Wu, S.; Yu, H.; Zhao, Y.; Fang, H.; Shen, J.; Zhou, C.; Fu, Y.; Li, R.; Wang, R.; Wang, J.; Zheng, K.; Fan, Q.; Chen, B.; Zhang, J. Deoxynivalenol Induces Oxidative Stress, Inflammatory Response and Apoptosis in Bovine Mammary Epithelial Cells. *J Anim Physiol Anim Nutr (Berl)* **2019**, *103* (6), 1663–1674. https://doi.org/10.1111/jpn.13180.

(102) Zhang, X.; Jiang, L.; Geng, C.; Cao, J.; Zhong, L. The Role of Oxidative Stress in Deoxynivalenol-Induced DNA Damage in HepG2 Cells. *Toxicon* **2009**, *54* (4), 513–518. https://doi.org/10.1016/j.toxicon.2009.05.021.

(103) Bony, S.; Carcelen, M.; Olivier, L.; Devaux, A. Genotoxicity Assessment of Deoxynivalenol in the Caco-2 Cell Line Model Using the Comet Assay. *Toxicol Lett* **2006**, *166* (1), 67–76. https://doi.org/10.1016/j.toxlet.2006.04.010.

(104) Hégarat, L. L.; Takakura, N.; Simar, S.; Nesslany, F.; Fessard, V. The in Vivo Genotoxicity Studies on Nivalenol and Deoxynivalenol. *EFSA Supporting Publications* **2014**, 33 pp. https://doi.org/10.2903/sp.efsa.2014.EN-697.

(105) Takakura, N.; Nesslany, F.; Fessard, V.; Le Hegarat, L. Absence of in Vitro Genotoxicity Potential of the Mycotoxin Deoxynivalenol in Bacteria and in Human TK6 and HepaRG Cell Lines. *Food and Chem Toxicol* **2014**, *66*, 113–121. https://doi.org/10.1016/j.fct.2014.01.029.

(106) Majtnerová, P.; Roušar, T. An Overview of Apoptosis Assays Detecting DNA Fragmentation. *Mol Biol Rep.* **2018**, *45* (5), 1469–1478. https://doi.org/10.1007/s11033-018-4258-9.

(107) Ren, Z.; Fan, Y.; Zhang, Z.; Chen, C.; Chen, C.; Wang, X.; Deng, J.; Peng, G.; Hu, Y.; Cao, S.; Yu, S.; Ma, X.; Shen, L.; Zhong, Z.; Zhou, Z.; Xu, Z.; Zuo, Z. Sodium Selenite Inhibits Deoxynivalenol-Induced Injury in GPX1-Knockdown Porcine Splenic Lymphocytes in Culture. *Sci Rep* **2018**, *8*, 17676. https://doi.org/10.1038/s41598-018-36149-x.

(108) Faixová, Z.; Faix, Š.; Bořutová, R.; Leng, Ľ. Efficacy of Dietary Selenium to Counteract Toxicity of Deoxynivalenol in Growing Broiler Chickens. *Acta Vet. Brno* **2007**, *76* (3), 349–356. https://doi.org/10.2754/avb200776030349.

(109) Wang, X.; Jin, Z.; Chen, M.; Duan, D.; Lammi, M. J.; Guo, X.; Chang, Y. Inhibiting the Aberrant Activation of Wnt/β-Catenin Signaling by Selenium Supplementation Ameliorates Deoxynivalenol-Induced Toxicity and Catabolism in Chondrocytes. *J Cell Physiol* **2020**, *235* (5), 4434–4442. https://doi.org/10.1002/jcp.29319.

(110) Faixová, Z.; Faix, Š.; Leng, L.; Váczi, P.; Renáta, S.; Maková, Z. Effects of Feeding Diet Contaminated with Deoxynivalenol on Plasma Chemistry in Growing Broiler Chickens and the Efficacy of Glucomannan Mycotoxin Adsorbent. *Acta Vet. Scand.* **2006**, *56* (5–6), 479–487. https://doi.org/10.2298/avb0606479f.

(111) Díaz-Llano, G.; Smith, T. K. Effects of Feeding Grains Naturally Contaminated with Fusarium Mycotoxins with and without a Polymeric Glucomannan Mycotoxin Adsorbent on Reproductive Performance and Serum Chemistry of Pregnant Gilts1. *J Anim Sci* **2006**, *84* (9), 2361–2366. https://doi.org/10.2527/jas.2005-699.

(112) Devreese, M.; Pasmans, F.; De Backer, P.; Croubels, S. An in Vitro Model Using the IPEC-J2 Cell Line for Efficacy and Drug Interaction Testing of Mycotoxin Detoxifying Agents. *Toxicol In Vitro* **2013**, *27* (1), 157–163. https://doi.org/10.1016/j.tiv.2012.09.020.

(113) Xiao, K.; Liu, C.; Qin, Q.; Zhang, Y.; Wang, X.; Zhang, J.; Odle, J.; Lin, X.; Hu, C.-A. A.; Liu, Y. EPA and DHA Attenuate Deoxynivalenol-Induced Intestinal Porcine Epithelial Cell Injury and Protect Barrier Function Integrity by Inhibiting

Necroptosis Signaling Pathway. *FASEB J* **2020**, *34* (2), 2483–2496. https://doi.org/10.1096/fj.201902298R.

(114) Kinser, S.; Li, M.; Jia, Q.; Pestka, J. J. Truncated Deoxynivalenol-Induced Splenic Immediate Early Gene Response in Mice Consuming (n-3) Polyunsaturated Fatty Acids. *J Nutr Biochem.* **2005**, *16* (2), 88–95. https://doi.org/10.1016/j.jnutbio.2004.10.003.

(115) Jia, Q.; Shi, Y.; Bennink, M. B.; Pestka, J. J. Docosahexaenoic Acid and Eicosapentaenoic Acid, but Not α-Linolenic Acid, Suppress Deoxynivalenol-Induced Experimental IgA Nephropathy in Mice. *J Nutr* **2004**, *134* (6), 1353–1361. https://doi.org/10.1093/jn/134.6.1353.

(116) Jia, Q.; Zhou, H.-R.; Bennink, M.; Pestka, J. J. Docosahexaenoic Acid Attenuates Mycotoxin-Induced Immunoglobulin A Nephropathy, Interleukin-6 Transcription, and Mitogen-Activated Protein Kinase Phosphorylation in Mice. *J Nutr* **2004**, *134* (12), 3343–3349. https://doi.org/10.1093/jn/134.12.3343.

(117) Wan, L. Y. M.; Turner, P. C.; El-Nezami, H. Individual and Combined Cytotoxic Effects of Fusarium Toxins (Deoxynivalenol, Nivalenol, Zearalenone and Fumonisins B1) on Swine Jejunal Epithelial Cells. *Food Chem Toxicol* **2013**, *57*, 276–283. https://doi.org/10.1016/j.fct.2013.03.034.

(118) Zhou, H.; George, S.; Hay, C.; Lee, J.; Qian, H.; Sun, X. Individual and Combined Effects of Aflatoxin B1, Deoxynivalenol and Zearalenone on HepG2 and RAW 264.7 Cell Lines. *Food Chem Toxicol* **2017**, *103*, 18–27. https://doi.org/10.1016/j.fct.2017.02.017.

(119) Lei, M.; Zhang, N.; Qi, D. In Vitro Investigation of Individual and Combined Cytotoxic Effects of Aflatoxin B1 and Other Selected Mycotoxins on the Cell Line Porcine Kidney 15. *Exp Toxicol Pathol* **2013**, *65* (7), 1149–1157. https://doi.org/10.1016/j.etp.2013.05.007.

Chapter 4: Investigating the extent to which the combination of DON and ZEN can induce damage on IPEC-J2 cells and feed supplements can protect against damage observed

Abstract

DON and ZEN are common mycotoxins that frequently co-occur, and so there is an urgent need to develop mycotoxin management strategies for combined mycotoxin exposures. The effects of combined mycotoxins have been shown to vary depending on the cell/animal used, the concentration of mycotoxins, the exposure time and type of damage analysed. The aim of this study was to investigate the combinatorial effects of DON and ZEN on IPEC-J2 cells and to examine the protective effects of Se-Y and My-A+ on DON + ZEN induced toxicity. When combined, DON and ZEN were shown to have a synergistic effect on cell viability. However, when genotoxicity was measured, it was found that the combined effect of both toxins was similar to that induced by ZEN alone. DON was not shown to add to the genotoxicity of ZEN. Additionally, with the TUNEL assay, it was found that 0.25 ppm ZEN did not add to the apoptotic effects of 0.9 ppm DON. When the combination of 0.9 ppm DON and 25 ppm ZEN was analysed, the combined apoptotic effect was not significantly different to that induced by 25 ppm ZEN alone. Protective effects of the feed supplements were observed against 0.9 ppm DON + 0.25 ppm ZEN and 0.9 ppm DON + 0.25 ppm DON induced cytotoxicity. The comet and TUNEL assays also exhibited a similar mitigation by the supplements against 0.9 ppm DON + 25 ppm ZEN induced DNA damage and apoptosis, respectively. In summary, the combination of DON and ZEN was shown to have a synergistic effect on the cytotoxicity of IPEC-J2 cells, but a less than additive effect was observed in terms of apoptosis and DNA damage. Pre-incubation with the feed supplements resulted in the mitigation of the damage induced by the combination of DON and ZEN.

4.1 Introduction

Most fungi produce more than one mycotoxin simultaneously, and thus humans and animals are often exposed to multiple mycotoxins [1,2]. The three main fungal genera responsible for the production of mycotoxins are *Aspergillus, Penicillium* and *Fusarium*. Some mycotoxins produced by the *Aspergillus* genus include, AFs, OTA and patulin [3,4]. *Penicillium* is also responsible for the production of patulin and OTA as well as cyclopiazonic acid and citrinin [4,5]. *Fusarium* produces some of the major mycotoxins, including fumonisins, DON, ZEN, T-2 toxin and HT-2 toxin [4,6,7].

In the 2020 world mycotoxin survey by BIOMIN, involving 21,709 samples collected from 79 countries, more than one mycotoxin was detected in 67% of the samples. In the samples from North America, 92% of the samples were found to be co-contaminated. In the Middle East and Africa, 80% and 86% of the samples analysed were contaminated by more than one mycotoxin, respectively [8]. Elsewhere, 22% of 176 finished feed samples were infected with more than one mycotoxin [9]. In another study, 67 out of 82 feed samples were contaminated by mycotoxins (mainly type B trichothecenes and fumonisins) and from the infected samples, 75% were shown to be co-contaminated [10]. In 50 samples of poultry feed samples from Slovakia, 44% of the samples were contaminated by four mycotoxins; T-2 toxin, HT-2 toxin, DON and ZEN [11]. These studies show that the occurrence of multiple mycotoxins simultaneously is a common occurrence.

Since DON and ZEN are both produced by *Fusarium culmorum*, they are frequently found in co-occurrence with each other [12]. In Portugal DON and ZEN was found to co-occur in 15% (46/307) of wheat and wheat-based products with a mean ZEN concentration of 170 µg/kg and mean DON concentration of 70 µg/kg [13]. In another study, DON and ZEN were seen to co-occur in Brazilian barley grain samples [14]. Co-occurrence was also reported in wheat from Brazil. A combination of DON, ZEN and NIV was found in 74% (2009) and 12% (2010) of wheat samples analysed. A higher occurrence of mycotoxins was observed in 2009 as compared to 2010 due to the weather conditions during wheat flowering in that year [15]. In a global mycotoxin survey, feed samples from 100 countries were collected over a ten-year period from January 2008 – December 2017 and analysed for the presence of mycotoxins. The survey showed that the combinations of DON + ZEN and DON + fumonisin had the highest percentage of co-occurrence in

finished feed (48%); DON and ZEN were found to co-occur in 39% and 28% of maize and wheat samples analysed, respectively [16]. Given the common co-occurrence of DON and ZEN, it is important to understand their combinatorial effects.

The effect of combined mycotoxins cannot be predicted by the toxic effects exhibited by the individual mycotoxins [1]. Combined mycotoxins generally are considered to result in one of three main interaction effects: exhibiting a synergistic effect, an antagonistic effect or an additive effect [1,17]. A synergistic effect is observed when the combined effect of mycotoxins is greater than the sum of their individual effects. However, in a review by Grenier and Oswald, synergistic interaction was further explored [1]. Of particular interest to the work presented in this chapter, Grenier and Oswald considered the situation where one mycotoxin has no effect and the combined effect is greater than that of the other mycotoxin alone, defining this effect as potentiation, a type of synergism. An additive effect is observed when the combined effect of the mycotoxins is equal to the sum of their individual effects. In contrast, an antagonistic effect is observed when the combined effect of mycotoxins is less than the sum of their individual effects. Where the effect of combined mycotoxins mostly reflects the effect of the more toxic mycotoxin, with no added effect of the other mycotoxin, the interaction can be defined as less than additive (Figure 4.1) [1,18].

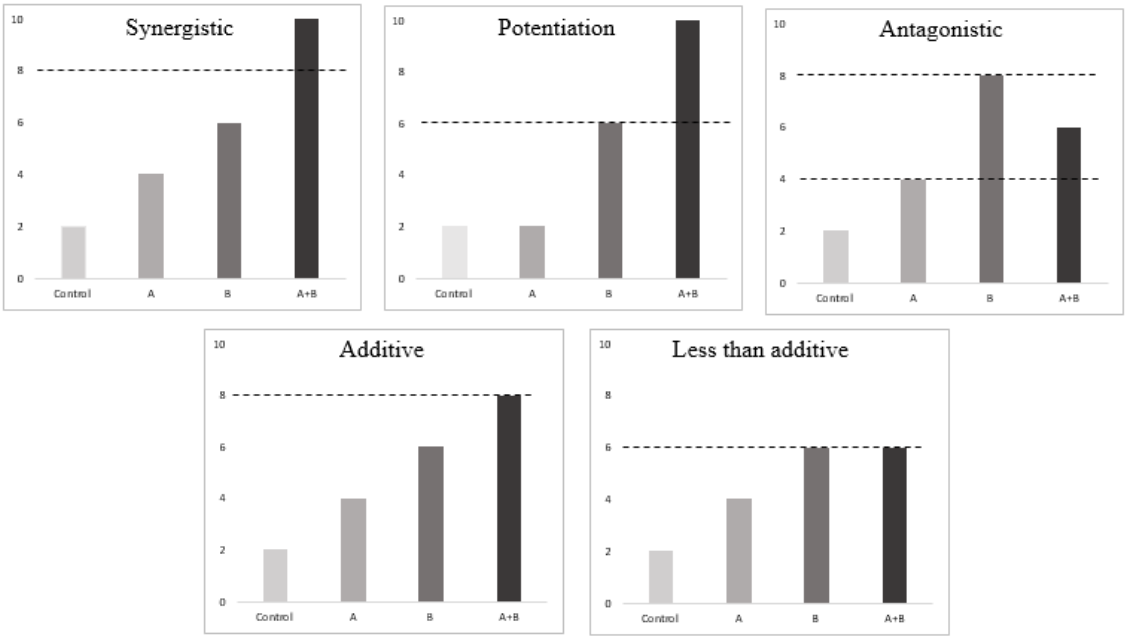

Figure 4.1: *The types of interactive effects of combined mycotoxins typically observed (adapted from Grenier and Oswald)* [1].

