Co-occurrence of DON and Emerging Mycotoxins in Worldwide Finished Pig Feed and their Combined Toxicity in Intestinal Cells and Liver Slices

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Résumé Français

Les mycotoxines sont des métabolites secondaires toxiques produits par certains champignons filamenteux. Selon leur toxicité et leur occurrence, certaines d'entre elles dont le déoxynivalénol (DON), l'une des toxines les plus répandues dans l'alimentation humaine et animale, sont été réglementées au sein de l'Union Européenne. D'autres métabolites secondaires découverts récemment ou encore peu étudiés sont appelées mycotoxines émergentes et ne sont ni détectés en routine ni réglementés. Les denrées alimentaires destinées à l'homme et à l'animal peuvent être naturellement contaminées par plusieurs mycotoxines et le risque lié à une exposition à des mélanges de mycotoxines est préoccupant.

Parmi les animaux d'élevage, le porc est une espèce très sensible aux mycotoxines. De par son alimentation riche en céréales, il peut être exposé à de fortes concentrations de ces contaminants. 524 échantillons d'aliments complets pour porcs prélevés dans le monde entier ont été analysés par une technique de chromatographie en phase liquide couplée à la spectrométrie de masse en tandem (LC-MS/MS) pour plus de 800 métabolites. 88 % des échantillons étaient co-contaminés avec du DON et d'autres mycotoxines réglementées et émergentes.

La toxicité du DON et des 10 mycotoxines émergentes les plus répandues a été évaluée en mesurant la viabilité de cellules épithéliales intestinales porcines (IPEC-1) après 48 h d'exposition. Trois mycotoxines émergentes (brevianamide F, cyclo-(L-Pro-L-Tyr) et tryptophol) n'ont pas eu d'impact sur la viabilité cellulaire. Les autres toxines ont été classées dans l'ordre de toxicité suivant: apicidine> enniatine A1> DON> beauvéricine> enniatine B> enniatine B1> émodine> aurofusarine.

La toxicité combinée du DON et des 10 mycotoxines émergentes a été évaluée en fonction de leurs concentrations réelles dans les aliments analysés. Nous avons observé que malgré la très forte fréquence des co-contaminations, la corrélation entre les concentrations de DON et des mycotoxines émergentes étudiées était faible. Nous avons donc évalué les effets toxiques de trois mélanges correspondant à des situations auxquelles les animaux peuvent être exposés. Le ratio n°1 a été calculé en utilisant la concentration P25 (1er quartile) de la mycotoxine émergente et la concentration P75 (3ème quartile) du DON. Le ratio n°3 correspondait au scénario inverse du ratio n°1. Le ratio n°2 a été calculé en utilisant la concentration médiane (2ème quartile) du DON et de chaque mycotoxine émergente.

Pour la plupart des mélanges, la cytotoxicité combinée était similaire à celle du DON seul. Pour ce qui concerne la santé des animaux, ces résultats ont montré que lorsque ces mycotoxines émergentes sont présentes avec le DON, elles n'exacerbent pas la toxicité du DON.

Outre l'intestin, le foie est le principal site de détoxification des xénobiotiques, y compris des mycotoxines, et représente un organe cible des contaminants alimentaires. Nous avons donc mis au point un nouvel outil, les Precision Cut Liver Slices (PCLS), des explants de foie d'épaisseur définie. Elles ont été utilisées pour évaluer la toxicité du DON (3 et 10 µM) à différents temps d'incubation (0 à 20 h), en étudiant l'expression génique, le contenu en ATP et en protéines totales.

Le milieu d'incubation a permis d'évaluer la qualité des PCLS en mesurant les marqueurs de dommage hépatique (phosphatase alcaline, lactate déshydrogénase, alanine aminotransférase, aspartate aminotransférase et protéines totales). Nous avons montré que ces marqueurs n'étaient impactés ni par le temps d'incubation, ni par le traitement. Les PCLS traitées avec 10 µM de DON pendant 4 h ou plus, montrent une altération de l'expression de certains gènes.

Ces expériences préliminaires ont montré que les PCLS représentent un modèle prometteur pour évaluer la toxicité hépatique des mycotoxines ou d'autres contaminants alimentaires.

Mots-clés: Mycotoxines émergentes, DON, Toxicité, Aliments pour porcs, IPEC-1, Slice de foie, Expression de gènes

Summary

Mycotoxins are toxic secondary metabolites, produced by several filamentous fungi. Depending on their toxicity and occurrence, some of them, including deoxynivalenol (DON), one of the most common toxin in food and feed, have been regulated in the European Union. Other secondary metabolites, which neither routinely determined nor regulated, are called emerging mycotoxins because they have been recently discovered or poorly investigated. Food and feed can be naturally contaminated by several mycotoxins and concern about the hazard of exposure to mycotoxin mixtures is increasing.

Among farm animals, pig is one of the most sensitive farm animal to mycotoxins and it can be exposed, through its rich cereal diet, to high concentrations of mycotoxins. In total, 524 finished pig feeds samples from worldwide were analyzed for more than 800 metabolites using, LC-MS/MS (liquid chromatography tandem-mass spectrometry) method. Eighty-eight percent of the samples were co-contaminated with DON and other regulated and emerging mycotoxins.

The toxicity of DON and the 10 most common emerging mycotoxins was analyzed on the viability of porcine intestinal epithelial cells (IPEC-1) over a 48 h period. Among the emerging mycotoxins, 3 of them (brevianamide F, cyclo-(L-Pro-L-Tyr), and tryptophol) did not alter cells viability. The other mycotoxins were ranked in the following order of toxicity: apicidin> enniatin A1> DON> beauvericin> enniatin B> enniatin B1> emodin> aurofusarin.

The combined toxicity of DON and the 10 emerging mycotoxins was assessed based on their actual ratios found in pig feed. We observed that, despite the very high frequency of co-contamination, there was a poor correlation between the concentrations of DON and emerging mycotoxins. Thus, we assessed the toxic effects of three mixtures corresponding to situations to which animals may be exposed. Ratio #1 was calculated using the P25 (1^{st} quartile) concentration of the emerging mycotoxin and P75 (3^{rd} quartile) concentration of DON. Ratio #2 was calculated using the median (2^{nd} quartile) concentration of DON and each emerging mycotoxin. Ratio #3 was the reverse scenario of ratio #1.

Cytotoxicity analyses showed that, in most of the mixtures, the combined toxicity was similar to the one of DON alone. These results demonstrated that, when these emerging mycotoxins are present with DON, in terms of pig health, it does not exacerbate the problem of the toxicity of DON.

In addition to intestine, liver is the main site of detoxification for xenobiotics, including mycotoxins and represents a target organ for food contaminants. Hence, we developed a new tool the Precision Cut Liver Slices (PCLs) an *ex vivo* explants of liver with a well-defined thickness. This tool was used to assess the toxicity of DON (3 and 10 µM) at different incubation times (0 to 20 h), by studying gene expression, ATP and total protein contents.

The incubation medium was used to assess the quality of PCLS by measuring liver damage markers (alkaline phosphatase, lactate dehydrogenase, alanine transaminase, aspartate transaminase and total proteins). We showed that these markers were not affected by either incubation time or treatment.

PCLS treated with 10 µM DON for 4 h or more, showed an alteration in the expression of certain genes. These preliminary experiments demonstrated that PCLS represent a promising model for assessing the hepatic toxicity of mycotoxins or other food contaminants.

Keywords: Emerging Mycotoxins, DON, Toxicity, Pig Feed, IPEC-1, Liver Slices, Gene Expression

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Abbreviations List

ABCG-8: ATP Binding Cassette
AFB1:Aflatoxin B1
AFN: Aurofusarin
AFs: Total Aflatoxins
AGS: Human Gastric Adenocarcinoma Cell Line
ALP: Alkaline Phosphatase
ALT: Alanine Transaminase
ANOVA: One-Way Analysis of Variance
API: Apicidin
AST: Aspartate Transaminase
ATP: Adenosine Tri Phosphate
B2-Microglobulin: Beta-2-Microglobulin
BEA: Beauvericin
BEAS-2B: Human Normal Lung Epithelial Cells
BRV-F: Brevianamide-F
CAST: Council For Agricultural Science and Technology
CAT: Catalase
CCL20: C-C Motif Chemokine Ligand
CCS: Copper Chaperone
CLDN-3: Claudin-3
CTG: Cell-Titer-Glo®
CONTAM: Panel On Contaminants in the Food Chain
Cyclo: Cyclo-(Pro-L-Tyr)
CYP: Cytochrome P450 Family
DMEM: Dulbecco's Modified Eagle Medium
DNA: Deoxyribonucleic Acid
DOM-1: Deepoxy-deoxynivalenol
DON: Deoxynivalenol
DUOX: Dual Oxidase
e.g: Example
EFSA: European Food Safety Authority
EMO: Emodin
ENN: Enniatins
EU: European Uniton
FAO: Food And Agriculture Organization
FDA: Food And Drug Administration
FOS: Fos Proto-Oncogene
FUM: Fumonisins
g: Gravitational Acceleration
GAP: Good Agricultural Practices
GLC-82: Lung Carcinoma Cell Line
GPx: Glutathione Peroxidase
h : Hour
HBL-100: Human Breast Cancer Cell Line
HCEC-1CT: Non-Tumorigenic Colon Cells
HEK: Human Embryonic Kidney Cells
HeLa: Human Cervix Cancer Cell Line
HepG2: Human Liver Cell Line
HKG: Housekeeping Gene
HT29: Adenocarcinoma Cell Line
HUVEC: Human Normal Endothelial Cells
IC_{50}: Half Maximal Inhibitory Concentration
PCLS: Precision Cut Liver Slices