The aim of this chapter was to study the combinatorial effects of the mycotoxins DON and ZEN on IPEC-J2 cell viability, genotoxicity and apoptosis, and to evaluate the effectiveness of potential mitigation strategies to ameliorate these effects. A synergistic effect was observed on cell viability. This was not observed however, when DNA damage and apoptosis were measured in that combining DON and ZEN did not add to the toxic effects of the individual mycotoxins and thus a 'less than additive' effect was to be concluded. In terms of mitigation, it was found that Se-Y and My-A+ effectively mitigated the toxic effects of the combined mycotoxins across all combinations investigated.

4.2 Materials and methods

4.2.1 Cell culture

Cell culture methods, ZEN and DON preparation and preparation of Se-Y and My-A+ were carried out as outlined in Chapters 2 and 3 (Sections 2.2.1, 2.2.2, 2.2.3 and 3.3.2).

4.2.2 Cell viability assay

Alamar Blue and CFDA-AM were used for the analysis of cell toxicity. Cells were seeded in a black flat-bottomed 96 well plate at 2 x 10^4 cells/mL in DMEM (see 2.2.1) with 2% porcine serum. Cells were incubated with ZEN and DON, individually and combined, and incubated for 24 h. The cell viability assay was then carried out as outlined in the Chapter 2 (Section 2.2.4). For analyses involving Se-Y and/or My-A+, the cells were incubated with digested Se-Y (0.4 ppm Se), My-A+ (2g/kg) or both for 48 h, prior to incubation with mycotoxins.

4.2.3 TUNEL Assay

Cells were pre-incubated with the feed supplements for 48 h, when required, followed by a 24 h incubation with ZEN and DON. The TUNEL assay was then carried out according to the manufacturer's instructions (Phoenix Flow Systems, San Diego, CA) as outlined in the previous Chapter 2 (Sections 2.2.5).

4.2.4 Alkaline Comet Assay

Cells were pre-incubated with the feed supplements for 48 h, when required, followed by a 24 h incubation with ZEN and DON. The comet assay was then carried out as outlined in the Chapter 2 (Sections 2.2.6).

4.2.5 Assessment of mycotoxin combination effect

Isobologram analysis using the Chou-Talalay model was used to assess the interaction of the combined mycotoxins. This model uses the Combination Index (CI) as a quantitative parameter to determine the interaction type. The CI value can be calculated by the following equation: [19]

$$^{n}(CI)_x = \sum_{j=1}^{n} \frac{(D)_j}{(D_x)_j} = \sum_{j=1}^{n} \frac{(D_x)_{1-n}\left\{[D]_j / \sum_1^n [D]\right\}}{(D_m)_j \left\{\frac{(f_{ax})_j}{[1-(f_{ax})_j}\right\}^{1/mj}}$$

Where $^{n}(CI)_x$ is the combination index for *n* drugs at *x%* inhibition, $(D_x)_{1-n}$ is the sum of the dose of *n* drugs that exerts *x%* inhibition in combination, $\left\{[D]_j / \sum_1^n [D]\right\}$ is the proportionality of the dose of each of the *n* drugs that exerts *x%* inhibition in combination, and $(D_m)_j \left\{\frac{(f_{ax})_j}{[1-(f_{ax})_j}\right\}^{1/mj}$ is the concentration of each toxin alone that exerts *x%* inhibition, where D_m is the median-effect concentration (e.g. IC_{50}), f_{ax} is the fractional inhibition at *x%* inhibition and *m* is the slope of the median-effect plot. The *m* value depicts the shape of the dose-effect curve, where $m = 1$ indicates a hyperbolic, $m > 1$ indicates a sigmoidal and $m < 1$ indicates a flat sigmoidal dose-effect curve. The CI value was defined as following, CI = 1 represents an additive effect, CI < 1 represents a synergistic effect and CI > 1 represents an antagonistic effect [19]. The CI value for the interaction of DON + ZEN was calculated using the Compusyn software (ComboSyn, Inc., Paramus, NJ, USA).

4.3 Results

4.3.1 DON + ZEN act synergistically to negatively affect IPEC-J2 cell viability

IPEC-J2 cells were exposed to DON, ZEN and DON + ZEN for 24 h. DON concentration of 0.9 ppm was chosen for this research, as this is the maximum allowable concentration in pig feed as set by EFSA. This is also the concentration at which cytotoxic and apoptotic effects were observed in IPEC-J2 cells following 24 h exposure (as shown in Chapter 3). ZEN concentrations of 0.25 and 25 ppm was chosen for this study. The lower concentration of 0.25 ppm ZEN was chosen as this is the maximum allowable concentration in pig feed as set by EFSA. The higher concentration of 25 ppm ZEN was chosen as this is the concentration at which the genotoxic and apoptotic effects of ZEN was observed in IPEC-J2 cells following 24 h exposure (as shown in Chapter 2).

Table 4.1: *The concentrations of DON and ZEN used to investigate their combinatorial effects.*

DON concentration (ppm)	ZEN concentration (ppm)	Reason
0.9	0.25	EFSA limits for the toxins in pig feed
0.9	25	Lowest concentration at which apoptosis and/or genotoxicity was detectable

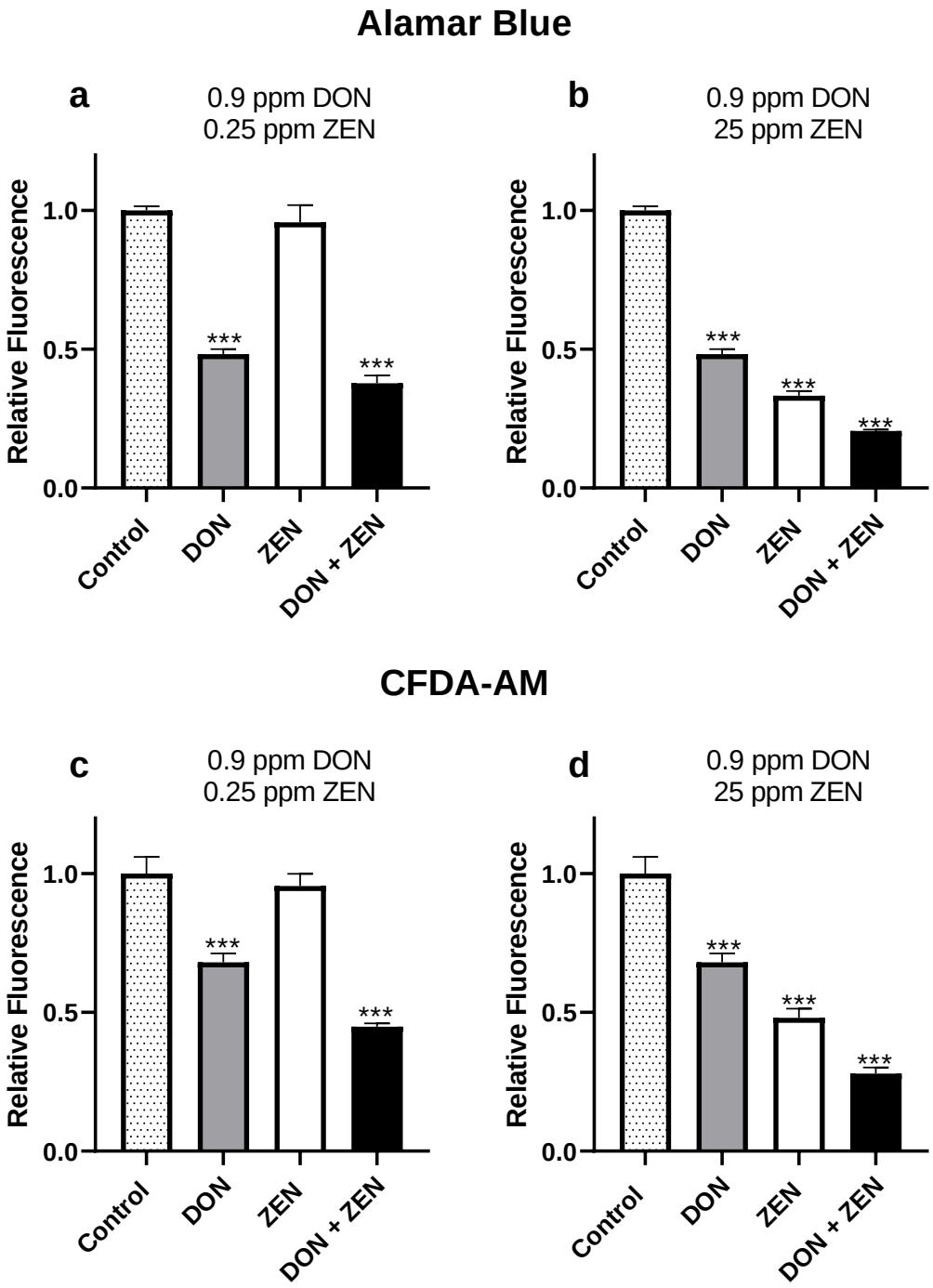

Figure 4.2: *The effect on IPEC-J2 viability following a 24 h exposure to DON and ZEN individually and combined. Results were obtained using the combined AB/CFDA-AM dual assay. Concentrations of 0.9 ppm DON and 0.25 ppm ZEN were used in graphs a and c as labelled in the graph. Concentrations of 0.9 ppm DON and 25 ppm ZEN were used for graphs c and d. Significant differences between control cells (0 ppm DON and ZEN) and cells insulted with DON and/or ZEN are highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

Following 24 h exposure to either 0.9 ppm DON (Figure 4.2 a-d) and 25 ppm ZEN (Figure 4.2 b and d) individually, a reduction in cell viability was observed, but there was no significant reduction in cell viability observed at 0.25 ppm ZEN (Figure 4.2 a and c). The combination of 0.9 ppm DON + 0.25 ppm ZEN resulted in a lower cell viability than that induced by 0.9 ppm DON alone. Similarly, lower cell viability was observed for the cells exposed to 0.9 ppm DON + 25 ppm ZEN than those exposed to the mycotoxins individually. The interaction between DON and ZEN was then assessed using the Chou-Talalay isobologram method. The Chou-Talalay method is one of the most commonly used for the measure of mycotoxin interaction [17]. The calculated CI values for the different combinations are presented in Table 4.2 below. The CI value for each of the combinations was found to be less than 1, thus indicating a synergistic effect for each combination with respect to cell viability, with a potentiation type of synergistic interaction observed for the combination of 0.9 ppm DON and 0.25 ppm ZEN.

According to Chou (2010) five data points is the hypothetical minimum required for combination study (two data points for toxin 1, two data points for toxin 2 and one for toxin 1 + 2). However, it is not recommended to use the minimum number of data points in order to have confidence in the outputs when using the method [20]. Therefore, in this study, at least 7 data points were entered for DON and ZEN individually and 4 data points with a constant ratio were entered for the combined mycotoxins. Only the graphs for the combinations of interest (0.9 ppm DON + 25 ppm ZEN and 0.9 ppm DON + 0.25 ppm ZEN) are shown in this Chapter (Figure 4.2). The graphs for the other combinations of the same ratios that were used to input into the Chou-Talalay model are shown in the appendix (Figures 6.1 – 6.4).

Table 4.2: *CI values for cells treated with DON and ZEN analysed by alamar blue and CFDA-AM, calculated using the Chou-Talalay method on CompuSyn.*

DON	ZEN	Alamar Blue CI value	CFDA-AM CI value
0.9	0.25	0.57298	0.56569
0.9	25	0.85081	0.70007

4.3.2 DON does not enhance ZEN-induced DNA damage in IPEC-J2

The comet assay was used to analyse the DNA damaging effects of DON and ZEN. As shown in Chapters 2 and 3, 0.25 ppm ZEN and 0.9 DON did not induce DNA damage in IPEC-J2 cells individually (Figures 2.3 and 3.4). DNA damage was also not induced following 24 h exposure to 0.9 ppm DON + 0.25 ppm ZEN. ZEN was shown to induce DNA damage in the cells at the higher concentration of 25 ppm after 24 h exposure. DNA damage was also induced by 25 ppm ZEN combined with 0.9 ppm DON. However, it was found that there was no significant difference between the tail DNA percentage following exposure to ZEN alone and ZEN combined with DON. This implies that DON did not exacerbate the genotoxicity induced by ZEN alone when combined with it.

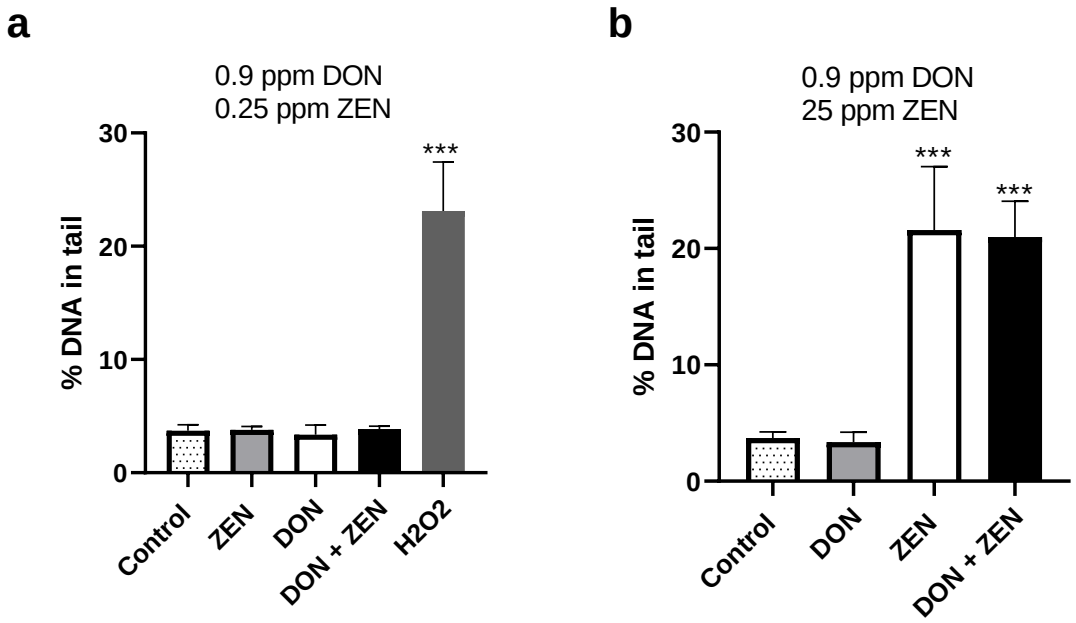

Figure 4.3: *Percentage tail DNA as determined by Comet Score software for IPEC-J2 cells incubated with DON and/or ZEN for 24 hours. The graphs above represent a) cells treated with 0.9 ppm DON + 0.25 ppm ZEN with H_2O_2 as the positive control b) cells treated with 0.9 ppm DON + 25 ppm ZEN. Data in the bar chart is presented as the mean of the median ± SD of triplicate samples. Significant difference between negative control cells and cells insulted with DON was highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

4.3.3 A combination of DON and ZEN did not add to apoptosis induced by either mycotoxins alone

Exposure to 0.9 ppm DON for 24 h was shown to induce apoptosis in IPEC-J2 cells, as seen in Chapter 3; however, exposure to 0.25 ppm ZEN did not result in any appreciable apoptosis being induced. In this study, the combined effect of 0.9 ppm DON and 0.25 ppm ZEN was examined by the TUNEL assay. The results showed that there was no significant difference between TUNEL negative values of the cells exposed to 0.9 ppm DON + 0.25 ppm ZEN and 0.9 ppm DON alone. Figure 4.4 shows that addition of 0.25 ppm ZEN did not exacerbate apoptosis induced by DON.