LC-MS/MS: Liquid Chromatography Mass Spectrometry/Mass Spectrometry
IL: Interleukin
IPEC-1: Intestinal Porcine Epithelial Cells
IPEC-J2: Intestinal Porcine Epithelial Cells from Jejunum
JRC: Joint Research Center
JUN: Jun Proto-Oncogene
Kg: kilogram
LD50: Lethal Dose of 50%
LDH: Lactate Dehydrogenase
LDLR: Low Density Lipoprotein Receptor
LOAEL: Lowest Observed Adverse Effect Level
LXR: Liver X Receptor
MCF-7: Human Breast Cancer Cell
mg: milligram
MAPK: Mitogen-Activated Protein Kinases
mn: Minute
mL: milliliter
MRC-5: Human Fibroblast-Like Cell Line
mRNA: Messenger RNA
MT: Metallothionein Member
MTL: Maximum Tolerable Level
N87: Human Gastric Cell Line
NFKB: Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B cells
nm: Nanometer
NOAEL: No Observable Adverse Effect Level
NR1H3: Nuclear Receptor Group H Member 3
NTC: Non-Template Control
OCLN: Occludin
PCTS: Precision-cut Tissue Slices
PDI: Probable Daily Intake
qRT-PCR: Quantitative Real Time-Polymerize Chain Reaction
RASFF: Rapid Alert System for Food and Feed
RIN: RNA Integrity Number
RNA: Ribonucleic Acid
RPL32: Ribosomal Protein L32
RPM: Round Per Minute
SCD1: Stearoyl-CoA Desaturase
SCRAB2: Scavenger Receptor Class B
SOD: Superoxide Dismutase
SP: Species
TBP: TATA-Box Binding Protein
TDI: Tolerable Daily Intake
TEER: Transepithelial Electrical Resistance
TNF-A: Tumor Necrosis Factor Alpha:
TRPT: Tryptophol
UK: United Kingdom
USA: United States Of America
WME: Williams' Medium E
ZEN: Zearalenone
$: Symbol of Dollar
'n': Total Number of Samples
£: Symbol of Pound
€: Symbol of Euro
°C: Symbol of Degree Celsius
µg: Microgram
µL: Microliter
µm: Micrometer
µM: Micromole
Ø: Symbol of diameter

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1. Introduction

1.1. Generalities on mycotoxins

In today's changing world, safety and security remains basic human needs. Ensuring food safety has been a major focus of national and international actions over the last years. Nowadays, international trade in agricultural commodities such as wheat, rice, barley, corn, sorghum, soybeans, groundnuts and oilseeds reaches hundreds of millions of tons each year (FAO 2019). In 2014, worldwide feed production was 964 million tons and among them 153 million tons were produced just in Europe (Kovalsky *et al.* 2016). As the occurrence of mycotoxins is widespread throughout the world, the global trade of agricultural commodities (e.g., animal feed) requires to monitor fungal toxins (Kovalsky *et al.* 2016). The worldwide contamination of food and feed with mycotoxins is a significant problem (Hussein and Brasel 2001). Recent surveys on the occurrence of mycotoxins have shown that 60 – 88% of the world's cereal grains are contaminated with mycotoxins (Gruber-Dorninger *et al.* 2019; Eskola *et al.* 2019). Mycotoxins can resist to high temperature and to chemicals like acids, both during storage/milling and cooking process and they are stable during food/feed processing like brewing, melting, hot drying or ensiling (Rodríguez-carrasco *et al.* 2016). Their presence in food and feed products is an important threat to human and animal health (Heshmati *et al.* 2017).

Origin of mycotoxins

Mycotoxins are toxic secondary metabolites, produced by toxigenic moulds in the *Aspergillus*, *Alternaria*, *Claviceps*, *Fusarium*, *Penicillium* and *Stachybotrys* genera occurring in food and feed commodities both pre- and post-harvest (Milićević *et al.* 2010) under appropriate environmental conditions (Jestoi 2008). Mycotoxins represent a potential health risk for humans and/or livestock. (García & Jarque, 2014). The exact number of mycotoxins is not known, but the number of potential toxic fungal metabolites has been estimated to be in the thousands (Council for Agricultural Science and Technology 2003). Traditionally, toxigenic fungi contaminating agricultural grains have been conventionally divided into two groups: those invading seed crops have been described as "field" fungi (e.g., *Cladosporium, Fusarium, Alternaria spp*.), which reputedly gain access to seeds during plant development, and those proliferating during storage, "storage" fungi, (e.g., *Aspergillus; Penicillium spp*) (Ismaiel and Papenbrock 2015; Alshannaq and Yu 2017). Among the field occurring mycotoxins, *Fusarium*

mycotoxins are the most frequently identified in grain and animal feed and it is important for farmers to manage cereals contamination by *Fusarium* species (Steffen and Graham 2017).

Occurrence and contamination

Over the last years, mycotoxins have been regularly classified in the top ten and top one causes (European Union (EU) members and non-members countries, respectively) for the Rapid Alert System for Food and Feed (RASFF) notifications. RASFF is an European tool that ensures the inter-countries flow of information when risks to public health are detected in the food chain (RASFF 2018). The evidence suggests that mycotoxins are occurring increasingly in agricultural products including grains destined for human and animal consumption (Steffen and Graham 2017). Food can be contaminated with mycotoxins at various stages of the food chain, in field, during storage, milling or at later points (Bennett and Klich 2003). In fact, there is a notable length of time between the harvest of agricultural commodity at the exporting country and its arrival at the distribution center of the importing country. Furthermore, storage conditions at the farm level as well as during transport under adverse weather conditions may not always be satisfactory. Therefore, there is considerable opportunity for mycotoxin contamination of agricultural commodities to take place throughout the food system (FAO and Miller 1991). On the other hands, feeding animal with contaminated feeds can lead to contaminated animal products (eggs, meat, milk…). For example, aflatoxin B1 in cattle feed can be metabolized by cows into aflatoxin M1, which is then secreted in milk (Younis *et al.* 2016). Furthermore, ochratoxin A in pig feed can accumulate in porcine tissues (Rutqvist 1978).

Human exposure to mycotoxins also results from the consumption of several sources of food such as plant-derived foods, animal products (meat, eggs and milk) and/ or the exposure to contaminated air and dust (Council for Agricultural Science and Technology 2003).

Regulations

Regulations on mycotoxins have been established in many countries in order to protect the consumers from the harmful effects of mycotoxins. Two factors are mainly taken into account in the decision-making process of setting limits for mycotoxins: (i) the toxicity and (ii) the exposure (van Egmond *et al.* 2007). International survey on mycotoxins legislation in foodstuffs and feedstuffs have shown that approximately 100 countries (covering approximately 85% of the world's inhabitants) had specific regulations or detailed guidelines for some mycotoxins (FAO 2004), whereas no data were available for about 50 countries, many of them were in

Africa (van Egmond 2002). In EU, mycotoxins regulation concern, ochratoxin A, patulin, deoxynivalenol (DON), zearalenone (ZEN), fumonisins (FUM) B1 & B2, T-2 & HT-2 toxin, aflatoxin B1 (AFB1), total aflatoxins (AFs) and patulin (Lerda 2011). In the following (Table 1), the maximum level of each above mentioned mycotoxins for pig feed is stated based on the EU recommendations.

Table 1: EU recommendation of mycotoxins in pig feed

Mycotoxins	Feedstuff	Guidance value in mg/kg (ppm) relative to pig feedingstuff with a moisture content of 12 %
DON	Complementary and complete feedingstuffs for pigs	0.9
ZEN	Complementary and complete feedingstuffs for piglets and gilts (young sows)	0.1
	Sows and fattening pigs	0.25
The sum of FUMs (B1 + B2)	Complementary and complete feedingstuffs for pig	5
The sum of T2 & HT2	Compound feed for pig	0.25
Ochratoxin A	Complementary and complete feedingstuffs for pigs	0.05

(European-Commission 2016)

Economical losses

The economic consequences of mycotoxins contamination are important. Commodities over the regulation should be destroyed. In addition, contaminated feed can induce losses in animal production. Due to the insufficient information, the economic costs of mycotoxins are impossible to determine accurately. However, the U.S. Food and Drug Administration (FDA)

has utilized a computer model to estimate the losses due to selected mycotoxins such as AFs, FUM and DON. Only for United States, the mean economic annual costs of crop losses from the above mentioned mycotoxins are estimated to be $932 million (Council for Agricultural Science and Technology 2003), whereas in UK they represent approximately £200/hectare and €450/hectare in Germany (Steffen and Graham 2017).

Toxicity

Acute and chronic dietary exposure to mycotoxins can induce a variety of adverse health effects in humans and animals, making these chemically diverse substances highly relevant agricultural contaminants (Eskola *et al.* 2019). Mycotoxins can exhibit acute toxicity as well as carcinogenic, mutagenic, teratogenic, immunotoxic, hepatotoxic, nephrotoxic, neurotoxic or estrogenic effects in human and animals (van Egmond *et al.* 2007). The European Food Safety Authority (EFSA), Panel on Contaminants in the Food Chain (CONTAM) calculated the acute and chronic exposure across the 22 European countries, using the highest reliable percentile of concentrations and individual consumption data for different age groups. Toddlers were in general the age group with the highest dietary chronic and acute exposure to the mycotoxins (EFSA 2014, 2018).

Unlike bacterial toxins, fungal toxins are not proteins and are usually barely detectable by the immune system of humans and animals. Most illnesses caused by mycotoxins are not reported to the doctors, whereas low levels can be dangerous to humans health (Steffen and Graham 2017). On the other hands, diagnosis of mycotoxicoses in human and animals is difficult as they may be similar to diseases with other causations. This is even more difficult in cases where more than one mycotoxin is involved (Alassane-Kpembi *et al.* 2017b).

Mycotoxins are more thoroughly studied in animals. Studies on animals have demonstrated that among farm animals, pig is one of the most sensitive species to the deleterious effects of mycotoxins. It can be exposed to high concentration of mycotoxins due to its rich cereal diets (Pinton *et al.* 2010). Previous studies on pig species have shown that, mycotoxins affect intestinal function. The gastrointestinal tract is the first physiological barrier against food contaminants, as well as the first target for these toxicants (Pinton and Oswald 2014). Indeed, after oral intake, the gastrointestinal tract is the first possible site of interaction (Fraeyman *et al.* 2018). In pigs, mycotoxins can decrease the feed consumption, reduce weight gain and alter reproduction and immunity (Korosteleva *et al.* 2007). Ruminants are less susceptible to

mycotoxins than monogastrics, because of the rumen microbiota. Ruminant's digestive system and especially resident bacteria and protozoa, are able to detoxify mycotoxins. Nevertheless, there is some evidence that ruminants can be poisoned by mycotoxins, causing lower animal production or even death of animals. Recent *in vitro* research showed that at conditions of rumen acidosis and lower microbial activity, a reduced detoxification in the rumen can take place (Debevere *et al.* 2020). Recent insights have generated an entirely new perspective where a bi-directional relationship exists between mycotoxins and gut microbiota, thus suggesting that gut microbiota might be involved in the development of mycotoxicosis (Jouany *et al.* 2009; Liew and Mohd-Redzwan 2018). Moreover, most of the toxicant can be detoxified in the liver, which is the main site of metabolism and detoxification of toxins and also a target organ of mycotoxins (Zain 2011; Pongratz and Bergander 2011). Nevertheless, the contamination of animal feed can have a major impact on dairy farming. Mycotoxins can be found in dairy products from two origins, indirect contamination, which results when dairy cows ingest feed that contains mycotoxins which pass into the milk such as aflatoxin M1 and direct contamination, which results from accidental growth of moulds secreting aflatoxins (Younis *et al.* 2016).

Mitigation strategies

Mycotoxins are still considered unavoidable contaminants in foods and feeds, because agronomic technology has not yet advanced to the stage at which pre-harvest contamination of susceptible crops by fungi can be eliminated (Wood 1992). In order to avoid harmful effects of contamination of food and feed caused by mycotoxins, three principles could be implemented; (i) prevention of contamination; (ii) decontamination of mycotoxin containing food and feed; and (iii) inhibition of absorption of mycotoxin from the digestive tract (Karlovsky 1999). There are three common strategies to mitigate toxicity of mycotoxins (Figure 1). One of the methodologies employed for mycotoxins detoxification is the physical strategy. Even though mycotoxins are stable compounds, some food processes including sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, nixtamalization and extrusion may affect their chemical structure (Bretz *et al.* 2006). Another common mitigation strategy is biotransformation. It consists in the use of enzymes that have been found to be effective in transforming mycotoxins into less toxic metabolites or completely inactivating them (Varga *et al.* 2010). For example, a mixed culture of two soil bacteria *Pseudomonas sp* and *Lysobacter sp* through formation of 3-keto-DON an enzymatic epimerization was capable of transforming DON into the non-toxic 3-epi-DON (Zhai *et al.* 2019). Likewise, a de-

epoxidase first isolated from *Eubacterium* isolated from bovine rumen fluid, is capable of converting DON into the nontoxic compound DOM-1 (Pierron *et al.* 2016c; Loi *et al.* 2017). Patulin was converted by *Saccharomyces cerevisiae* into two isomers of ascladiol, E-ascladiol and Z-ascladiol which are nontoxic to human cell lines (Tannous *et al.* 2017; Wang *et al.* 2019). Detoxification of fumonisin B1 was carried out in two steps. Initial step was de-esterification reaction followed by deamination of the resulting hydrolyzed fumonisin B1 (Heinl *et al.* 2010; Grenier *et al.* 2012). Furthermore, isothiocyanates are natural reactive compounds that were found to reduce the toxicity of some mycotoxins (Varga *et al.* 2010). In addition, yeast cell wall, probiotics, prebiotics, fibers and protein ingredients have been used to reduce mycotoxins bioaccessibility in the gastrointestinal tract (Mallebrera *et al.* 2013) (Figure 1).

Control should begin as early as possible, starting at the farm level with primary agricultural production. Good agricultural practices (GAP) is the first line against contamination of cereals with mycotoxins (Awad *et al.* 2010). Furthermore, preventive strategies in storage level such as; storage at low moisture levels and prevention of grain damage during processing and prevention of the formation of mycotoxins in feed help to reduce mycotoxins content (Dawson, 2001).

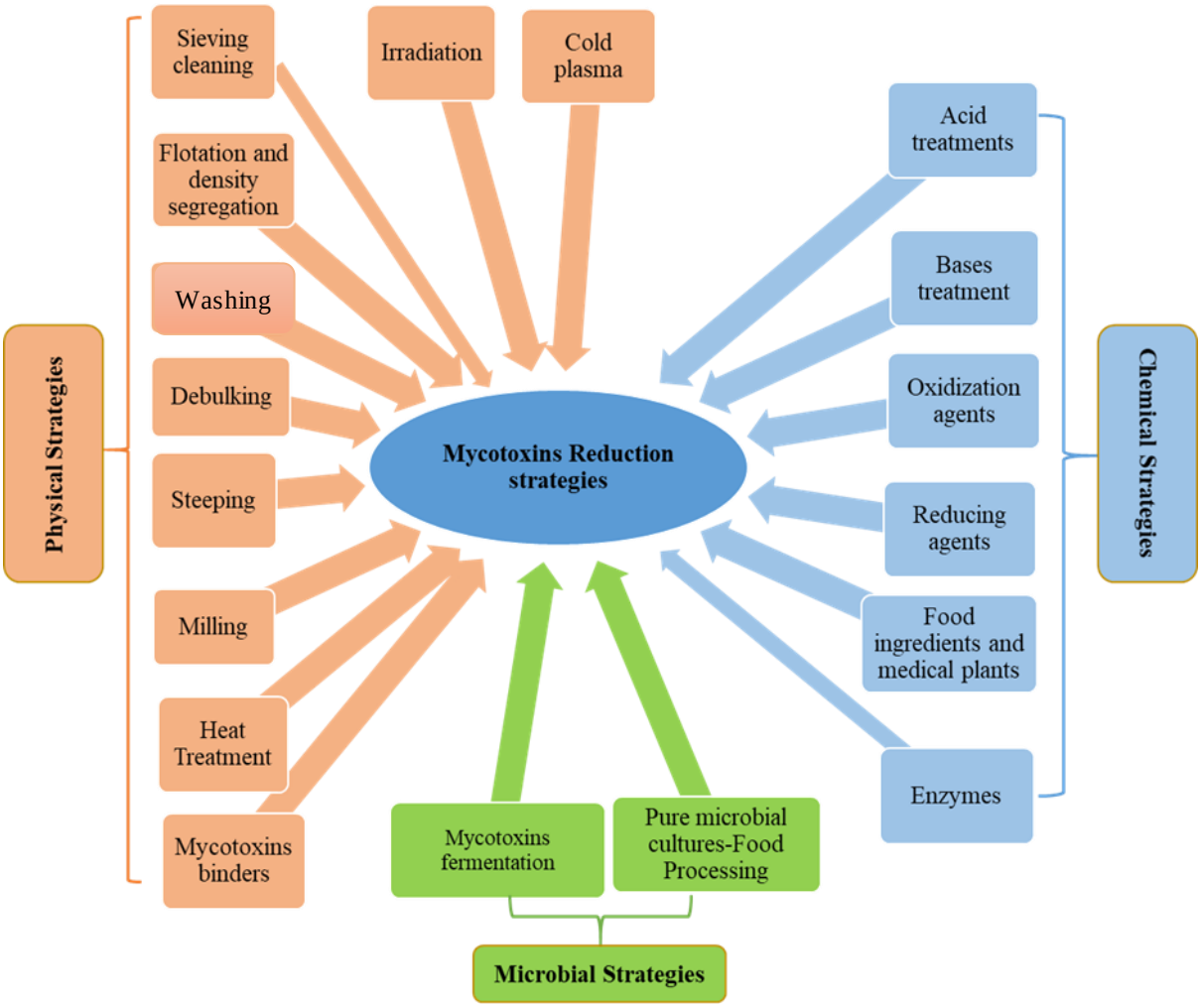


Figure 1: General schematization of physical, chemical and microbial strategies used for mycotoxin reduction in food and feed chains (Luz *et al.* 2017).

Co-contamination

Another issue that pays attention of researchers is the co-contamination of food and feed by several mycotoxins. Food commodities are commonly contaminated with various fungal species at a time. In addition, most fungi are able to simultaneously produce number of mycotoxins. Therefore feed commodities can be contaminated by several mycotoxins, and completed feed is made from various commodities (Streit *et al.* 2012). The simultaneous presence of mycotoxins in products intended for human consumption is of high importance, because mycotoxins could interact with each other, potentially enhancing their toxic effects (Ruiz *et al.* 2011). Maize is an example where several mycotoxins have been reported to occur simultaneously (Gonçalves and Cubero-leon 2017).