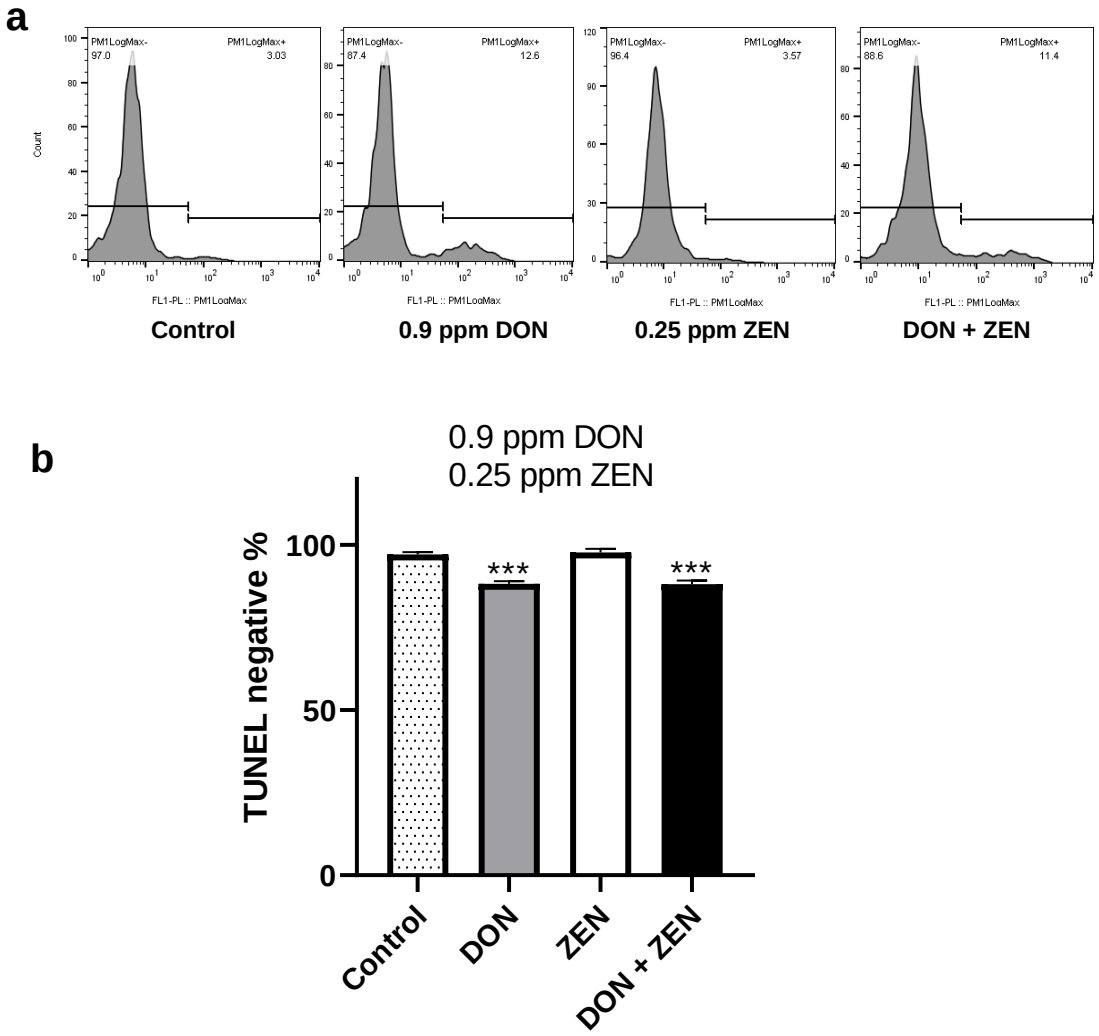

Figure 4.4: *TUNEL assay on IPEC-J2 cells exposed to 0.9 ppm DON and/or 0.25 ppm ZEN for 24 h. A) Representative histogram plots of cells treated 0 ppm DON/ZEN, 0.9 ppm DON, 0.25 ppm ZEN, DON + ZEN. B) TUNEL negative values for cells incubated with 0.9 ppm DON and/or 0.25 ppm ZEN. Data in the bar chart is presented as the mean ± SD of triplicate samples. Significant difference between control cells ((0 ppm DON/ZEN) and cells insulted with DON/ZEN are highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

The lowest concentration at which apoptosis was previously shown (Chapter 2) to be induced by ZEN was at 25 ppm. As both 0.9 ppm DON and 25 ppm ZEN were previously shown to induce apoptosis individually, their combined effect was evaluated here. Figure 4.5 shows the TUNEL negative values of cells exposed to 0.9 ppm DON and 25 ppm ZEN. As shown in Chapters 2 and 3, the TUNEL negative population of cells treated with

ZEN was found to be lower than of that treated by DON. The combination of DON and ZEN resulted in apoptosis similar to that induced by ZEN alone, thus indicating a less than additive effect.

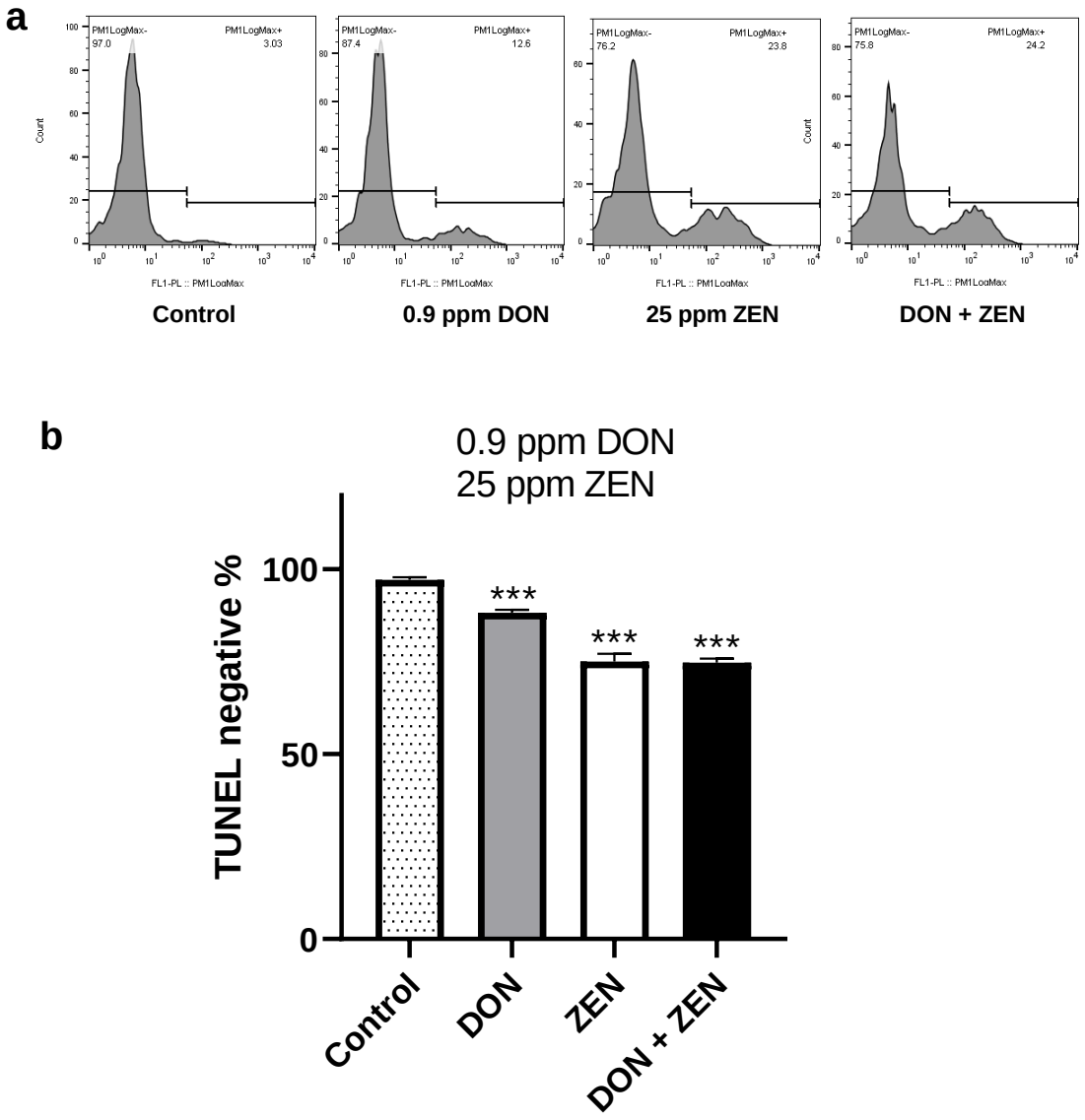

Figure 4.5: *TUNEL assay on IPEC-J2 cells exposed to 0.9 ppm DON and/or 25 ppm ZEN for 24 h. A) Representative histogram plots of cells treated 0 ppm DON/ZEN, 0.9 ppm DON, 25 ppm ZEN, DON + ZEN. B) TUNEL negative values for cells incubated with 0.9 ppm DON and/or 25 ppm ZEN. Data in the bar chart is presented as the mean ± SD of triplicate samples. Significant difference between control cells ((0 ppm DON/ZEN) and cells insulted with DON/ZEN are highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

4.3.4 Sel-Plex and Mycosorb A+ exhibit protective effects against cytotoxicity induced by DON + ZEN

IPEC-J2 cells were incubated with Se-Y, My-A+ and Se-Y + My-A+ for 48 h prior to exposure to the combined mycotoxins DON + ZEN. Figure 4.6 shows that a significant protective effect was observed against the cytotoxicity induced by DON + ZEN. This protective effect was observed in both of the combinations that were tested. Se-Y and My-A+ both exhibited a similar level of mitigation against DON + ZEN induced toxicity. Additionally, the protective effect provided by the combination of Se-Y and My-A+ was found to be similar to that by the individual compounds. Therefore, the interaction effect of the supplements can be defined as ‘less than additive’.

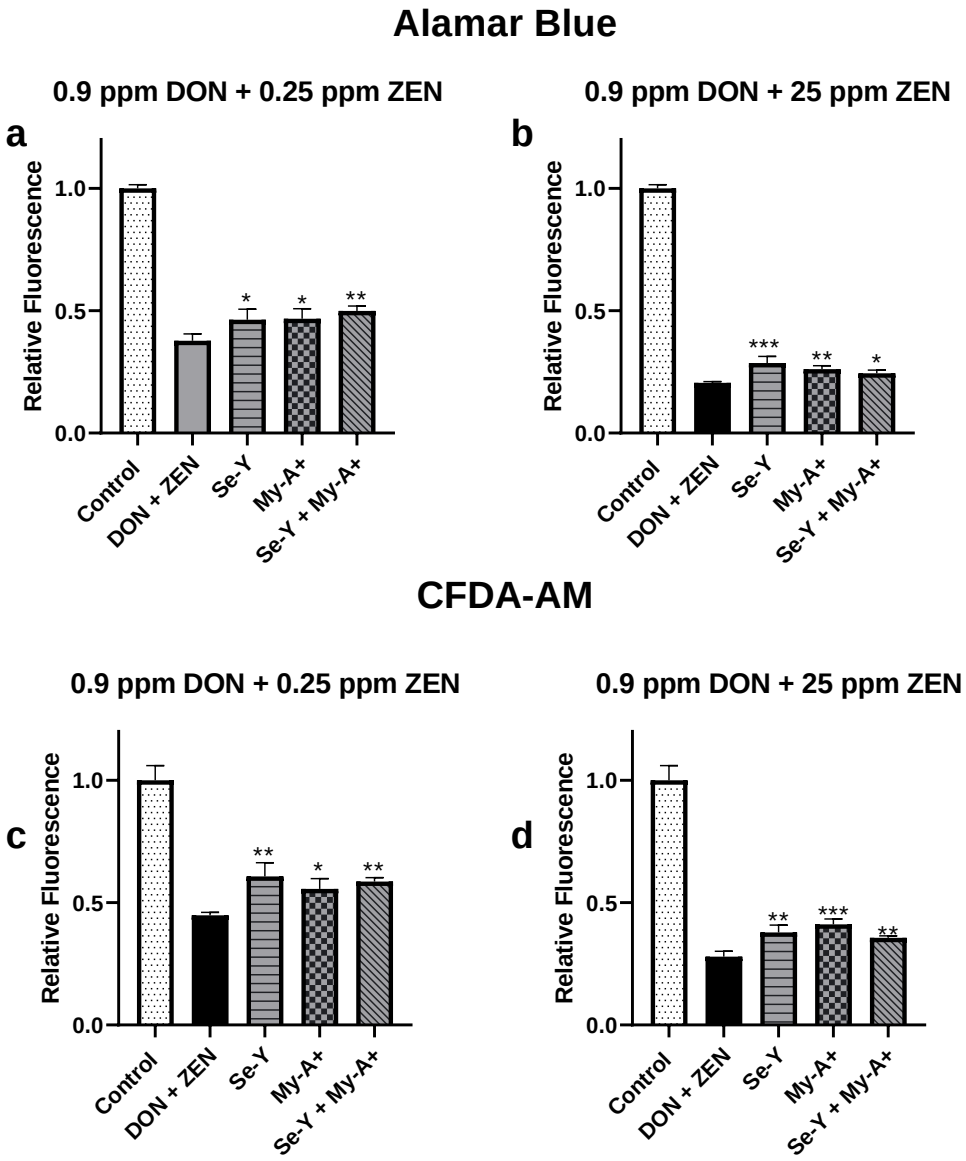

Figure 4.6: *Se-Y and My-A+ mitigate against DON/ZEN induced cytotoxicity in IPEC-J2 cells. The graphs above represent a) cells treated with 0.9 ppm DON + 0.25 ppm ZEN analysed by alamar blue b) cells treated with 0.9 ppm DON + 25 ppm ZEN analysed by alamar blue c) cells treated with 0.9 ppm DON + 0.25 ppm ZEN analysed by CFDA-AM and d) cells treated with 0.9 ppm DON + 25 ppm ZEN analysed by CFDA-AM. In all cases cells were pre-incubated for 48h with supplement (as indicated underneath) followed by insult with DON + ZEN for 24 h. Results are expressed as the mean of triplicates ± SD and are presented as relative to the negative control (0 ppm ZEN, no supplements). Significant differences between cells insulted with DON + ZEN with and without supplement pre-treatment are highlighted by one way ANOVA followed by Dunnett's test (*P<0.05, **P<0.01, ***P<0.001).*

4.3.5 Sel-Plex and Mycosorb A+ exhibit protective effects against DON + ZEN induced DNA damage

The protective effect of the feed supplements Se-Y and My-A+ against the damage induced by the combination of ZEN and DON was analysed. Pre-incubating the cells with the supplements prior to exposure to DON + ZEN resulted in reduced DNA damage. This was measured by the significant reduction in the percentage of DNA in tail in the cells that were treated with Se-Y, My-A+ and Se-Y and My-A+. As with the previous chapters, there was no significant difference observed in the protective effects provided by the different supplements.

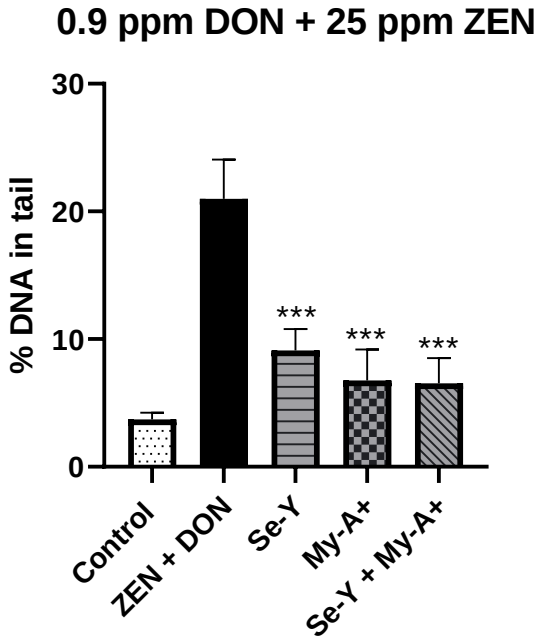

Figure 4.7: *Se-Y and My-A+ mitigate against DON + ZEN induced DNA damage in IPEC-J2 cells. IPEC-J2 cells were pre-treated with Se-Y, My-A+ or a combination of both for 48 h followed by a 24 h exposure to 0.9 ppm DON + 25 ppm ZEN and analysed for DNA damage by comet assay. Data in the bar chart is presented as the mean of median ± SD of triplicate samples. Significant differences between cells insulted with DON + ZEN with and without supplement pre-treatment are highlighted by one way ANOVA followed by Dunnett's test (*P<0.05, **P<0.01,***P<0.001).*

4.3.6 Sel-Plex and Mycosorb A+ mitigate DON + ZEN induced apoptosis in IPEC-J2 cells

The TUNEL assay also showed that pre-treating the cells with the feed supplements resulted in the mitigation of apoptosis induced by DON + ZEN. An increase in the TUNEL negative values were observed in the samples that underwent the pre-treatment. Cells treated with DON + ZEN with no pre-treatment had a TUNEL negative value of 74.8%. The cells pre-treated with Se-Y, My-A+ and Se-Y + My-A+ had a TUNEL negative value of 82.53%, 81.2% and 80.47% respectively. The increase in the TUNEL negative value shows the decrease in apoptosis in the cells pre-incubated with the supplements. No significant difference was observed between the cells treated with Se-Y, My-A+ and Se-Y + My-A+. The combination of the feed supplements did not result in increased mitigation against DON+ZEN induced apoptosis. Thus, the combined interaction of the supplements was found to be less than additive.

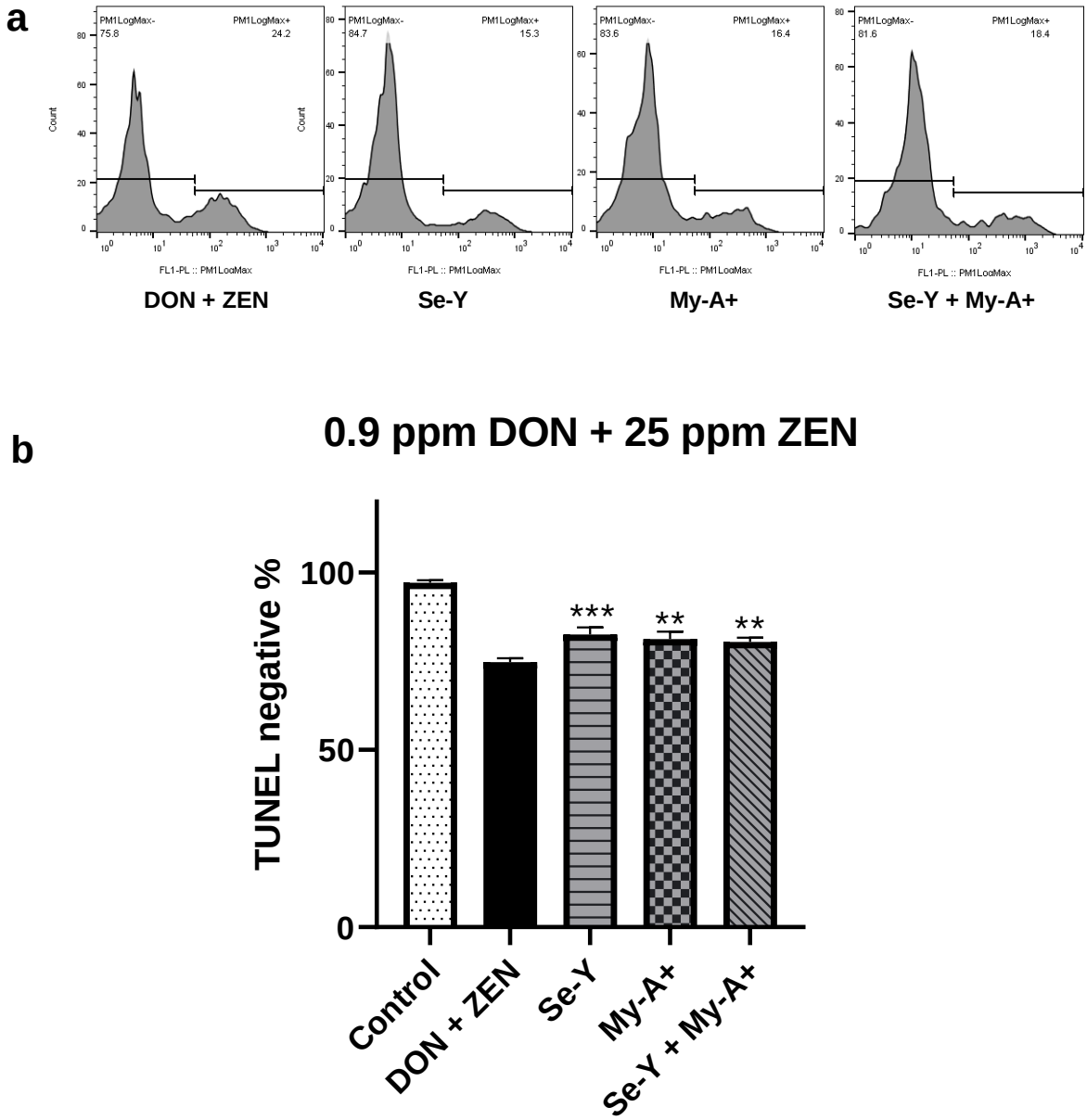

Figure 4.8: *Se-Y and My-A+ mitigate against DON + ZEN induced apoptosis in IPEC-J2 cells. IPEC-J2 cells were pre-treated with Se-Y, My-A+ or a combination of both for 48 h followed by a 24-h exposure to 0.9 ppm DON + 25 ppm ZEN and analysed by TUNEL assay. Data in the bar chart is presented as the mean ± SD of triplicate samples. Significant differences between cells insulted with DON + ZEN with and without supplement pre-treatment are highlighted by one way ANOVA followed by Dunnett's test (*P<0.05, **P<0.01,***P<0.001).*

4.4 Discussion

Animals are often exposed to more than one mycotoxins simultaneously [21]. ZEN and DON are among the most ubiquitous mycotoxins in cereal grains and are also found to co-occur frequently [13,15,22]. Therefore, the aim of this chapter was to study the combined effects of the two *Fusarium* mycotoxins DON and ZEN on IPEC-J2 cells. The cells were exposed to the mycotoxins, individually and combined for 24 h. The alamar blue and CFDA-AM assays showed a larger reduction in cell viability in cells that were exposed to DON + ZEN than the cells exposed to the mycotoxins individually. A synergistic effect was observed with each of the concentration combinations that were tested. DON was shown to induce cytotoxicity in IPEC-J2 cells at 0.9 ppm; however, 0.25 ppm ZEN did not result in any significant change in cell viability. When the cells were exposed to a combination of 0.9 ppm DON and 0.25 ppm ZEN, the cell viability was lower than when treated with DON alone. This shows that 0.25 ppm ZEN exacerbated the cytotoxic effects of DON in IPEC-J2 cells, thus indicating a potentiating effect. ZEN induced significant cytotoxicity at a concentration of 25 ppm. When 25 ppm ZEN was combined with 0.9 ppm DON, the resulting cytotoxicity was also more than that of ZEN and DON individually. The Chou-Talalay method was used to calculate the CI values of the combined effects. This mathematical model verified that the combination of DON + ZEN resulted in a synergistic effect on cell viability.

The synergistic effect of DON + ZEN was also observed in mouse brain in another study in which DON and ZEN combinations were shown to have dose-dependent and synergistic effects on apoptosis in mouse brain cells and their protein and antioxidant levels [23]. Synergism was also reported regarding the viability of HepG2 and RAW 264.7 cell lines following 48 h exposure to DON + ZEN [24]. DON + ZEN combined were also shown to have a synergistic effect on lipid peroxidation in Caco-2 cells following 24 h exposure [25]. However, in contrast, another study reported an antagonistic effect on the viability of Caco-2 cells [26]. Antagonistic effects were also observed on the cytotoxicity, mitochondrial apoptosis and cell cycle analysis of HCT116 cells following exposure to DON + ZEN [27]. One study reported a synergistic effect following 1 h exposure to the same mycotoxin combination on the proteome of HepaRG cells. However, following 24 h exposure to the same doses an antagonistic effect was observed [28]. These studies show that the effects of combined mycotoxins cannot be predicted from their individual effects.

Additionally, the effect of combined mycotoxins can vary considerably depending on the dose, exposure time and the cell/animal type used.

The DNA damaging effects of the combination of DON and ZEN were examined using the alkaline comet assay. As DON did not induce DNA damage at 0.9 ppm, it was combined with 0.25 ppm ZEN, which also did not induce DNA damage. However, no DNA damage was detected with the combination of both mycotoxins. With 25 ppm ZEN, which was the lowest concentration at which ZEN induced DNA damage, DON did not add to the damage induced. The tail DNA percentage for the combination was not significantly different to that of 25 ppm ZEN. As no synergy was observed this differed from the results of the cell viability assay.

As with the comet assay, synergism was not observed with the TUNEL assay. The combination of 0.9 ppm DON and 0.25 ppm ZEN was also examined here. Although DON induced apoptosis at this concentration, ZEN did not, in agreement with what was previously observed in Chapters 2 and 3. The results showed that the cocktail of mycotoxins did not induce more apoptosis than that induced by DON alone. Thus, 0.25 ppm ZEN did not add to the apoptotic effects of 0.9 ppm DON. Higher concentration of ZEN at 25 ppm and 0.9 ppm DON were both previously shown to induce apoptosis in IPEC-J2 cells. The TUNEL negative value (healthy cells) was lower for the cells insulted with ZEN than that seen with DON. When the toxins were combined, the level of apoptosis was found to be similar to that induced by ZEN only. Thus, combining with DON did not add to the damage induced by ZEN and there was no significant difference in the cells treated with ZEN alone and DON + ZEN in combination leading to the conclusion of 'a less than additive effect'. A synergistic effect was not observed here and this could be because DON and ZEN may compete for the same target or receptor site in the induction of apoptosis [29].

Similar results were observed by Smith *et al.* regarding the viability of HepaRG cells under conditions of chronic exposure to mycotoxins. The cytotoxic effect of DON + ZEN was shown to be similar to that of DON alone and therefore having a combination of DON + ZEN did not add to the toxic effects of DON [30]. Elsewhere, it was shown that ZEN alone induced apoptosis in HepaRg cells but that DON alone did not. In that instance, it was found that DON + ZEN induced the same level of apoptosis as that of ZEN alone and that DON did not add to or reduce the level of apoptosis induced by ZEN

[31]. With the PK15 cell line, however, it was reported that the combination of DON and ZEN resulted in a higher percentage of apoptosis than that by DON and ZEN alone [32]. A synergistic effect was also observed on the apoptosis of porcine splenic lymphocytes following 48 h exposure to DON and ZEN [33]. These results show that the effect of the mycotoxin combinations can vary between different cell types.

The protective effect of the feed supplements Se-Y, My-A+ and Se-Y + My-A+ on toxicity induced by DON and ZEN combined was also examined. Cell viability, comet and TUNEL assays all showed that the feed supplements could mitigate the toxicity induced by the combined mycotoxins. Although a synergistic effect of co-exposure to DON and ZEN was observed with the cell viability assay, the feed supplements were still found to have an ameliorative effect. Combining the mycotoxins did not inhibit the protective effects of the supplements. With the cell viability assay, reduced cytotoxicity was observed in cells that were pre-treated with the feed supplements. This was observed with both of the DON and ZEN combinations tested (0.9 ppm DON with 0.25/25 ppm ZEN). The comet assay showed a decrease in the percentage of tail DNA of the cells that underwent the pre-treatment when compared to those that did not. With the TUNEL assay, an increase in the TUNEL negative population was observed in the cells that were pre-treated prior to exposure to the combined mycotoxins.

Each of the feed supplements, individually and combined, were shown to have a protective effect on the IPEC-J2 cells against DON and ZEN induced toxicity. However, there was no significant difference observed between the ameliorative effects of Se-Y and My-A+. Additionally, the observed protective effects of the combination of Se-Y and My-A+ was similar to that of the supplements individually. Combining the two protective agents did not increase the level of protection against the mycotoxins. This may be as pre-incubation with the products individually had already resulted in the maximum level of protection achievable. Thus, combining the products does not result in higher levels of protection as it has already reached the extent to which the damage can be reversed. The combined effect of the feed supplements can be defined as less than additive. Our results differ from that of another a study by Dvorska *et al.*, where the protective effect of Mycosorb (modified glucomannan mycotoxin binder without the algae component) and Se-Y against T-2 toxin was analysed in chicken liver. Here, the results showed that Mycosorb resulted in a protective effect against T-2 toxin. The protective effect was

further enhanced when the mycotoxin binder was combined with Se-Y. However, that study is very different to the work carried out here as the mycotoxin binding capacity of the Mycosorb was analysed in that study, whereas the protective effects observed here are likely due to the PUFAs present in the algal component of My-A+ as the binding capacity of the feed additive has been removed following the digestion simulation.

Algae is an important source of long chain PUFAs such as EPA and DHA, which are known for their antioxidant and anti-inflammatory properties [34]. DHA, a long chain n-3 PUFA has been shown to reduce oxidative stress and lower levels of pro-inflammatory cytokines in macrophages from patients with abdominal aortic aneurysm [35]. Elsewhere, hepatotoxicity induced by paracetamol in rats was shown to be mitigated by pre-treatment with n-3 PUFAs. It was found that PUFAs exhibited protection against liver damage as anti-oxidant and anti-inflammatory effects and improved serum lipid profile were observed [36]. Se is also known to have antioxidant properties and its protective effects against various mycotoxins have been analysed previously. In one study, Se was shown to reduce hepatic dysfunction and apoptosis induced by AFB1 in the liver of ducklings [37]. Elsewhere, Se was shown to protect PK15 cells against OTA induced nephrotoxicity by improving selenoenzyme expression and promoting antioxidant capacity [38]. Se and PUFAs were both shown to mitigate the toxic effects of DON + ZEN in this study. However, their combination did not result in additional protective effects, potentially as the maximum level of protection may have been achieved individually.