There are reports of a combination of many mycotoxins, such as DON, AF (B1, B2 and M1), FUM (A, B1, B2 and B3), ZEN and other fungal secondary metabolites in maize seeds and

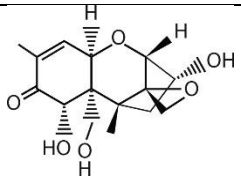
grains, as well as in animal feed formulated (Streit *et al.* 2013; Anjorin *et al.* 2016; Abdallah *et al.* 2017). DON co-occurs with other regulated mycotoxins as well as with emerging mycotoxins (Borutova *et al.* 2012; FVaclavikova *et al.* 2013; Streit *et al.* 2013). Multi-mycotoxin studies have reported that 75%–100% animal feed was co-contaminated by DON and other mycotoxins (Streit *et al.* 2012; Kovalsky *et al.* 2016; Novak *et al.* 2019). In food and feed ingredients sourced in Southern Europe, DON was found in 94% of the samples at maximum 365 µg/kg concentration (total number of samples 'n' = 416) (Griessler *et al.* 2010).

1.2. Deoxynivalenol (DON)

Origin

Deoxynivalenol (DON) is a regulated mycotoxin belonging to the group of type B trichothecenes (Knutsen *et al.* 2017), mainly produced by the fungi of the *Fusarium* genus, (Lewczuk *et al.* 2016). DON is one of the most common contaminants of wheat, corn, and barley worldwide. Second, DON is a very stable compound, during both storage and the processing/cooking of food, and does not degrade at high temperatures. On the other hands, DON is chemically also stable and to some extent resistant to food and feed processing (Table 2) (Wood 1992; Kabak 2009). DON occurs in cereal grains alone or in combination with its most relevant acetylated derivatives, such as 3-acetyl-DON, 15-acetyl-DON and DON-3-glucoside (10–20% of the DON-levels) and either with other fungal secondary metabolites so called emerging mycotoxins (Eskola *et al.* 2019; Schatzmayr and Streit 2013). It is produced in fields prior to harvest and its occurrence in food cannot be completely avoided due to the major impact of weather conditions as well as the high chemical and thermal resistance, both during storage/milling and processing/cooking of food (Lewczuk *et al.* 2016). DON is an undesirable substance in animal feed; in particular pigs were identified as the most sensitive animal species (EFSA 2013). In addition, DON can affect all animal species, with the following rank order of sensitivity: pigs > mice > rats > poultry ≈ ruminants (Rotter 1996).

Table 2: Characteristics of DON

Property	**Information**
Name	Deoxynivalenol (DON) vomitoxin
Chemical structure	
IUPAC name	12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8on
Molecular formula	$H_{15}O_{20}O_6$
Molar mass	296.32 g/mol
CAS number	51481-10-8
Physical state	Colourless fine needles
Soluble in:	Polar organic solvents (*e.g.*, aqueous methanol, ethanol, chloroform, acetonitrile, and ethyl acetate) and water

Occurrence

DON is one of the most widely distributed trichothecene (Sprando *et al.* 2005). It contaminates grains and cereal-based food and feed such as wheat, barley, oats, rye and maize, and less often in rice, sorghum and triticale (Kabak 2009). A worldwide ten years survey on more than 70,000 different commodities reported that DON was found in finished feed (70%), maize (67%), maize dried distillers grains with soluble (DDGS) (83%), maize silage (62%), soybean grains (29%), soybean meal (31%), wheat (65%) barley (61%), and rice (27%) samples with maximum concentrations of 32,890 µg/kg, 51,370 µg/kg, 84,860 µg/kg, 34,860 µg/kg, 5,500 µg/kg, 5,600 µg/kg, 49,300 µg/kg, 35,000 µg/kg and 3,860 µg/kg respectively (Gruber-Dorninger *et al.* 2019). As well, in more than 25,000 samples collected from 28 European countries between 2007 and 2014, DON was found in food (52%, n= 21,916), feed (47%, n=4,000) and in unprocessed grains of undefined end-use (45%, n= 15,943) samples, with mean concentrations of 95.5 µg/kg, 1,815 µg/kg and 357 µg/kg respectively (Knutsen *et al.* 2017). A global survey performed in 2004 covering 19,000 of food and feed samples showed that DON was found in Central Europe (56%), North Asia (78%) and North America (68%) in mean concentration of 1,009 µg/kg, 1,060 µg/kg and 1,418 µg/kg respectively (Schatzmayr and Streit 2013). DON was found simultaneously with 16-35 other metabolites in 79% of 1113 finished feed, maize and maize silage samples collected between 2012 to 2015 from worldwide (Kovalsky *et al.* 2016).

Toxicity

DON is one of the least lethal trichothecenes, but at high dose, acute exposure to DON elicits abdominal distress, increased salivation, malaise, diarrhea, necrosis of bone marrow, lymphoid tissue and both kidney and heart lesion (Pestka 2010; Ruiz *et al.* 2011). Based on adverse gastrointestinal effects of DON on Chinese people, the CONTAM identified that vomiting occurred within 30 minutes after an eating occasion and a non-observed adverse effect level (NOAEL) for acute effect was calculated, 26 µg DON/kg b.w for a single eating occasion. The highest acute dietary exposure to DON was identified in the young children and infants. Human outbreaks from acute exposure to DON have been repeatedly reported in Asia. Indeed, vomiting is a critical acute effect of DON in humans (Knutsen *et al.* 2017). The evidence of adverse health effects in humans due to chronic exposure to DON is lacking, but the CONTAM panel has identified reduced body weight gain in experimental animals as the critical chronic effect for human risk assessment.

Furthermore, the CONTAM Panel calculated a lowest observed adverse effect level (LOAEL) and NOAEL of DON for pig. It is noted that naturally contaminated feed had a stronger effect on the feed intake and weight gain than pure DON, and this lead to the assumption that other toxic compound are present (EFSA 2004). Overall, NOAELs for reduced feed intake and reduced body weight gain (0.7–5.0 mg DON/kg feed) was observed overlapping with even a wider range of LOAELs (0.35–13 mg DON/kg feed) (Knutsen *et al.* 2017). Impaired immune response, reproductive, neurological, hematological, and molecular effects are also reported from *in vivo* or *in vitro* studies (Sobrova *et al.* 2010). Previous findings have shown that, if the concentration of DON increases over than 12.5 mg/kg feed, it causes feed refusal and vomiting in animals (Fink-Gremmels 1999). However, the oral bioavailability and absorption of DON in animals depends on several parameters including species, age and gender. For example after oral ingestion, within 15–30 min, 7 % of DON was detected in ruminants (sheep and cow), 25 % in rat's, and up to 89 % in pig's blood (Goyarts and Dänicke 2006; Payros *et al.* 2016). A major part of the ingested DON in pigs was absorbed quickly from the proximal segments of the small intestine (Eriksen and Pettersson 2004; Dänicke *et al.* 2004). In mice, DON was rapidly distributed to the tissues, e.g. liver, kidney, spleen and heart, following oral exposure to a 25 mg/kg b.w dose, reaching the maximum concentrations at about the same time as in plasma. Concentration of DON in liver was (12.1 - 19.6 µg/g,), in kidney (7.6 - 9.0 µg/g,) and in spleen was observed (7.9 µg/g) respectively (Pestka *et al.* 2008). Since nutrients are absorbed in the small intestine, the gastrointestinal tract is the first barrier against food contaminants and it may be exposed to high concentration of mycotoxins (Pinton and Oswald 2014; Alassane-Kpembi *et al.* 2015; Fraeyman *et al.* 2018).

Effects of DON were assessed on human intestinal epithelium using *in vitro* approach and on porcine intestinal epithelium using *in vitro* and *in vivo* approaches. DON decreased the transepithelial electrical resistance (TEER) in a time and dose-dependent manner in Caco-2 and IPEC-1 cells *in vitro*. Exposure of Caco-2 to DON 5 or 20 µM decreased only 7% of TEER, but the decrease reached 58% after treatment with DON 100 µM. In longer treatment, during 14 days of exposure TEER was decreased by 19, 29, 77 and 79% for cells treated with DON 5, 10, 50 and 100 µM respectively. DON also decreased TEER in a time and dose dependent manner in IPEC-1 *in vitro.* In short time of exposure (one day), the TEER was decreased only by 25% and 60% due to DON 10 µM and 50 µM, whereas following a longer incubation it was significantly decreased by 58, 69, 75 and 97% for cells treated with DON 5, 10, 20 and 50 µM respectively. Furthermore, a significant decrease in cell viability was observed when cells were

exposed to highest concentration of DON (200 µM in IPEC-1 cells and 500 µM in Caco-2 cells). DON decreased by 40% the amount of the tight junction protein claudin-4 (CLDN-4), when pigs received DON contaminated feed (2.85 mg/kg DON/kg feed) during 5 weeks compared to the control diet. Overall, the porcine intestinal epithelial cells IPEC-1 showed more sensitivity than human cells Caco-2 (Pinton *et al.* 2009).