There are very few published studies on the mitigation of toxic effects following exposure to multiple mycotoxins. Most of these focus on reversing the toxicity of single mycotoxins. One study investigated the protective effect of the antioxidant N-acetylcysteine (NAC) against toxicity induced by combined *Fusarium* mycotoxins. Treatments with NAC was shown to mitigate against the up-regulation of caspase-3, cytochrome c and *p53* mRNA levels caused by DON + ZEN [32]. Another study found that supplementation with *Bacillus subtilis* biodegradation product reversed the toxic effect of AF + ZEN on the laying performance and egg quality in birds [39]. Due to the unpredictability of mycotoxin interactions, it cannot be assumed that the compounds that can mitigate the effects of individual mycotoxins can also mitigate the effects of combination of mycotoxins.

4.5 Conclusion

From the results presented, it was found that a combination of DON and ZEN resulted in a synergistic effect on the viability of IPEC-J2 cells, following 24 h exposure. A mixture of the mycotoxins caused more cytotoxicity that the mycotoxins did individually. However, when the genotoxic effects of the combinations were analysed, synergism was not observed. Although DON did not induce any genotoxic effects on the cells, it also did not exacerbate the genotoxic effects induced by ZEN. Therefore, the DNA damage induced by DON + ZEN was similar to that by ZEN alone. Similarly, the TUNEL assays showed that combining the mycotoxins did not exacerbate the apoptotic effects of the individual mycotoxins. These results show that the combined effects cannot be determined by the individual effects induced by the mycotoxins. Additionally, the protective effect of the feed supplements against DON + ZEN induced toxicity was also investigated. The results showed that pre-treatment with Se-Y, My-A+ and Se-Y + My-A+ resulted in the reduction of the toxicity induced by DON + ZEN. Thus, the feed supplements were able to mitigate the effects of the combined mycotoxins as well as the individual mycotoxins. However, no significant difference was observed between the protective effects of the feed supplements, individually or in combination. It is therefore important to research potential routes of mitigation of combined mycotoxins, as it cannot be assumed that an agent that mitigates the effect of a single mycotoxin can mitigate the effects of co-exposure to mycotoxins, and neither can it be assumed that increasing the number of ameliorative feed supplements will result in an enhanced mitigation of their effects.

4.6 References

(1) Grenier, B.; Oswald, I. P. Mycotoxin Co-Contamination of Food and Feed: Meta-Analysis of Publications Describing Toxicological Interactions. *World Mycotoxin J.* **2011**, *4* (3), 285–313. https://doi.org/10.3920/WMJ2011.1281.

(2) Palumbo, R.; Crisci, A.; Venâncio, A.; Cortiñas Abrahantes, J.; Dorne, J.-L.; Battilani, P.; Toscano, P. Occurrence and Co-Occurrence of Mycotoxins in Cereal-Based Feed and Food. *Microorganisms* **2020**, *8* (1), 74. https://doi.org/10.3390/microorganisms8010074.

(3) Ráduly, Z.; Szabó, L.; Madar, A.; Pócsi, I.; Csernoch, L. Toxicological and Medical Aspects of Aspergillus-Derived Mycotoxins Entering the Feed and Food Chain. *Front. Microbiol.* **2020**, *10*, 2908. https://doi.org/10.3389/fmicb.2019.02908.

(4) Ismaiel, A. A.; Papenbrock, J. Mycotoxins: Producing Fungi and Mechanisms of Phytotoxicity. *Agriculture* **2015**, *5* (3), 492–537. https://doi.org/10.3390/agriculture5030492.

(5) Perrone, G.; Susca, A. Penicillium Species and Their Associated Mycotoxins. In *Mycotoxigenic Fungi: Methods and Protocols*; Moretti, A., Susca, A., Eds.; Methods in Molecular Biology; Springer: New York, NY, 2017; pp 107–119. https://doi.org/10.1007/978-1-4939-6707-0_5.

(6) Bertero, A.; Moretti, A.; Spicer, L. J.; Caloni, F. Fusarium Molds and Mycotoxins: Potential Species-Specific Effects. *Toxins (Basel)* **2018**, *10* (6). https://doi.org/10.3390/toxins10060244.

(7) Ji, F.; He, D.; Olaniran, A. O.; Mokoena, M. P.; Xu, J.; Shi, J. Occurrence, Toxicity, Production and Detection of Fusarium Mycotoxin: A Review. *Food Prod Process and Nutr* **2019**, *1* (1), 6. https://doi.org/10.1186/s43014-019-0007-2.

(8) World Mycotoxin Survey: Impact 2021 https://www.biomin.net/science-hub/world-mycotoxin-survey-impact-2021/ (accessed 2021 -04 -23).

(9) Griessler, K.; Rodrigues, I.; Handl, J.; Hofstetter, U. Occurrence of Mycotoxins in Southern Europe. *World Mycotoxin J.* **2010**, *3* (3), 301–309. https://doi.org/10.3920/WMJ2009.1198.

(10) Monbaliu, S.; Van Poucke, C.; Detavernier, C.; Dumoulin, F.; Van De Velde, M.; Schoeters, E.; Van Dyck, S.; Averkieva, O.; Van Peteghem, C.; De Saeger, S. Occurrence of Mycotoxins in Feed as Analyzed by a Multi-Mycotoxin LC-MS/MS Method. *J Agric Food Chem* **2010**, *58* (1), 66–71. https://doi.org/10.1021/jf903859z.

(11) Labuda, R.; Parich, A.; Berthiller, F.; Tancinová, D. Incidence of Trichothecenes and Zearalenone in Poultry Feed Mixtures from Slovakia. *Int J Food Microbiol* **2005**, *105* (1), 19–25. https://doi.org/10.1016/j.ijfoodmicro.2005.06.005.

(12) Streit, E.; Schatzmayr, G.; Tassis, P.; Tzika, E.; Marin, D.; Taranu, I.; Tabuc, C.; Nicolau, A.; Aprodu, I.; Puel, O.; Oswald, I. P. Current Situation of Mycotoxin Contamination and Co-Occurrence in Animal Feed-Focus on Europe. *Toxins (Basel)* **2012**, *4* (10), 788–809. http://dx.doi.org/10.3390/toxins4100788.

(13) Marques, M. F.; Martins, H. M.; Costa, J. M.; Bernardo, F. Co-Occurrence of Deoxynivalenol and Zearalenone in Crops Marketed in Portugal. *Food Addit Contam Part B Surveill* **2008**, *1* (2), 130–133. https://doi.org/10.1080/02652030802253983.

(14) Piacentini, K. C.; Rocha, L. O.; Savi, G. D.; Carnielli-Queiroz, L.; Almeida, F. G.; Minella, E.; Corrêa, B. Occurrence of Deoxynivalenol and Zearalenone in Brewing Barley Grains from Brazil. *Mycotoxin Res* **2018**, *34* (3), 173–178. https://doi.org/10.1007/s12550-018-0311-8.

(15) Calori-Domingues, M. A.; Bernardi, C. M. G.; Nardin, M. S.; de Souza, G. V.; dos Santos, F. G. R.; Stein, M. de A.; Gloria, E. M. da; Dias, C. T. dos S.; de Camargo, A. C. Co-Occurrence and Distribution of Deoxynivalenol, Nivalenol and Zearalenone in Wheat from Brazil. *Food Addit Contam Part B Surveill* **2016**, *9* (2), 142–151. https://doi.org/10.1080/19393210.2016.1152598.

(16) Gruber-Dorninger, C.; Jenkins, T.; Schatzmayr, G. Global Mycotoxin Occurrence in Feed: A Ten-Year Survey. *Toxins* **2019**, *11* (7), 375. https://doi.org/10.3390/toxins11070375.

(17) Kifer, D.; Jakšić, D.; Šegvić Klarić, M. Assessing the Effect of Mycotoxin Combinations: Which Mathematical Model Is (the Most) Appropriate? *Toxins (Basel)* **2020**, *12* (3), 153. https://doi.org/10.3390/toxins12030153.

(18) Smith, M.-C.; Madec, S.; Coton, E.; Hymery, N. Natural Co-Occurrence of Mycotoxins in Foods and Feeds and Their in Vitro Combined Toxicological Effects. *Toxins (Basel)* **2016**, *8* (4), 94. https://doi.org/10.3390/toxins8040094.

(19) Chou, T.-C. Theoretical Basis, Experimental Design, and Computerized Simulation of Synergism and Antagonism in Drug Combination Studies. *Pharmacol Rev* **2006**, *58* (3), 621–681. https://doi.org/10.1124/pr.58.3.10.

(20) Chou, T.-C. Drug Combination Studies and Their Synergy Quantification Using the Chou-Talalay Method. *Cancer Res* **2010**, *70* (2), 440–446. https://doi.org/10.1158/0008-5472.CAN-09-1947.

(21) Zain, M. E. Impact of Mycotoxins on Humans and Animals. *J Saudi Chem Soc* **2011**, *15* (2), 129–144. https://doi.org/10.1016/j.jscs.2010.06.006.

(22) Reddy, K. E.; Lee, W.; Jeong, J. young; Lee, Y.; Lee, H.-J.; Kim, M. S.; Kim, D.-W.; Yu, D.; Cho, A.; Oh, Y. K.; Lee, S. D. Effects of Deoxynivalenol- and Zearalenone-Contaminated Feed on the Gene Expression Profiles in the Kidneys of Piglets. *Asian-Australas J Anim Sci* **2018**, *31* (1), 138–148. https://doi.org/10.5713/ajas.17.0454.

(23) Ren, Z. H.; Deng, H. D.; Deng, Y. T.; Deng, J. L.; Zuo, Z. C.; Yu, S. M.; Shen, L. H.; Cui, H. M.; Xu, Z. W.; Hu, Y. C. Effect of the Fusarium Toxins, Zearalenone and Deoxynivalenol, on the Mouse Brain. *Environ Toxicol Pharmacol* **2016**, *46*, 62–70. https://doi.org/10.1016/j.etap.2016.06.028.

(24) Zhou, H.; George, S.; Hay, C.; Lee, J.; Qian, H.; Sun, X. Individual and Combined Effects of Aflatoxin B1, Deoxynivalenol and Zearalenone on HepG2 and RAW 264.7 Cell Lines. *Food Chem Toxicol* **2017**, *103*, 18–27. https://doi.org/10.1016/j.fct.2017.02.017.

(25) Kouadio, J. H.; Dano, S. D.; Moukha, S.; Mobio, T. A.; Creppy, E. E. Effects of Combinations of Fusarium Mycotoxins on the Inhibition of Macromolecular Synthesis, Malondialdehyde Levels, DNA Methylation and Fragmentation, and Viability in Caco-2 Cells. *Toxicon* **2007**, *49* (3), 306–317. https://doi.org/10.1016/j.toxicon.2006.09.029.

(26) Ji, J.; Wang, Q.; Wu, H.; Xia, S.; Guo, H.; Blaženović, I.; Zhang, Y.; Sun, X. Insights into Cellular Metabolic Pathways of the Combined Toxicity Responses of Caco-2 Cells Exposed to Deoxynivalenol, Zearalenone and Aflatoxin B1. *Food and Chemical Toxicology* **2019**, *126*, 106–112. https://doi.org/10.1016/j.fct.2018.12.052.

(27) Bensassi, F.; Gallerne, C.; Sharaf el dein, O.; Hajlaoui, M. R.; Lemaire, C.; Bacha, H. In Vitro Investigation of Toxicological Interactions between the Fusariotoxins Deoxynivalenol and Zearalenone. *Toxicon* **2014**, *84*, 1–6. https://doi.org/10.1016/j.toxicon.2014.03.005.

(28) Smith, M.-C.; Timmins-Schiffman, E.; Coton, M.; Coton, E.; Hymery, N.; Nunn, B. L.; Madec, S. Differential Impacts of Individual and Combined Exposures of Deoxynivalenol and Zearalenone on the HepaRG Human Hepatic Cell Proteome. *J Proteomics* **2018**, *173*, 89–98. https://doi.org/10.1016/j.jprot.2017.11.025.

(29) Lu, H.; Fernández-Franzón, M.; Font, G.; Ruiz, M. J. Toxicity Evaluation of Individual and Mixed Enniatins Using an in Vitro Method with CHO-K1 Cells. *Toxicol In Vitro* **2013**, *27* (2), 672–680. https://doi.org/10.1016/j.tiv.2012.11.009.

(30) Smith, M.-C.; Madec, S.; Pawtowski, A.; Coton, E.; Hymery, N. Individual and Combined Toxicological Effects of Deoxynivalenol and Zearalenone on Human Hepatocytes in in Vitro Chronic Exposure Conditions. *Toxicol Lett* **2017**, *280*, 238–246. https://doi.org/10.1016/j.toxlet.2017.08.080.

(31) Smith, M.-C.; Hymery, N.; Troadec, S.; Pawtowski, A.; Coton, E.; Madec, S. Hepatotoxicity of Fusariotoxins, Alone and in Combination, towards the HepaRG Human Hepatocyte Cell Line. *Food Chem Toxicol* **2017**, *109* (Pt 1), 439–451. https://doi.org/10.1016/j.fct.2017.09.022.

(32) Zhang, W.; Zhang, S.; Zhang, M.; Yang, L.; Cheng, B.; Li, J.; Shan, A. Individual and Combined Effects of Fusarium Toxins on Apoptosis in PK15 Cells and the Protective Role of N-Acetylcysteine. *Food Chem Toxicol* **2018**, *111*, 27–43. https://doi.org/10.1016/j.fct.2017.10.057.

(33) Ren, Z.; Deng, H.; Deng, Y.; Liang, Z.; Deng, J.; Zuo, Z.; Hu, Y.; Shen, L.; Yu, S.; Cao, S. Combined Effects of Deoxynivalenol and Zearalenone on Oxidative Injury and Apoptosis in Porcine Splenic Lymphocytes in Vitro. *Exp Toxicol Pathol* **2017**, *69* (8), 612–617. https://doi.org/10.1016/j.etp.2017.05.008.

(34) Harwood, J. L. Algae: Critical Sources of Very Long-Chain Polyunsaturated Fatty Acids. *Biomolecules* **2019**, *9* (11), 708. https://doi.org/10.3390/biom9110708.

(35) Meital, L. T.; Windsor, M. T.; Perissiou, M.; Schulze, K.; Magee, R.; Kuballa, A.; Golledge, J.; Bailey, T. G.; Askew, C. D.; Russell, F. D. Omega-3 Fatty Acids Decrease Oxidative Stress and Inflammation in Macrophages from Patients with Small Abdominal Aortic Aneurysm. *Sci Rep* **2019**, *9* (1), 12978. https://doi.org/10.1038/s41598-019-49362-z.

(36) El-Gendy, Z. A.; El-Batran, S. A.; Youssef, S.; Ramadan, A.; Hotaby, W. E.; Bakeer, R. M.; Ahmed, R. F. Hepatoprotective Effect of Omega-3 PUFAs against Acute Paracetamol-Induced Hepatic Injury Confirmed by FTIR. *Hum Exp Toxicol* **2021**, *40* (3), 526–537. https://doi.org/10.1177/0960327120954522.

(37) Liao, S.; Shi, D.; Clemons-Chevis, C. L.; Guo, S.; Su, R.; Qiang, P.; Tang, Z. Protective Role of Selenium on Aflatoxin B1-Induced Hepatic Dysfunction and Apoptosis of Liver in Ducklings. *Biol Trace Elem Res* **2014**, *162* (1), 296–301. https://doi.org/10.1007/s12011-014-0131-4.

(38) Gan, F.; Xue, H.; Huang, Y.; Pan, C.; Huang, K. Selenium Alleviates Porcine Nephrotoxicity of Ochratoxin A by Improving Selenoenzyme Expression In Vitro. *PLoS One* **2015**, *10* (3), e0119808. https://doi.org/10.1371/journal.pone.0119808.

(39) Jia, R.; Ma, Q.; Fan, Y.; Ji, C.; Zhang, J.; Liu, T.; Zhao, L. The Toxic Effects of Combined Aflatoxins and Zearalenone in Naturally Contaminated Diets on Laying Performance, Egg Quality and Mycotoxins Residues in Eggs of Layers and the Protective Effect of Bacillus Subtilis Biodegradation Product. *Food Chem Toxicol.* **2016**, *90*, 142–150. https://doi.org/10.1016/j.fct.2016.02.010.

Chapter 5: Conclusion and future work

5.1 Overall conclusion and future work

Mycotoxin contamination is a major problem in the agri-food industry. The common occurrence of mycotoxins in raw materials that are used in animal feed results in an easy assimilation route into the food chain [1]. The effects of mycotoxins are not only detrimental to animal health but also to the global economy due to crop losses and costly management strategies [2]. DON and ZEN are two frequently occurring *Fusarium* mycotoxins and their ingestion can result in various negative health effects in animals, especially pigs [3]. Therefore, it is necessary to understand the implications of these compounds as foodstuff contaminants and to research mitigation strategies against demonstrated toxic effects. The research detailed in this thesis investigates the toxicity of DON and ZEN, both individually and combined, on intestinal pig cells, specifically IPEC-J2 cells. Additionally the protective effects of two feed supplements, Se-Y, a selenium enriched yeast product, and My-A+, a mycotoxin binder rich in PUFAs, against mycotoxin induced toxicity was investigated.

The aim of the research presented in Chapter 2 was to investigate the toxic effects of ZEN on IPEC-J2 cells and the potential protective effects of Se-Y and My-A+ against its toxicity. It was found that, following 24 h exposure, ZEN did not reduce cell viability at a concentration of 0.25 ppm, which is the maximum EU allowable ZEN in feed for sows and fattening pigs. This is reassuring in that it supports the current legislative viewpoint that this is a safe maximal contamination level. Here, studies to determine whether ZEN could cause negative effects at concentrations above the maximum allowable level found that following 24 h exposure, a reduction in cell viability was observed with a concentration of 3 ppm and above. In fact, exposure to ZEN was found to have a time and dose dependent effect on the viability of IPEC-J2 cells. ZEN was further shown to induce DNA damage and apoptosis at a concentration of 25 ppm after 24 h exposure. Following the exploration of the detrimental impacts of ZEN, the potential protective effects of Se-Y and My-A+ on ZEN induced toxicity were studied. Pre-treatment of the cells for 48 h with Se-Y and My-A+, alone and in combination, prior to ZEN exposure, was shown to reduce its cytotoxic effects, genotoxic and apoptotic effects. However, combination of the these two feed supplements did not result in increased mitigation, as no significant difference was observed between treatment with Se-Y individually, My-A+ individually, and a combination of both Se-Y and My-A+.

The effect of DON on IPEC-J2 cells was investigated in Chapter 3, including the potential for mitigation by Se-Y and My-A+ against DON induced toxicity. It was found that DON had a detrimental effect on cell viability as it was shown to reduce the viability of IPEC-J2 cells at a concentration of 0.15 ppm following 24 h exposure. This concentration is below the maximum EU allowable concentration of 0.9 ppm, highlighting that, in contrast to what was determined with ZEN, the results obtained here do not support the regulatory position that DON concentrations up to 0.9 ppm are safe for pig feeds. The cytotoxicity induced by DON was time and dose dependent as determined with the combined alamar blue and CFDA-AM dual assays. The TUNEL assay showed a decrease in TUNEL negative values with increasing DON concentration, an indication of increased DNA fragmentation due to apoptosis. A significant level of apoptosis was detected in cells exposed to 0.9 ppm DON. However, the comet assay, which detects levels of DNA strand breaks, did not detect DNA damage following exposure of cells to up to 5 ppm DON. In the second part of the chapter, the potential for mitigation by the feed supplements Se-Y and My-A+, both individually and combined, against damage induced by exposure to DON, was investigated. As with Chapter 2, a protective effect was observed in the cells treated with the feed supplements with the cell viability and TUNEL assays and again, there was no significant difference observed between the protective effects of Se-Y and My-A+. Additionally, it was found that combining the two products did not result in an increase in the level of protection. The comet assay was not employed here, as DON-induced DNA damage was not observed at the outset.

From the results obtained in Chapters 2 and 3 it can be concluded that DON is more toxic to IPEC-J2 cells than ZEN, and that whilst the maximum allowable concentration of ZEN is supported by the results in this work, this was not the case for DON, where cell viability was compromised at concentrations below the current maximum allowable concentration. After 24 h exposure, 0.15 ppm DON was shown to reduce cell viability significantly, however 3 ppm ZEN was the lowest concentration at which cytotoxicity was observed. Similarly, DON was shown to induce apoptosis at 0.9 ppm (the current allowable limit), but 25 ppm ZEN (100 times the current permissible limit) was required to induce observable apoptosis and genotoxicity.

ZEN and DON are frequently found to co-occur in grains and thus animals may be exposed to both mycotoxins simultaneously. However, the combinatorial effects of the mycotoxins cannot be predicted by their individual effects. Therefore, in Chapter 4, the

combinatorial effect of DON and ZEN combined on IPEC-J2 cells was investigated. Two concentration combinations were used for the study. The first combination was 0.9 ppm DON and 0.25 ppm ZEN as these are the maximum allowable concentrations of DON and ZEN in pig feed as set by the EU commission. The second combination used was 0.9 ppm DON and 25 ppm ZEN as these were the lowest concentrations at which genotoxicity and/or apoptosis was observable following mycotoxin exposure. The combination of DON and ZEN was shown to be more cytotoxic to the cells than the individual mycotoxins, thus exhibiting a synergistic effect. This effect was observed with both of the combinations tested. However, a synergistic effect was not observed with the TUNEL or comet assay. There was no DNA damage detected by the comet assay with 0.9 ppm DON and 0.25 ppm ZEN individually and, DNA damage was still not detected following co-exposure. For the second combination, the combined effect of 25 ppm ZEN + 0.9 ppm DON was found to be the same as that for 25 ppm ZEN alone, and thus DON did not add to the genotoxic effects of ZEN. Similarly, with the TUNEL assay, 0.25 ppm ZEN did not induce any observable apoptosis and when it was combined with 0.9 ppm DON, the effect of the mixture was similar to that seen with DON alone. The combined effect of 0.9 ppm DON and 25 ppm ZEN was shown to be similar to that of ZEN alone. Thus, co-exposure did not lead to an increase in the level of apoptosis seen with the individual mycotoxins. Additionally, combining the mycotoxins did not affect the mitigation capacity of the feed supplements. Se-Y and My-A+ were used to investigate their mitigation effects against combined mycotoxins. As the effects of combined mycotoxins are unpredictable, it cannot be assumed that the products that mitigated the effects of DON and ZEN alone can mitigate the effects of DON and ZEN combined. As with the individual toxins, pre-treatment with the feed supplements ameliorated the toxicity of DON and ZEN combined. There was no significant difference between the protective effects of Se-Y and My-A+ and their combined protective effect was found to be similar to their individual effects. The results of this chapter showed that the co-exposure effects differed in terms of their impact on IPEC-J2 cells, with synergistic effects observed regarding cytotoxicity, but less than additive effects determined during genotoxic and apoptosis evaluations.

The research outlined in this thesis adds to our knowledge of individual and combinatorial toxicities of mycotoxins, and provides a greater insight into the effectiveness of potential mycotoxin mitigation strategies. The effects of the mycotoxins and protective effects of

Se and PUFAs from Se-Y and My-A+, respectively, was investigated in this study *in vitro*. *In vitro* testing is cost and time effective and provides an alternative to animal testing [4,5]. However, it is important to note that the results obtained from *in vitro* experiments may not be reflected during *in vivo* testing. Many other external and internal factors could influence the results including temperature, nutrients and digestive enzymes amongst others. Nonetheless, *in vitro* experimentation is still an efficient and useful method for initial assessment of mycotoxins and feed additives.

From the literature review carried out in Chapter 1, it can be concluded that the effect of combined mycotoxins cannot be predicted by their individual effects. It was also observed from the literature review that the combined effect differs depending on the cell type, concentrations used, exposure time and the toxic effect being analysed. This effect was observed in the results of Chapter 4, where synergistic effects were observed with the cell viability assay but not with comet or TUNEL assay, which measure genotoxicity and apoptosis. Two concentration combinations were used for these assays. These concentrations were chosen, as they are key concentration cut-offs for European food legislation. In order to further study the combinatorial effects of DON and ZEN, different concentration combinations should be used as it has been shown that the combinatorial effects may vary depending on the concentrations used. Combinations varying from non-cytotoxic concentrations (below 3 ppm ZEN and below 0.15 ppm DON, as per Chapters 2 and 3) to cytotoxic concentrations can give a good insight of the interaction between the two mycotoxins.

Two feed additives Se-Y and My-A+ were used in this study for their Se and PUFA content respectively, both as individual mitigating agents, and to explore whether in combination they might afford an additive protective effect. From the results observed in Chapters 2 to 4, it was found that the two feed supplements protected IPEC-J2 cells against DON and ZEN induced toxicity. However, it was observed that combining the two supplements did not increase their overall protective effects. This could be due to a maximum level of mitigation having already been achieved by the feed supplements individually and thus the same protective effect was observed when the two compounds were combined. Therefore, to test this theory future work could involve first varying the concentrations of either Se-Y and My-A+ in order to examine the threshold levels of protection that they might provide when used individually. Following this, using various concentrations tested individually, the combined protective effect of the two supplements

can be studied. This experiment can give an enhanced insight into the mitigating effects of Se-Y and My-A+, both individually and in combination.

This study specifically evaluated the effect of two Alltech products. These products were chosen both because of their position as market leaders in this industry and because of the extent to which research on their effects has been published in scientific literature. Alltech's Se-Y is the first form of selenium-enriched yeast and the first strain-specific form to be approved by the EU. It has been proven to improve the performance and efficiency of animals. It was the first form of organic selenium to be reviewed by the FDA. Alltech is the world's largest producer of natural selenium and the use of Sel-Plex has been thoroughly researched for over 30 years [6]. Se supplementation is usually added to pig feed to tackle Se deficiency and to improve growth performance and organic Se has shown to be less toxic and more bioavailable than inorganic sources [7,8]. My-A+ is the successor to Europe's best selling mycotoxin binder. This product has shown to have increased efficacy and reaches high quality standards and has been shown to reduce the impact of mycotoxins [9]. Therefore, whilst the issue of Se deficiency and mycotoxin contamination are not related, they can both be addressed by co-supplementation of the two associated feed additives. An important consideration as a result is to demonstrate that the combination of the feed additives is non-toxic. As feed supplementation regimens become increasingly complex, it would be prudent to carry out a cost-benefit analysis, such as the recent study initiated by Teagasc with regard to enzymatic supplementation in pigfeed [10]. To date however, no comparable analysis has been carried out for co-supplementation with Se-Y and My-A+.

Following the digestion simulation carried out with the My-A+, the adsorbent capacity of the mycotoxin binder was removed. Therefore, it has been hypothesised that it is the PUFAs present in the algae component of My-A+ that is providing the protective effect against DON and ZEN. In order to show that it is the PUFAs providing the protective effect an experiment could be designed as part of future work. The cells can be pre-incubated with PUFAs such as DHA, EPA and ARA for 48 h, followed by 24 h exposure to the mycotoxins. The results of this experiment can then be compared to the results obtained in Chapters 2, 3 and 4 where My-A+ was used.

The research presented in this thesis clearly illustrates that where two mycotoxins co-occur, their impact cannot effectively be predicted and may be more damaging to the

cells than the effects of each mycotoxin when combined. However, DON and ZEN are not the only two mycotoxins that have been demonstrated to co-occur. The two major metabolites of ZEN, α-ZOL and β-ZOL, are found to be contaminants of plant-based products and they are also found to frequently occur with their parent compound [11–13]. For example, a study was carried out to analyse the occurrence of DON, ZEN and their metabolites in various food and feed products. From 52 samples of fibre-enriched bread, 25%, 17% and 25% were contaminated with ZEN, α-ZOL and β-ZOL respectively. ZEN was found to contaminate 56% of bran-enriched bread and 20% and 25% of the 2010 samples were contaminated with α-ZOL and β-ZOL respectively. ZEN, α-ZOL and β-ZOL were found in 52%, 50% and 40% of the 61 cornflakes samples tested. From 13 oatmeal samples, 62% were contaminated with ZEN. From the 2010 samples, 50% and 63% of the samples were contaminated with α-ZOL and β-ZOL, respectively. From the feed materials analysed, it was found that maize based products were highly contaminated with ZEN and its metabolites. The presence of ZEN and its metabolites were also detected in wheat and its by-products [14]. These studies highlight the frequent co-occurrence of α-ZOL and β-ZOL, consequently highlighting the importance in investigating their toxic effects using simultaneous exposure models.

As with DON and ZEN, the impact of ZEN metabolites are also known to be dependent on the cell type, but the reasons for these varying effects are poorly understood, to the extent that potential effects cannot accurately be predicted for different cell types based on the individual effects. The effect of ZEN and its metabolites individually has been studied on various cell types. One study showed the cytotoxicity of ZEN, α-ZOL and β-ZOL on Vero cells (monkey kidney cells). Both metabolites were shown to be cytotoxic to the cells with β-ZOL more toxic to the cells than α-ZOL [15]. Elsewhere, ZEN was not shown to induce DNA damage in CHO-K1 (Chinese hamster ovary) cells but α-ZOL and β-ZOL did, with α-ZOL more genotoxic than β-ZOL [16]. The combined effects of ZEN and its metabolites have also been studied. One such study reported additive effects with binary combinations of ZEN, α-ZOL and β-ZOL on CHO-K1 cells. When all three compounds were combined, an antagonistic effect was observed at low concentrations and a synergistic effect was observed at higher concentrations [17]. Another study using HepG2 cell line showed that β-ZOL was the most cytotoxic followed by ZEN and α-ZOL. With binary combinations of the compounds, a synergistic effect was observed [18]. In a different study also using HeG2 cells, the combined effect of ZEN and α-ZOL was

investigated. It was found that with lower concentrations, an antagonistic effect was observed and synergism was evident at higher concentrations [19]. However, there are no studies to date investigating the combinatorial effects of ZEN and its two major metabolites on IPEC-J2 cells, even though these cells, as a pig intestinal cell line, are a highly relevant *in vitro* model for evaluation of ingestion of feedstuffs by pigs. Therefore, future work should include the investigation of the toxic effects of the two metabolites on the cells and compare it to the effects induced by ZEN. Additionally, the combined effects of ZEN and its metabolites should be studied to understand their interactive effects on the toxicity of IPEC-J2 cells.