Mode and mechanism of action

Concerning the mode of action, DON binds to ribosomes, leading to a ribotoxic stress and the inhibition of protein synthesis and subsequently also RNA and DNA synthesis (Sobrova *et al.* 2010). This ribotoxic stress also activates different mitogen-activated protein kinases (MAPKs). Activation of MAPKs explains several effects of DON, such as apoptosis or survival of cells, inflammatory effect and oxidative stress. Two major mediators of DON-induced anorexia/emesis have been described, pro-inflammatory cytokines and secretion of satiety hormones, which activate receptors in the abdominal vagus afferent (Knutsen *et al.* 2017). Furthermore, DON regulates tight junction proteins such as CLDN through signaling molecules as activation of MAPKs (Ghareeb *et al.* 2015).

Regulation

Exposure to DON usually originates from the consumption of contaminated plant commodities, but might occur also via a secondary route following the consumption of meat, milk and eggs, containing residual amounts of mycotoxins ingested by food-producing animal (Fink-Gremmels 1999). The highest exposure of DON comes from grain-based products, especially ‘bread and rolls’, ‘fine bakery wares’ and ‘pasta (raw) (Knutsen *et al.* 2017). In order to protect public health, to keep contaminants at levels that are toxicologically acceptable, many countries have established the maximum level for DON in food and feed. According to the EU commission regulation a tolerable daily intake (TDI) of 1 µg/kg b.w per day was established for DON. Although, young children were chronically exposed to DON at levels close to or even higher than the TDI (EFSA 2013). For example; the assessment of chronic dietary exposure of the German population resulted that young children 4-6 year-old received 2.7 fold higher DON than the TDI. Chronic exposure level of the Norwegian population was estimated on average 2.0 µg/kg b.w (2 year-old children) per day, which is twofold higher than the TDI. Toddlers and other children are the most exposed groups considering chronic exposure. Chronic dietary exposure of children to DON is estimated between 0.54 and 1.02 µg/kg b.w per day. Chronic

dietary exposure of adolescents, adults, elderly and very elderly to DON is estimated between 0.22 and 0.58 μg/kg b.w. per day. The acute effects of DON in humans are similar to those in animals (EFSA 2013). EU commission established maximum level for food and feedstuff as stated following (Table 3 and Table 4).

Table 3: EU regulation for DON in foodstuff

	Foodstuff	**Maximum recommendation level (mg/kg)**
1	Unprocessed cereals other than durum wheat, oats and maize	1.25
2	Unprocessed durum wheat and oats	1.75
3	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling	1.75
4	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs	0.75
5	Pasta (dry)	0.75
6	Bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals	0.5
7	Processed cereal-based foods and baby foods for infants and young children	0.2
8	Milling fractions of maize with particle size > 500 micron falling within and other maize milling products with particle size > 500 micron not used for direct human consumption	0.75
9	Milling fractions of maize with particle size ≤ 500 and other maize milling products with particle size ≤ 500 micron not used for direct human consumption	1.25

(European-Commission 2007)

Table 4: EU regulation for DON in feedstuff

	Feedstuff	**Guidance value in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12 %**
1	Feed Materials : ➢ Cereals and cereal products with the exception of maize by-products ➢ Maize by-products	 8 12
2	Complementary and complete feedingstuffs with the exception of: ➢ complementary and complete feedingstuffs for pig ➢ complementary and complete feedingstuffs for calves (< 4 months), lambs and kids	5 0.9 2

(European-Commission 2006)

1.3. Fungal toxic secondary metabolites (Emerging Mycotoxins)

Emerging mycotoxins are fungal secondary metabolites that are neither routinely determined, nor legislatively regulated (Vaclavikova *et al.* 2013). Emerging Mycotoxins are usually co-produced with other well-known mycotoxins (Hussein and Brasel 2001). The most relevant and frequently occurring emerging mycotoxins are *Fusarium* toxins including Enniatins (ENNs), Beauvericin (BEA), Apicidin (API), Aurofusarin (AFN), Moniliformin and Fusaproliferin (Gruber-Dorninger *et al.* 2016; Jajić *et al.* 2019). ENNs and BEA belong to a group of cyclic hexadepsipeptides, AFN is a dimeric naphthoquinone, and API is a cyclic tetra peptide (Frandsen *et al.* 2006; Niehaus *et al.* 2014; Rodríguez-Carrasco *et al.* 2016). Furthermore, other fungal species such as *Alternaria, Aspergillus* and *Penicillium* produce some emerging mycotoxins such as Brevianamid-F (BRV-F), Emodin (EMO) and Cyclo-Pro-L-Tyr (Cyclo) (Streit *et al.* 2013). In addition, *Acremonium* which is perceived to be a heterogeneous taxon, also produce some emerging mycotoxins such as Tryptophol (TRPT) (Glenn *et al.* 1996).

As these mycotoxins have only been discovered over the last few decades, they are to date poorly investigated (Springler *et al.* 2016b). Although recent sensitive analytical methods via LC-MS/MS has assisted the discovery of new fungal secondary metabolites (Malachová *et al.* 2014), but still the toxicology, toxicokinetics and toxicodynamics data of these metabolites are fragmentary (Taevernier *et al.* 2015).

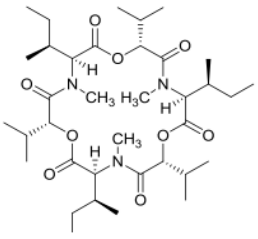
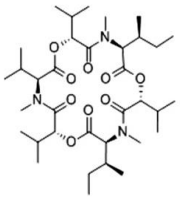
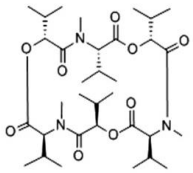
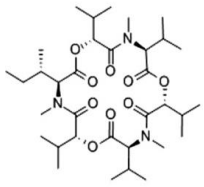
Emerging mycotoxins can occur in high frequency and sometimes also in high concentrations in cereals and in other grain-based products (Rodríguez-Carrasco *et al.* 2016; Gruber-Dorninger *et al.* 2016). In addition, their presence was reported in mold of water-damaged houses in milder climate regions such as North America and Western Europe (Taevernier *et al.* 2016). Thus, emerging mycotoxins gain growing interest due to their rapidly increasing presence across the food chain (Lucioli *et al.* 2013; Fraeyman *et al.* 2018). Although emerging mycotoxins are concerned, maximum permitted levels may not be proposed in the immediate future. This is primarily due to the lack of data related to their occurrence, contamination level, and toxicity. In order to better assess the risk of these mycotoxins, regular surveillance is a prerequisite to understand their significance as natural contaminants in human and animal nutrition (Jestoi 2008). Among them, the ten most prevalent emerging mycotoxins in feed are presented.

1.3.1. Enniatins (ENNs)

Origin

ENNs (A, A-1, B and B-1) are cyclic hexadepsipeptides (Table 5) secondary metabolites that have been known since few decades (Ivanova *et al.* 2006), and produced by several *Fusarium* species, such as *F. avenaceum, F. oxysporum, F. poae, or F. tricinctum* (Gruber-Dorninger *et al.* 2016). So far 29 species of ENNs have been isolated and characterized, either as a single compound or mixtures of inseparable homologs (Sy-Cordero *et al.* 2012). The most frequently detected ENNs in food and feed are ENN A, A-1, B and B-1 (Kamyar *et al.* 2004; Fraeyman *et al.* 2017). Naturally occurring ENNs commonly consist of three d-2-hydroxycarboxylic acid residues linked alternatively to three l-*N*-methyl-amino acid residues (Table 5) (Uhlig *et al.* 2006).

Table 5: Characteristics of ENNs

Property	**Information**			
Chemical structures	**ENN-A**  *Molecular Formula* **C36H63N3O9**	**ENN-A1**  *Molecular Formula* C35H61N3O9	**ENN-B**  *Molecular Formula* C33H57N3O9	**ENN-B1**  *Molecular Formula* C34H59N3O9
Molar mass g/mol	ENN-A 681.9	ENN-A1 667.9	ENN-B 639.8	ENN-B1 653.9
CAS number	2503-13-1	4530-21-6	917-13-5	19914-20-6
Physical state	Colourless fine needles			
Soluble in:	Polar organic solvents (*e.g.*, aqueous methanol, ethanol, chloroform) DMP, DMSO and poor solubility in water			
Chemical structure and characteristics of ENNs (ENN-A, A1, B and B1) (Uhlig *et al.* 2006; Escrivá *et al.* 2015).				

Occurrence

ENNs were detected in food (63%, n= 4,251), feed (32%, n = 3,640) and unprocessed grains (76%, n = 2,647) samples collected between 2010 and 2013 in 12 EU countries (EFSA 2014). ENNs (A, A-1, B and B-1) were also detected in more than 90% of feed and feed raw materials (n=83) with maximum concentrations of 1,745 µg/kg, 2,216 µg/kg, 780 µg/kg and 2,690 µg/kg respectively (Streit *et al.* 2013). In pig feed (n=1,141, worldwide) samples, ENNs (A, A-1, B and B-1) were found in 50%, 77%, 82% and 82% with maximum concentration of 307 µg/kg, 549 µg/kg, 1,514 µg/kg and 1,846 µg/kg respectively. ENN-B and ENN-B1 were the most

prevalent than of ENN-A and ENN-A1 as well the concentrations of ENN-B and ENN-B1 were higher than of ENN-A and ENN-A1 (Novak *et al.* 2019). ENNs (A, A-1, B and B-1) were also found in Egyptian feed (87%, 91%, 93% and 91%) samples (n=77) and maize (32%, 35%, 44% and 28%) samples (n=79) respectively. Concentration of ENNs was higher in feed (1.9 – 25 µg/kg) rather than maize (<1 µg/kg). Since feeds are composed of several raw materials, therefore compound feeds might be higher contaminated than maize (Abdallah *et al.* 2017). ENNs (A, A-1, B and B-1) were also found in Finland's grain samples (wheat, barley, rye and oats, n=38) collected in 2001 and 2002. The incidence of positive samples and the concentrations of ENNs were quite high in both years, especially for ENN-B and B1 that were detected in all samples (maximum concentrations of 18,300 µg/kg and 5,720 µg/kg respectively). ENN-A and A1 were detected in 68 % of the samples with the highest levels of 950 µg/kg and 2,000 µg/kg respectively (Jestoi *et al.* 2004). ENN-B was also found in 70 % of the baby food samples at levels up to 1,100 µg/ kg and in 44 % of pasta samples at levels of up to 106 µg/kg while other authors reported contamination rates of between 50 – 90 % of wheat, maize and barley samples with total concentrations of ENNs of up to 500 mg/kg (Juan *et al.* 2013).