Similarly, metabolites of DON such as 3-ADON, 15-ADON and DON-3G are often found to contaminate cereal grains. One study investigated the presence of these compounds in various food and feed samples. From 52 samples of fibre-enriched bread, 3-A-DON, 15-ADON and DON-3G were found in 38%, 33% and 50% of samples respectively, with parent compound DON present in 85% of the samples. In 36 bran-enriched bread, DON contaminated 75% of samples, with 3-ADON, 15-ADON detected in 44% of the products and DON-3G was found in 53%. From 61 cornflakes samples, DON was found to contaminate 66% and its metabolites 3-ADON and 15-ADON contaminated 70% and 65% of the samples. In popcorn, DON, 3-ADON and 15-ADON was found in 50%, 83% and 67% of the 12 samples. Feed for fattening pigs made with wheat, barley and maize, was also analysed and 62%, 100% and 69% of the samples were contaminated with 3-ADON, 15-ADON and DON-3-G. Horse feed (barley, maize and wheat) had 68%, 21%, 71% and 36% of DON, 3-ADON, 15ADON and DON-3G [14]. Although these metabolites are detected in grains, often in co-occurrence with DON, the toxic effects induced by these compounds have not been studied as extensively as their parent compound both *in vivo* and *in vitro* [20].

The occurrence of the derivatives of DON highlights the importance of understanding both their individual and combined effects. Although there are some studies on the toxicity of the metabolites individually, there are very few studies in the literature on their combined effects with each other and the parent compound DON. One study investigated the effect of DON and its two acetylated metabolites, 3-ADON and 15-ADON on the IPEC-1 cell line. 15-ADON was found to be the most toxic to proliferating cells, followed by DON and 3-ADON was the least toxic. However, on differentiated cells, DON and 3-ADON did not have an effect on the barrier function which 15-ADON was shown to

impair [21]. Elsewhere, IPEC-J2 cells were used to assess the toxicity of DON and its metabolites. It was shown that, following 72 h exposure, DON-3G was the least toxic to the cells followed by 3-ADON. DON and 15-ADON were shown to be the most toxic and had similar toxicities to each other [22]. In HepG2 cells however, 3-ADON was found to be more toxic than 15-ADON [23]. A synergistic interaction was observed when 3-ADON and 15-ADON were combined. The combination of the two acetylated derivatives were shown to be more toxic than the compounds individually when tested on HepG2 cells [24]. Elsewhere, the toxicity of DON, 3-ADON and 15-ADON, individually and combined, on IPEC-1 cells was investigated. The combination of DON + 15-ADON and 15-ADON and 3-ADON resulted in a synergistic interaction. However, for the combination of DON + 3-ADON antagonism was reported at lower concentrations and synergy at higher concentrations [25]. In human gastric epithelial GES-1 cells, the combination of DON + 15-ADON resulted in synergistic effects [26]. In summary, the effects of DON have been studied extensively, but the effects of its derivatives have not received the same attention. As DON is often found to co-occur with its derivatives, the combined effects of DON and its derivatives on IPEC-J2 cells should be studied, to continue the work carried out in this study. This experiment will provide greater insight into the interactive effects of DON and its metabolites on model cell systems.

One of the most interesting observations during this study was the extent to which impacts on cell viability were determined but no adverse effect was recorded from a genotoxic perspective, as measured by the comet assay. The alkaline comet assay can detect single and double DNA strand breaks and alkali-labile sites [27]. However, the standard comet assay cannot detect other lesions that may be induced including oxidised bases [28]. In contrast, the enzyme-modified comet assay can be used to detect these specific DNA lesions [29]. Endonuclease III (EndoIII) and formamidopyrimidine DNA glycosylase (FPG) are two of the most commonly used enzymes for the detection of oxidative DNA damage. Digestion with the enzymes, following the lysis step, coverts the oxidative DNA lesions to strand breaks [28]. Endo (III) can recognise oxidised pyrimidines and FPG recognises oxidised purines [30]. The level of oxidised purines and pyrimidines are measured by subtracting the percentage tail intensity of buffer treated from FPG or Endo III treated respectively [31]. Therefore, in order to determine if DON and ZEN induce oxidative DNA damage, the enzyme modified comet assay can be employed. This method has been used previously to determine oxidative DNA damage induced by

various mycotoxins. In one study, FPG was used to measure oxidative DNA damage induced by ZEN in Chang liver cells. ZEN was shown to induce oxidative stress in a dose-dependent manner [32]. Elsewhere, the comet assay showed that AFB1 induced DNA strand breaks in HepG2 cells, following 24 h exposure. However, with the enzyme-modified comet assay, DNA damage was detected after digestion with FPG but not with endo III. In the same study, OTA did not induce any DNA damage in HepG2 cells and enzymatic digestion showed that oxidative damage was also not induced [33]. The genotoxicity of DON was analysed in TK6 cells and in seven mice organs (duodenum, colon, blood, liver, spleen, kidney, bone marrow). DON was not shown to induce DNA damage in the organs or TK6 cells using the comet assay with or without FPG [34]. In another study, following 3 h exposure, OTA did not induce any strand breaks and oxidative DNA damage in human renal proximal tubular epithelial cell line (HK-2). However, with a longer exposure time of 6 h, strand breaks were observed at 400 µM and 600 µM, additionally, oxidative damage was detected by FPG and endo III at a concentration as low as 50 µM [35]. This shows that although strand breaks were not induced at the lower concentration, oxidised purines and pyrimidines were detected. The enzyme-modified comet assay has not been used to detect oxidative DNA damage induced by DON and ZEN in IPEC-J2 cells. As a continuation of the work carried out in this thesis, the enzyme modified comet assay could be used to determine if oxidised purines and pyrimidines are produced following exposure to DON and/or ZEN. As the alkaline comet assay detects strand breaks, inclusion of the enzyme modified comet assay can provide additional insight into oxidative DNA damage induced by the mycotoxins.

Overall, this thesis tackles the major problem of mycotoxin contamination in the agri-food industry with a focus on animal feed. The work carried out in this study also highlights the importance of mycotoxin management due to the detrimental health effects associated with mycotoxins. The toxic effects induced by two of the most common mycotoxins were analysed, both individually and combined. ZEN and DON, individually, were shown to induce cytotoxicity and apoptosis. ZEN was also shown to induce DNA damage in IPEC-J2 cells, but the same effect was not observed with DON. The combination of ZEN and DON resulted in increased cytotoxicity at each of the combinations that were tested. However, apoptosis and DNA damage induced by the individual mycotoxins was not exacerbated following co-exposure. Thus, this study emphasises the need for more research on the combined toxicological effects of

mycotoxins as it is clear that they are not predictable based on their individual effects. Additionally, the mitigation of the toxic effects induced by mycotoxins was also examined in this study. The two feed supplements Se-Y and My-A+ were used to examine the protective effects of Se and PUFAs respectively. The results of this study exhibited the capacity of organic Se and PUFAs for protection against both individual and combined mycotoxin-induced toxicity, though no additive impact was detected on combining the feed supplements. Overall, however, the feed supplements were shown to provide protection against cytotoxicity, DNA damage and apoptosis induced by both DON and ZEN. Therefore, the findings of this study are a significant step towards developing mitigation strategies to counter the adverse effects of mycotoxins on animal health.

5.2 References

(1) Alshannaq, A.; Yu, J.-H. Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. *Int J Environ Res Public Health* **2017**, *14* (6), 632. https://doi.org/10.3390/ijerph14060632.

(2) Zain, M. E. Impact of Mycotoxins on Humans and Animals. *J Saudi Chem Soc* **2011**, *15* (2), 129–144. https://doi.org/10.1016/j.jscs.2010.06.006.

(3) Gruber-Dorninger, C.; Jenkins, T.; Schatzmayr, G. Global Mycotoxin Occurrence in Feed: A Ten-Year Survey. *Toxins* **2019**, *11* (7), 375. https://doi.org/10.3390/toxins11070375.

(4) Xu, R.; Karrow, N. A.; Shandilya, U. K.; Sun, L.; Kitazawa, H. In-Vitro Cell Culture for Efficient Assessment of Mycotoxin Exposure, Toxicity and Risk Mitigation. *Toxins (Basel)* **2020**, *12* (3), 146. https://doi.org/10.3390/toxins12030146.

(5) Tran, V. N.; Viktorova, J.; Augustynkova, K.; Jelenova, N.; Dobiasova, S.; Rehorova, K.; Fenclova, M.; Stranska-Zachariasova, M.; Vitek, L.; Hajslova, J.; Ruml, T. In Silico and In Vitro Studies of Mycotoxins and Their Cocktails; Their Toxicity and Its Mitigation by Silibinin Pre-Treatment. *Toxins (Basel)* **2020**, *12* (3), 148. https://doi.org/10.3390/toxins12030148.

(6) Sel-Plex® https://www.alltech.com/node/9636 (accessed 2021 -09 -05).

(7) Cao, J.; Guo, F.; Zhang, L.; Dong, B.; Gong, L. Effects of Dietary Selenomethionine Supplementation on Growth Performance, Antioxidant Status, Plasma Selenium Concentration, and Immune Function in Weaning Pigs. *J Anim Sci Biotechnol* **2014**, *5* (1), 46. https://doi.org/10.1186/2049-1891-5-46.

(8) Lynch, S. J.; Horgan, K. A.; White, B.; Walls, D. Selenium Source Impacts Protection of Porcine Jejunal Epithelial Cells from Cadmium-Induced DNA Damage, with Maximum Protection Exhibited with Yeast-Derived Selenium Compounds. *Biol Trace Elem Res* **2017**, *176* (2), 311–320. https://doi.org/10.1007/s12011-016-0828-7.

(9) Mycosorb A+ https://www.alltech.com/mycosorb (accessed 2021 -09 -05).

(10) PIG-ZYME - Teagasc | Agriculture and Food Development Authority https://www.teagasc.ie/animals/pigs/research/research-projects/pig-zyme/ (accessed 2021 -09 -05).

(11) Tiemann, U.; Viergutz, T.; Jonas, L.; Schneider, F. Influence of the Mycotoxins α- and β-Zearalenol and Deoxynivalenol on the Cell Cycle of Cultured Porcine Endometrial Cells. *Reprod Toxicol* **2003**, *17* (2), 209–218. https://doi.org/10.1016/S0890-6238(02)00141-7.

(12) Palumbo, R.; Crisci, A.; Venâncio, A.; Cortiñas Abrahantes, J.; Dorne, J.-L.; Battilani, P.; Toscano, P. Occurrence and Co-Occurrence of Mycotoxins in Cereal-Based Feed and Food. *Microorganisms* **2020**, *8* (1), 74. https://doi.org/10.3390/microorganisms8010074.

(13) Schollenberger, M.; Müller, H.-M.; Rüfle, M.; Suchy, S.; Plank, S.; Drochner, W. Natural Occurrence of 16 Fusarium Toxins in Grains and Feedstuffs of Plant Origin from Germany. *Mycopathologia* **2006**, *161* (1), 43–52. https://doi.org/10.1007/s11046-005-0199-7.

(14) De Boevre, M.; Di Mavungu, J. D.; Landschoot, S.; Audenaert, K.; Eeckhout, M.; Maene, P.; Haesaert, G.; De Saeger, S. Natural Occurrence of Mycotoxins and Their Masked Forms in Food and Feed Products. *World Mycotoxin J* **2012**, *5* (3), 207–219. https://doi.org/10.3920/WMJ2012.1410.

(15) Othmen, Z. O.-B.; Golli, E. E.; Abid-Essefi, S.; Bacha, H. Cytotoxicity Effects Induced by Zearalenone Metabolites, α Zearalenol and β Zearalenol, on Cultured Vero Cells. *Toxicology* **2008**, *252* (1), 72–77. https://doi.org/10.1016/j.tox.2008.07.065.

(16) Tatay, E.; Font, G.; Ruiz, M.-J. Cytotoxic Effects of Zearalenone and Its Metabolites and Antioxidant Cell Defense in CHO-K1 Cells. *Food Chem Toxicol* **2016**, *96*, 43–49. https://doi.org/10.1016/j.fct.2016.07.027.

(17) Tatay, E.; Meca, G.; Font, G.; Ruiz, M.-J. Interactive Effects of Zearalenone and Its Metabolites on Cytotoxicity and Metabolization in Ovarian CHO-K1 Cells.

Toxicology in Vitro **2014**, *28* (1), 95–103. https://doi.org/10.1016/j.tiv.2013.06.025.

(18) Marin, D. E.; Pistol, G. C.; Bulgaru, C. V.; Taranu, I. Cytotoxic and Inflammatory Effects of Individual and Combined Exposure of HepG2 Cells to Zearalenone and Its Metabolites. *Naunyn-Schmiedeberg's Arch Pharmacol* **2019**, *392* (8), 937–947. https://doi.org/10.1007/s00210-019-01644-z.

(19) Wang, H. W.; Wang, J. Q.; Zheng, B. Q.; Li, S. L.; Zhang, Y. D.; Li, F. D.; Zheng, N. Cytotoxicity Induced by Ochratoxin A, Zearalenone, and α-Zearalenol: Effects of Individual and Combined Treatment. *Food Chem Toxicol* **2014**, *71*, 217–224. https://doi.org/10.1016/j.fct.2014.05.032.

(20) Eckard, S.; Wettstein, F. E.; Forrer, H.-R.; Vogelgsang, S. Incidence of Fusarium Species and Mycotoxins in Silage Maize. *Toxins (Basel)* **2011**, *3* (8), 949–967. https://doi.org/10.3390/toxins3080949.

(21) Pinton, P.; Tsybulskyy, D.; Lucioli, J.; Laffitte, J.; Callu, P.; Lyazhri, F.; Grosjean, F.; Bracarense, A. P.; Kolf-Clauw, M.; Oswald, I. P. Toxicity of Deoxynivalenol and Its Acetylated Derivatives on the Intestine: Differential Effects on Morphology, Barrier Function, Tight Junction Proteins, and Mitogen-Activated Protein Kinases. *Toxicol Sci* **2012**, *130* (1), 180–190. https://doi.org/10.1093/toxsci/kfs239.

(22) Broekaert, N.; Devreese, M.; Demeyere, K.; Berthiller, F.; Michlmayr, H.; Varga, E.; Adam, G.; Meyer, E.; Croubels, S. Comparative in Vitro Cytotoxicity of Modified Deoxynivalenol on Porcine Intestinal Epithelial Cells. *Food Chem Toxicol* **2016**, *95*, 103–109. https://doi.org/10.1016/j.fct.2016.06.012.

(23) Juan-García, A.; Juan, C.; Tolosa, J.; Ruiz, M.-J. Effects of Deoxynivalenol, 3-Acetyl-Deoxynivalenol and 15-Acetyl-Deoxynivalenol on Parameters Associated with Oxidative Stress in HepG2 Cells. *Mycotoxin Res* **2019**, *35* (2), 197–205. https://doi.org/10.1007/s12550-019-00344-0.

(24) Juan-García, A.; Juan, C.; Manyes, L.; Ruiz, M.-J. Binary and Tertiary Combination of Alternariol, 3-Acetyl-Deoxynivalenol and 15-Acetyl-Deoxynivalenol on

HepG2 Cells: Toxic Effects and Evaluation of Degradation Products. *Toxicol In Vitro* **2016**, *34*, 264–273. https://doi.org/10.1016/j.tiv.2016.04.016.

(25) Alassane-Kpembi, I.; Puel, O.; Oswald, I. P. Toxicological Interactions between the Mycotoxins Deoxynivalenol, Nivalenol and Their Acetylated Derivatives in Intestinal Epithelial Cells. *Arch Toxicol* **2015**, *89* (8), 1337–1346. https://doi.org/10.1007/s00204-014-1309-4.

(26) Yang, Y.; Yu, S.; Tan, Y.; Liu, N.; Wu, A. Individual and Combined Cytotoxic Effects of Co-Occurring Deoxynivalenol Family Mycotoxins on Human Gastric Epithelial Cells. *Toxins (Basel)* **2017**, *9* (3), 96. https://doi.org/10.3390/toxins9030096.

(27) Langie, S. A. S.; Azqueta, A.; Collins, A. R. The Comet Assay: Past, Present, and Future. *Front. Genet.* **2015**, *6*, 266. https://doi.org/10.3389/fgene.2015.00266.

(28) Muruzabal, D.; Collins, A.; Azqueta, A. The Enzyme-Modified Comet Assay: Past, Present and Future. *Food and Chem Toxicol* **2021**, *147*, 111865. https://doi.org/10.1016/j.fct.2020.111865.

(29) Ding, W.; Bishop, M. E.; Lyn-Cook, L. E.; Davis, K. J.; Manjanatha, M. G. In Vivo Alkaline Comet Assay and Enzyme-Modified Alkaline Comet Assay for Measuring DNA Strand Breaks and Oxidative DNA Damage in Rat Liver. *J Vis Exp* **2016**, No. 111. https://doi.org/10.3791/53833.

(30) Azqueta, A.; Shaposhnikov, S.; Collins, A. R. DNA Oxidation: Investigating Its Key Role in Environmental Mutagenesis with the Comet Assay. *Mutat Res* **2009**, *674* (1), 101–108. https://doi.org/10.1016/j.mrgentox.2008.10.013.

(31) Gedik, C. M.; Boyle, S. P.; Wood, S. G.; Vaughan, N. J.; Collins, A. R. Oxidative Stress in Humans: Validation of Biomarkers of DNA Damage. *Carcinogenesis* **2002**, *23* (9), 1441–1446. https://doi.org/10.1093/carcin/23.9.1441.

(32) Kang, C.; Lee, H.; Yoo, Y.-S.; Hah, D.-Y.; Kim, C. H.; Kim, E.; Kim, J. S. Evaluation of Oxidative DNA Damage Using an Alkaline Single Cell Gel Electropho-

resis (SCGE) Comet Assay, and the Protective Effects of N-Acetylcysteine Amide on Zearalenone-Induced Cytotoxicity in Chang Liver Cells. *Toxicol Res* **2013**, *29* (1), 43–52. https://doi.org/10.5487/TR.2013.29.1.043.

(33) Corcuera, L. A.; Arbillaga, L.; Vettorazzi, A.; Azqueta, A.; López de Cerain, A. Ochratoxin A Reduces Aflatoxin B1 Induced DNA Damage Detected by the Comet Assay in Hep G2 Cells. *Food Chem Toxicol* **2011**, *49* (11), 2883–2889. https://doi.org/10.1016/j.fct.2011.07.029.

(34) Hégarat, L. L.; Takakura, N.; Simar, S.; Nesslany, F.; Fessard, V. The in Vivo Genotoxicity Studies on Nivalenol and Deoxynivalenol. *EFSA Supporting Publications* **2014**, 33 pp. https://doi.org/10.2903/sp.efsa.2014.EN-697.

(35) Arbillaga, L.; Azqueta, A.; Ezpeleta, O.; López de Cerain, A. Oxidative DNA Damage Induced by Ochratoxin A in the HK-2 Human Kidney Cell Line: Evidence of the Relationship with Cytotoxicity. *Mutagenesis* **2007**, *22* (1), 35–42. https://doi.org/10.1093/mutage/gel049.

Chapter 6: Appendix

Appendix A: Constant ratios used for Chou Talalay method (1:3.6)

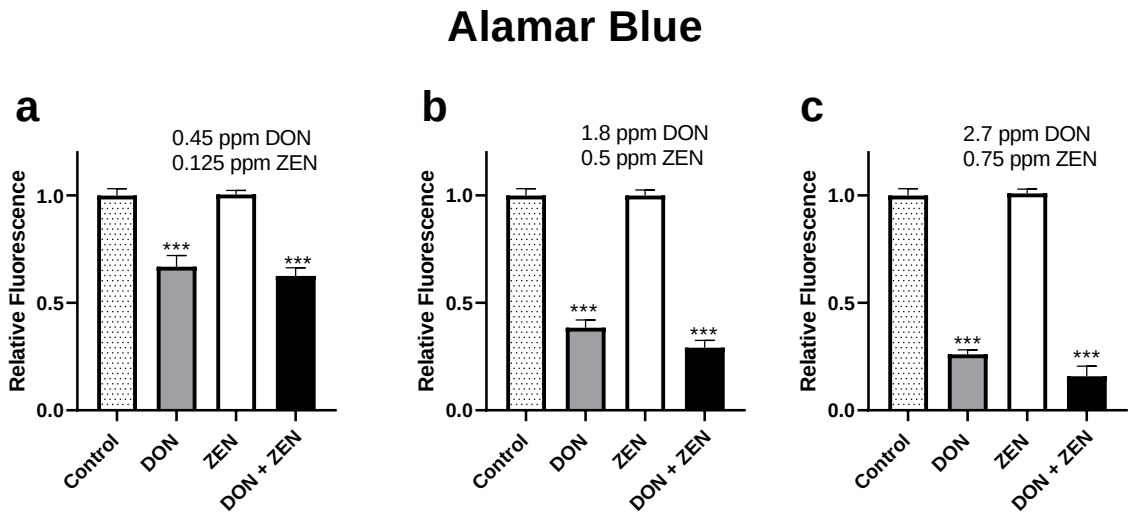

Figure 6.1: *The effect on IPEC-J2 viability following 24 h exposure to DON and ZEN individually and combined analysed by alamar blue. Cells were exposed to ZEN and DON ratio of 1:3.6. Significant differences between control cells (0 ppm DON and ZEN) and cells insulted with DON and/or ZEN are highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

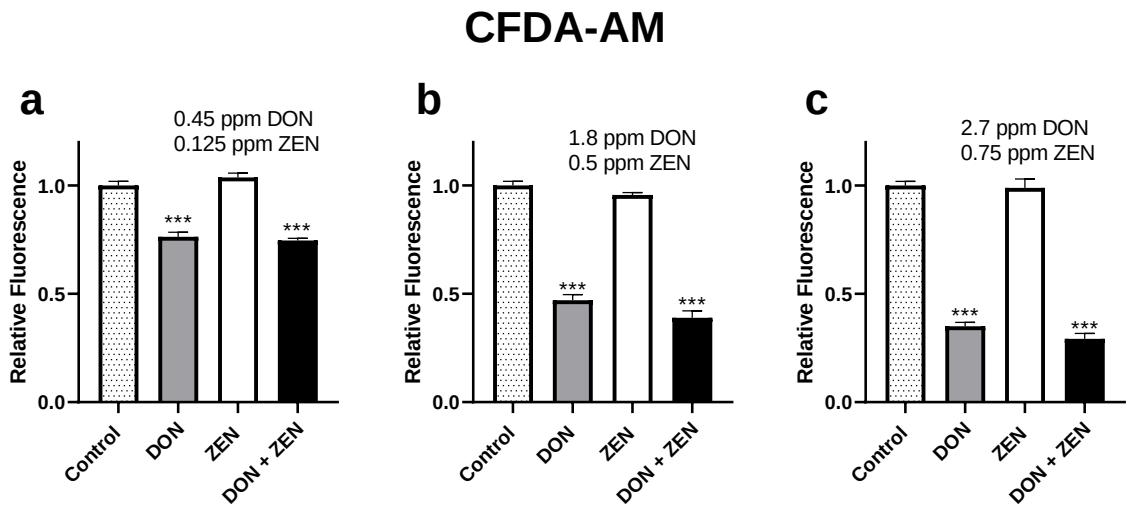

Figure 6.2: *The effect on IPEC-J2 viability following 24 h exposure to DON and ZEN individually and combined analysed by CFDA-AM. Cells were exposed to ZEN and DON ratio of 1:3.6. Significant differences between control cells (0 ppm DON and ZEN) and cells insulted with DON and/or ZEN are highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

Appendix B: Constant ratios used for Chou Talalay method (1:27.78)

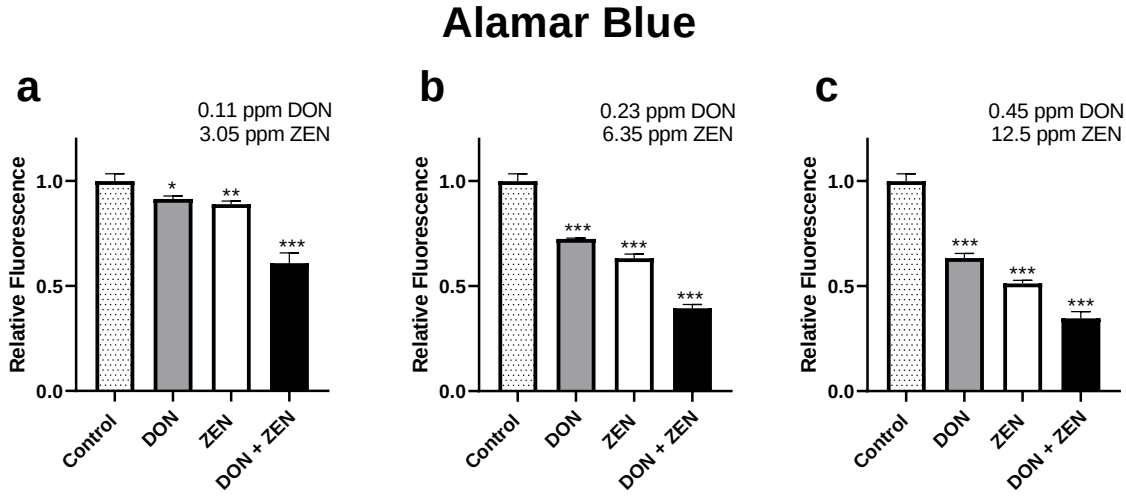

Figure 6.3: *The effect on IPEC-J2 viability following 24 h exposure to DON and ZEN individually and combined analysed by alamar blue. Cells were exposed to ZEN and DON ratio of 1:27.78. Significant differences between control cells (0 ppm DON and ZEN) and cells insulted with DON and/or ZEN are highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

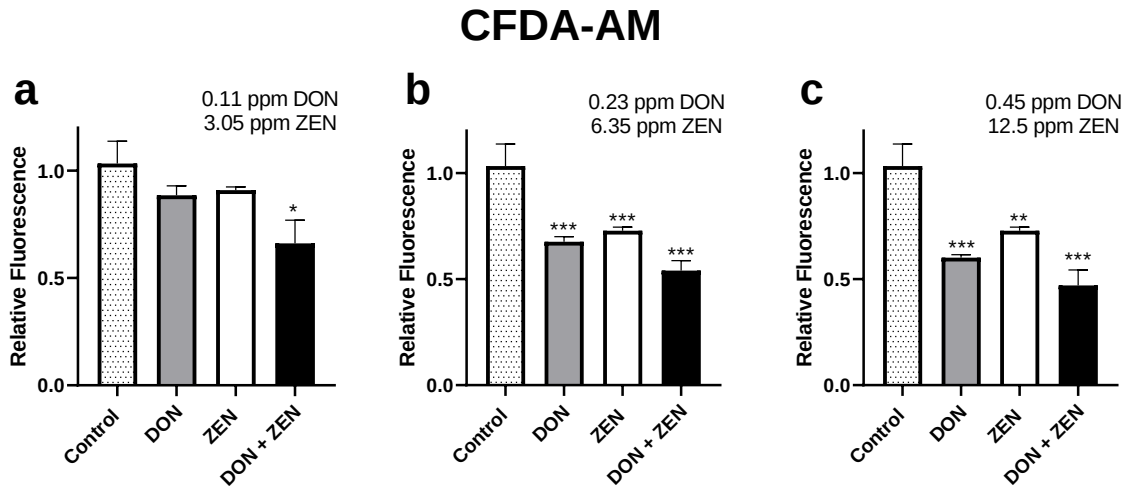

Figure 6.4: *The effect on IPEC-J2 viability following 24 h exposure to DON and ZEN individually and combined analysed by CFDA-AM. Cells were exposed to ZEN and DON ratio of 1:27.78. Significant differences between control cells (0 ppm DON and ZEN) and cells insulted with DON and/or ZEN are highlighted by one way ANOVA (*P<0.05, **P<0.01,***P<0.001).*

Appendix C: Presentations and Publications

C1: Publications

- Thapa A, Horgan KA, White B, Walls D. Deoxynivalenol and Zearalenone - Synergistic or Antagonistic Agri-Food Chain Co-Contaminants? *Toxins*. 2021; 13(8):561. https://doi.org/10.3390/toxins13080561
- Individual and combined effect of Deoxynivalenol and Zearalenone on IPEC-J2 cells (in preparation)

C2: Oral presentations

- **School of Chemical Sciences 3rd Chemistry Day, 4th May 2018**
  Dublin City University
  An investigation of mycotoxin-induced DNA damage in porcine intestinal cells
- **30th Irish Environmental Researchers Colloquium, 20th – 22nd October 2020**
  Dublin City University
  An investigation of mycotoxin-induced DNA damage in porcine intestinal cells

C3: Poster presentations

- **9th Conference of Analytical Science Ireland, 16th – 18th May 2018**
  National University of Ireland, Maynooth
  The use of comet assay in the detection of DNA damage in IPEC-J2 cells.
- **School of Biotechnology 11th Annual Research Day, 25th January 2019**
  Dublin City University
  An investigation of mycotoxin induced DNA damage in IPEC-J2 porcine intestinal cells
- **School of Chemical Sciences 4th Chemistry day 10th May 2019**
  Dublin City University
  An investigation of mycotoxin induced DNA damage in IPEC-J2 porcine intestinal cells
- **71st Irish Universities Chemistry Research Colloquium, 20th – 21st June 2019**
  Technological University Dublin, Royal College of Surgeons in Ireland
  An investigation of mycotoxin induced DNA damage in IPEC-J2 porcine intestinal cells