Toxicity

ENNs possess a wide range of biological properties, such as; cytotoxicity, hemolysis, permeability and skin damage and decrease of TEER. ENNs have ionophoric properties and can form complexes with cations (Kolf-Clauw *et al.* 2013; Taevernier 2016; Fraeyman *et al.* 2016; Springler *et al.* 2016b; Olleik *et al.* 2019). Cytotoxicity of ENNs (A, A1, B and B1) was assessed during 24 h incubation in both proliferating and differentiated IPEC-J2 cells. ENNs were ranked in the following order of decreasing toxicity ENN-A > > ENN-A1 > ENN-B1> > >ENN-B respectively (Fraeyman *et al.* 2018). In similar cell line, after 48 h incubation all ENNs (A, A1, B and B1) showed almost similar absolute IC_{50} values of 3.40 µM, 4.15 µM, 3.25 µM and 3.67 µM respectively (Novak *et al.* 2019). In a similar time of exposure, cytotoxic effect of ENNs (0 to 100 µM) were studied in different cell types of human origin such as Caco-2, human normal lung epithelial cells (BEAS-2B), human normal epidermal keratinocytes (HEK), human liver cell line HepG2, human normal endothelial cells (HUVEC) and human gastric cell line (N87). Effect of ENNs in different cell types was dissimilar (Table 6). Among them, N87 cells were more sensitive than other cell lines (Olleik *et al.* 2019).

Table 6: Cytotoxicity of ENNs in different cell lines

Organ	Cell Type	Toxicity	Range of IC50
Human intestinal cells	Caco-2	ENN-A > ENN-A1 > ENN-B1 >ENN- B	1.1 µM to 4.6 µM
Human lung epithelial cells	BEAS-2B		5.7 µM to 43.7 µM
Human epidermal keratinocytes	HEK		2 µM to 54 µM
Human endothelial cells	HUVEC		2.8 µM to 17.3 µM
Human liver cells	HepG2	ENN-A > ENN-B > ENN-A1 ≈ENN- B1	3 µM to 5.6 µM
Human gastric carcinoma cell line	N87	ENN-A1 > ENN-B1 > ENN-A >ENN- B	0.003 µM to 1.7 µM

Furthermore, hemolytic activity of ENNs was assessed on human erythrocytes. According to their hemolytic activity, ENNs were ranked in the following order of toxicity; ENN-A> ENN-A1> ENN-B1> ENN-B respectively (Olleik *et al.* 2019).

In another study performed on Caco-2 cells, ENN-B decreased cell viability from 2.5 to 10 µM up to 30% 24 h whereas at 48 and 72 h it decreased the viability from 1.25 to 10 µM up to 50% and over 90% respectively. The obtained IC_{50} were of 9.2 µM, 6.9 µM and 5.09 µM after 24 h, 48 h and 72 h respectively (Fernández-Blanco *et al.* 2016). For tumor cells (neuronal origin), ENN-B was highly toxic. In a short term treatment (8 h) and at a very low concentration, ENN-B (0.1-1 µM) had tumor promoting functions based on growth stimulation (Dornetshuber *et al.* 2007).

Taking into account the above results, ENNs are toxic *in vitro*, whereas the *in vivo* studies that have been carried out in rodent and mice, have shown very low or no toxicity. Oral doses of 0.5–1 g/kg body weight (b.w) per day over 6 days to mice and single oral doses of up to 50 mg/kg b.w/day in rats did not produce toxic effects (Kolf-Clauw *et al.* 2013). In a study of (Rodríguez-carrasco *et al.* 2016), 5 mg/kg b.w ENN-B administrated to the mice for 9 days did not affect body weight, food intake and behavior of animals. In another study, 20.91 mg/kg b.w/day of ENN-A was administrated in the feed to a 2-month-old female Wistar rats, during the 28 day experiment and no adverse effect were seen in organ weight and histology of duodenum (Manyes *et al.* 2014). In similar treatment period 465 mg/kg feed, ENN-A was assessed in rats, no significant difference in feed intake and no gross illness was observed (Juan *et al.* 2014).

Concerning the metabolism of ENNs, it depends on the species or compound. A toxicokinetic study was performed in one piglet, in order to investigate the simultaneous detection and quantification of ENN-A, A1, hB and B1 in animal plasma. The pig received a single oral intra-

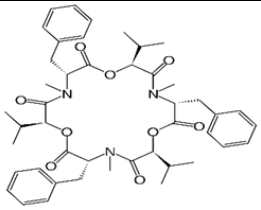
gastric bolus of 0.05 mg/kg b.w of all these mycotoxins including BEA. Blood samples were collected before (0 min) and at 10, 20, 30 and 40 min, 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 h post-administration. The authors claimed that despite of their similar chemical structure, there was a big difference in oral absorption between the different ENNs. ENN-B seemed to have the highest oral absorption, followed by ENN-B1, A1 and finally ENN-A. The maximal plasma concentrations (C_{max}) for ENN-B, B1, A1 and A, were 73.4, 35.2, 11.6 and 6.8 ng/mL respectively. The time to maximal plasma concentration (T_{max}) was 20 min after bolus administration for ENN-B1, B and A1, whereas the T_{max} for ENN-A was 30 min post-administration. The authors claimed that the elimination rate of all ENNs was fast and comparable to DON (Devreese *et al.* 2013). Furthermore, toxicokinetic properties and absolute oral bioavailability of ENN-B1 was evaluated in pig. Pigs were administered ENN-B1 (0.05 mg/kg b.w), either by oral gavage or by intravenous (IV) injection in the ear vein. ENN-B1 was rapidly absorbed after oral administration. The absolute oral bioavailability was 91% after 2 h. After IV administration, ENN-B1 was distributed and eliminated in accordance with oral administration (Devreese *et al.* 2014).

1.3.2. Beauvericin (BEA)

Origin

BEA is a cyclic hexadepsipeptide (Table 7) synthesized by several *Fusarium* species, including *F. subglutinans, F. proliferatum, F. oxysporum, F. moniliforme, F. anthophilum* and *F. beauvrecin* (Fotso and Smith 2003). BEA was firstly isolated from *Beauveria bassiana* (Hamill *et al.* 1969) and reported for its insecticidal properties and its toxicity to *Artemia salina* (Randazzo *et al.* 1993). BEA is known to exert a broad spectrum of biological effects such as, antibiotic, anti-inflammatory activities, as well as anticancer effects in various cancer cell lines (Heilos *et al.* 2017).

Table 7: Characteristics of BEA

Property	**Information**
Chemical structures	 $C_{45}H_{57}N_3O_9$
Molar mass	784.0 g/mol
Physical state	Colourless fine needles
CAS number	26048-05-5
Soluble in:	Polar organic solvents (*e.g*., aqueous methanol, ethanol, chloroform) DMP, DMSO and poor solubility in water
Chemical structure and characteristics of BEA (Logrieco *et al.* 1998; Liuzzi *et al.* 2017).	

Occurrence

BEA was reported in food (80%, n=732), feed (79%, n=861) and unprocessed grains (46%, n=554) samples collected between 2010 and 2013 in 12 EU countries. For food, the highest mean concentrations were measured in dried fruits (29.5 µg/kg), followed by oilseeds (8.86 µg/kg) and cereal-based food for infants and young children (8 µg/kg). For feed and unprocessed grains, BEA was present in maize gluten (EFSA 2014). In a worldwide survey of pig feed, BEA was reported in 68% of total samples (n=1,141) with maximum concentration of 413 µg/kg (Novak *et al.* 2019). BEA was found in high prevalence (98%, n=83) with maximum concentration of 2,326 µg/kg in feed and feed raw materials collected in EU (Streit *et al.* 2013). Furthermore, occurrence of BEA was reported from different countries in various commodities. BEA was found 33-95% in several food commodities, such as wheat based products, rice, maize based products and barley based products, collected in Italy, Spain, Morocco and Tunisia (n=265), with maximum concentrations 844 µg/kg, 57.4 µg/kg, 73.9 µg/kg and 82 µg/kg respectively (Serrano *et al.* 2012). In Italian corn (n=94) and oat (n=7) samples BEA was found 27-57% in maximum concentrations 41 µg/kg (Ritieni *et al.* 1997; Juan *et al.* 2013). All the wheat samples (n=13), from Finland and all the rice samples (n=70) from morocco were contaminated by BEA with maximum concentration 3,500 µg/kg and 12,810 µg/kg respectively. Even, 33% of baby food (n=68) were contaminated by BEA with maximum concentration of 10,600 µg/kg in Morocco (Logrieco *et al.* 2002b; Meca *et al.* 2010; Sifou *et al.* 2011). In addition, BEA was reported in Egyptian maize (63%, n=79) and feed (88%, n=77). Concentration of BEA in feed (88 µg/kg) was higher than maize (63 µg/kg) (Abdallah *et al.* 2017).

Toxicity

Acute exposure to BEA is not a concern to human health (EFSA 2014). However, in human and mammalian cell lines, BEA demonstrated toxic effects as it induces apoptosis, increases cytoplasmic calcium concentration and leads to DNA fragmentation (Logrieco *et al.* 2002a; Luz *et al.* 2017). The toxicity of BEA is primarily regarded to its ionophoric properties that alters the normal physiological concentrations of cations in and out of cells, acting as an ion carrier through the plasma membrane (Hilgenfeld and Saenger 1982; Fotso and Smith 2003). It also induces lipid peroxidation and alters kinetics in heart metabolism (Ruiz *et al.* 2011). Cytotoxic effect of BEA was assessed on different human cell lines: Caco-2, BEAS-2B, HEK, HepG2, HUVEC and N87 for 48 h. They were ranked based on the following order of decreasing sensitivity: HUVEC> HepG2> Caco-2> HEK> BEAS-2B>N87, with IC_{50} of 2.4 µM> 3.4 µM> 3.9 µM> 5.4 µM> 6.3 µM> 27.5 µM respectively (Olleik *et al.* 2019). In addition, BEA revealed toxic effect on human fibroblast-like cell line (MRC-5) After 24 h, the IC_{50} was of 5.0 µM and 1.1 µM via Alamar Blue™ and BrdU assay respectively (Ivanova *et al.* 2006). Furthermore, a toxicogenomic study was performed to investigate gene expression changes triggered by BEA exposure (1.5, 3 and 5 µM) during 24 h in Jurkat cells, a human lymphoblastic T cell line, through RNA-sequencing and differential gene expression analysis. The results demonstrated BEA-induced mitochondrial damage affecting the respiratory chain and pointing to apoptosis through the caspase cascade. The most significantly altered pathways genes, involved in the respiratory chain, were significantly down-regulated. These results bring greater relevance to mitochondria as a target site for BEA induced cytotoxicity in cellular models (Escrivá *et al.* 2018).

Nevertheless, BEA is identified toxic *in vitro*, but is reported to be not toxic *in vivo.* Toxicity and pharmacological behavior of BEA was investigated in mice during 9 days, no effect on body weight, food intake and behavior was observed at 5 mg/kg b.w (Rodríguez-carrasco *et al.* 2016). However, in a brain influx study performed in mice, BEA crossed the blood-brain barrier (Taevernier *et al.* 2016). Therefore, no conclusion could be drawn with respect to chronic exposure due to the lack of relevant *in vivo* toxicity data.

Metabolization of BEA was studied in pig. A toxicokinetic study was performed in one piglet, in order to investigate detection and quantification of BEA in plasma. The pig received a single oral intra-gastric bolus of 0.05 mg/kg b.w of BEA along with ENNs (ENN-A, A1, B and B1). Blood samples were drawn before (0 min) and at 10, 20, 30 and 40 min, 1, 1.5, 2, 3, 4, 6, 8, 10

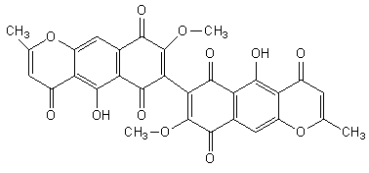
and 12 h post-administration. The authors claimed that for BEA, no plasma concentration-time profile was designed as the plasma concentrations were above the limit of quantification (LOQ) level at only 2 time points, i.e. 0.51 ng/mL at 30 min and 0.82 ng/mL at 40 min post-administration. Thus, BEA revealed a lower oral bioavailability compared to ENNs (Devreese *et al.* 2013).

1.3.3. Aurofusarin (AFN)

Origin

AFN is a dimeric naphthoquinone belonging to the naphthoquinone group of polyketides (Table 8) (Frandsen *et al.* 2006). AFN is a yellow-red pigment produced by *Fusarium* head-blight disease caused by several species of *Fusarium*, such as *F. graminearum*, *F. culmorum*, and *F. crookwellense* (Kim *et al.* 2006).

Table 8: Characteristics of AFN

Property	**Information**
Chemical structures	 $C_{30}H_{18}O_{12}$
Molar mass	570.4 g/mol
Physical state	Black and brown fine needles
CAS number	88360-87-6
Physical state	Dependent on the pH value of the solvent, ranging from golden yellow in acidic solvents to red/purple in alkaline solvents
Soluble in:	Polar organic solvents (*e.g.*, aqueous methanol, ethanol, chloroform) DMP, DMSO and poor solubility in water
Chemical structure and characteristics of AFN (Tanaka and Tamura 1961).	

Occurrence

AFN has been identified in feed ingredients in particular in contaminated grains and known as a new crop pollutant (Dvorska *et al.* 2003). AFN was found in feed raw materials 84% (n=83, Europe), finished feed 80.7% (n=1,141, worldwide), 73% (n=77, Egypt) and maize 9% (n=79, Egypt) with maximum concentration of 17,659 µg/kg, 85,360 µg/kg, 3,005 µg/kg and 1,858 µg/kg respectively (Streit *et al.* 2013; Abdallah *et al.* 2017; Novak *et al.* 2019). In addition, AFN was found in settled dust of Polish poultry farms (n=13), among 27 other detected chemicals, with the highest concentration of 281.44 µg/kg (Skóra *et al.* 2016).

Toxicity

AFN was investigated on several endpoints of toxicological importance such as cytotoxicity, genotoxicity and oxidative stress. AFN at 10 µM significantly decreased the cellular proliferation of colon adenocarcinoma cell line (HT29) and non-tumorigenic colon cells (HCEC-1CT) after 72 h of exposure at 12% and 7% respectively. Exposure to AFN 5 µM for 24 h significantly reduced mitochondrial activities of HT29 and HCEC-1CT (24 % and 23 %), whereas AFN 10 µM reduced up to 30 % and 40% respectively. Furthermore, the genotoxic impact of AFN (5 µM and 10 µM) after 1 h was investigated in the comet assay. AFN significantly increased a tail intensity of DNA damage. The authors claimed significant DNA damage indicating the potential involvement of other mechanisms in the genotoxic impact of AFN. Overall, AFN was found to be cytotoxic in both tumorigenic and non-tumorigenic colon cells (Jarolim *et al.* 2018). Likewise, AFN 10 µM reduced the mitochondrial activity of Caco-2 cells (Dvorska *et al.* 2001). AFN showed similar potential toxicity as DON, in terms of mitochondrial activity determined by the WST-1 assay. Although the results obtained with 1 and 5 µM has indicated that AFN may show a steeper dose response curve compared to DON (Vejdovszky *et al.* 2016).

AFN was either toxic *in vivo* to poultry and reduced the nutritional quality of quail meat and eggs. A dose 26.4 mg AFN /kg feed administrated to 45 days old quails caused a significant decrease of vitamins A and E, total carotenoid, lutein and zeaxanthin concentrations in the meat and significantly increased egg yolk susceptibility to lipid peroxidation. AFN also compromised immune function of laying chickens and quail and decreased fertility and hatchability (Dvorska and Surai 2004). Accumulation of AFN in egg yolk stimulated lipid peroxidation directly, causing a decrease in antioxidant concentration (Dvorska 2014) and also caused a significant decrease in vitamins A and E and fatty acids in egg yolk (Kim *et al.* 2008). Another possibility proposed to explain the detrimental effect of AFN on antioxidants is a disruption of nutrients absorption in intestine (Dvorska 2014).

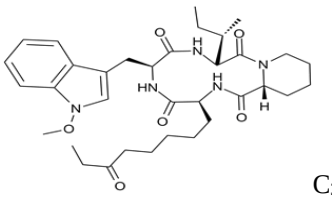
In spite of a great variety of toxic effects, the molecular mechanisms of action are poorly understood and information about AFN is still limited (Dvorska *et al.* 2001).

1.3.4. Apicidin (API)

Origin

API is a cyclic tetrapeptide built up of four amino acids; N-methoxy-L-tryptophan, L-isoleucine, D-pipecolic acid, and L-2-amino-8-oxodecanoic acid (Table 9) produced by some *Fusarium* species (Niehaus *et al.* 2014). It was firstly isolated in 1996 from *F. pallidoroseum* (later known as *F. semitectum*) as a new agent showing histone deacetylase inhibiting activity in apicomplexan parasites such as *Plasmodium berghei* (Singh *et al.* 1996; von Bargen *et al.* 2013). Derivatives of API have been identified, but their structure, mode of action and biological activities are almost similar. The most frequent derivatives of APIs are API-E, API-D_2 and API-B (Jin *et al.* 2010).

Table 9: Characteristics of API

Property	**Information**
Chemical structures	 $C_{34}H_{49}N_5O_6$
Molar mass	623.8 g/mol
CAS number	183506-66-3
Physical state	Colourless fine needles
Soluble in:	Polar organic solvents (*e.g.*, aqueous methanol, ethanol, chloroform) DMP, DMSO and poor solubility in water
Chemical structure and characteristics of API (Singh *et al.* 1996).	

Occurrence

API was found in feed raw materials 66% (n=83 Europe), finished feed 52% (n=1,141 worldwide) and 17% (n=77 Egypt) with maximum concentrations of 160 µg/kg, 1,568 µg/kg and 5.4 µg/kg respectively (Streit *et al.* 2013; Abdallah *et al.* 2017; Novak *et al.* 2019).

Toxicity

API is known as a potential toxic agent (Zhang *et al.* 2017). API revealed strongest cytotoxic effect on IPEC-J2 with an absolute IC_{50} value of 0.52 µM during 48 h (Novak *et al.* 2019). In the same cell line, API 2.5 µM decreased the TEER over 93%, after 24 h of exposure (Springler *et al.* 2016b). API inhibited proliferation of different human cancer cell lines (MCF-7, a human breast cancer cell; HBL-100, a human breast cancer cell line, HeLa, a human cervix cancer cell

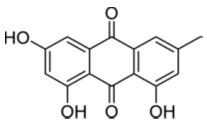
line; and AGS, a human gastric adenocarcinoma cell line) dose dependently. The half maximal effect (IC_{50}) were calculated; 1.17, 0.57, 0.51 and 0.13 µM after 48 h respectively. In the same study, API showed toxicity in mouse cancer cell lines (v-*ras*-transformed NIH3T3, a mouse fibroblast cell line and Colon 3.1-M26, a mouse colon carcinoma cell line). The IC_{50} were obtained 0.18 and 0.17 µM respectively (Han *et al.* 2000). Human pancreatic cancer cell lines (Capan-1 and Panc-1) were exposed to API for 48 h. For concentrations above 100 nM, the cytotoxicity and a sustained loss of cell viability were significantly increased (Bauden *et al.* 2015). API showed potent inhibitory activity against lung carcinoma (GLC-82) cells with the IC_{50} value of 6.94 µM after 48 h. API 14.0 µM induced apoptosis via mitochondrial pathway in GLC-82 cells by 7.2 %, 16.6 %, 26.4 % and 37.8 %, after 12 h, 24 h, 36 h, and 48 h respectively (Zhang *et al.* 2017). API was incorporated into complete rat diets during 14 days at concentrations of 0.5 and 1 g/kg feed and fed to 21 day-old female rats. API induced toxic effects including body weight loss, hemorrhage in the stomach, intestine and bladder and finally death. API at 0.5 g/kg caused death after 10 to 14 days of exposure and at 1 g/kg animal died within 7 days (Park *et al.* 1999).

1.3.5. Emodin (EMO)

Origin

EMO, an orange-red crystalline compound, is a 1,3,8-trihydroxy-6-methylanthraquinone, extracted from *Aspergillus wentii* isolated from weevil-damaged Chinese chestnuts (Table 10) (Wells *et al.* 1975). It belongs to the anthraquinones group, mainly produced by several *Aspergillus species,* including *A. wentii, A. flavus* and *A. ochraceus* (Nandani *et al.* 2013; Streit *et al.* 2013).

Table 10: Characteristics of EMO

Property	**Information**
Chemical structures	 $C_{15}H_{10}O_5$
Molar mass	270.24 g/mol
CAS number	518-82-1
Physical state	The orange-red, crystalline
Soluble in:	Polar organic solvents DMP, DMSO and in water
Chemical structure and characteristics of EMO (Wells *et al.* 1975).	

Occurrence

EMO was originally reported as being more common in bark and roots, but it is clear now that EMO occurs in vegetative organs (stem, foliage) as well in reproductive organs (flower, fruit, seeds and pods). EMO is occurring in tropical and subtropical plant families as well in the plant families from temperate regions (Izhaki 2002). EMO was found in 89% sample of feed and feed raw material (n=83, Europe), finished feed 69% (n=1141, worldwide) and 92% (n=79, Egypt) and maize 57% (n=77, Egypt) with maximum concentrations of 1,570 µg/kg, 1,170 µg/kg, 76 µg/kg and 66.6 µg/kg respectively (Streit *et al.* 2013; Abdallah *et al.* 2017; Novak *et al.* 2019).

Toxicity

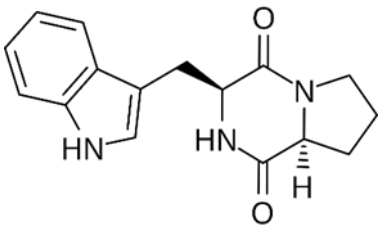
The toxicity of EMO has been very poorly investigated either *in vitro* or *in vivo*. Cytotoxic effect of EMO was assessed on the viability of IPEC-J2 over 48 h. The maximum toxicity of EMO was reached up to 50 µM and IC_{50} was obtained 13.09 µM (Novak *et al.* 2019). The cytotoxic effect of EMO was assessed on mouse embryos at the blastocyst stage, subsequent embryonic attachment and outgrowth *in vitro,* and *in vivo* implantation by embryo transfer. Blastocysts treated with EMO 25–75 µM exhibited significantly increased apoptosis and a corresponding decrease in total cells number. Furthermore, EMO in drinking water of mouse led to apoptosis and decreased cell proliferation in embryo and inhibited early embryonic development to the blastocyst stage (Chang *et al.* 2012). EMO was also toxic, when it was given orally to redwing blackbirds (*Agelaius phoeniceusin*) and starlings (*Sternus vulgaris)*. The oral administration of EMO to cockerels caused moderate diarrhea and mortality within 5 days of ingestion (LD_{50} = 3.7 mg/ kg) (Wells *et al.* 1975).

1.3.6. Brevianamide-F (BRV-F)

Origin

BRV-F is a precursor of the biosynthetic pathway of fumitremorgins and the tryprostatins, produced by *Penicillium brevicompactum, Aspergillus versicolor* and *A. fumigatus* (Table 11) (Frisvad *et al.* 2009).

Table 11: Characteristics of BRV-F

Property	**Information**
Chemical structures	 $C_{16}H_{17}N_3O_2$
Molar mass	283.3 g/mol
CAS number	38136-70-8
Physical state	White solid fine needless
Soluble in:	Polar organic solvents (*e.g*., aqueous methanol, ethanol, chloroform) DMP, DMSO
Chemical structure and characteristics of BRV-F (Wilson *et al.* 1973).	

Occurrence

BRV-F was found in feed and as well in feed raw materials. The proportion of the contaminated samples were 65.2% (n= 1,141, worldwide), 69% (n=83, Europe), 86% (n= 77, Egypt), maize 6% (n= 79, Egypt) and processed cassava 79.6% (n=373, Nigeria) with maximum concentration of 1,170 µg/kg, 2,043 µg/kg, 353 µg/kg , 5 µg/kg and 44 µg/kg respectively (Novak *et al.* 2019; Streit *et al.* 2013; Abdallah *et al.* 2017; Abass *et al.* 2017).

Toxicity

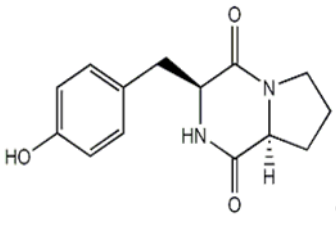
The toxic effect of BRV-F is poorly investigated. In a recent study, effect of BRV-F was examined on the viability of IPEC-J2 cells over 48 h, but did not show negative effect up to 150 µM (Novak *et al.* 2019). Likewise, a preliminary toxicity study of BRV (BRV-A3) showed no acute toxicity in mice upon oral or intraperitoneal administration. However, the molecule induced cytotoxicity and inflammatory lung response in intratracheally exposed mice (Wauters 2015).

1.3.7. Cyclo-(L-Pro-L-Tyr) (Cyclo)

Origin

Cyclic dipeptide Cyclo-(L-Pro-L-Tyr) is a diketopiperazine formed by the fusion of tyrosine and proline (Table 12), reported as a secondary metabolite of fungi (Capon *et al.* 2007). Cyclo was firstly isolated from *Alternaria alternata* decades ago (Stierle *et al.* 1988).

Table 12: Characteristics of Cyclo-Pro-L-Tyr

Property	**Information**
Chemical structures	 $C_{14}H_{16}N_2O_3$
Molar mass	260.3 g/mol
CAS number	357-67-5
Physical state	White solid fine needless
Soluble in:	Soluble in ethanol, methanol, DMF or DMSO. Limited water solubility.
Chemical structure and characteristics of Cyclo (Holden *et al.* 1999).	

Occurrence

Occurrence of Cyclo is reported in finished feed 87.6% (n=1,141 worldwide), 100% (n=77, Egypt) and maize 43% (n=79, Egypt) with a maximum concentration of 34,910 µg/kg, 4,244 µg/kg and 21 µg/kg respectively (Abdallah *et al.* 2017; Novak *et al.* 2019).

Toxicity

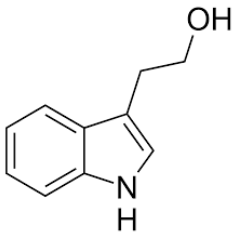
Toxicity of Cyclo is not reported yet. In a recent study, effect of Cyclo was assessed up to 150 µM on the viability of IPEC-J2 over 48 h, but was not toxic (Novak *et al.* 2019).

1.3.8. Tryptophol (TRPT)

Origin

TRPT (indole-3-ethanol) is an aromatic alcohol (Table 13) produced by *Acremonium species* and formed as the end product of tryptophan catabolism (Kosalec *et al.* 2008; Streit *et al.* 2013). TRPT was firstly isolated from cucumber seed as a plant growth regulator (Rayle and Purves 1967). However, it is an endogenous plant constituent and growth regulator, but is also produced as a secondary metabolite by certain bacteria, yeast and fungi (Kosalec *et al.* 2011).

Table 13: Characteristics of TRPT

Property	**Information**
Chemical structures	 $C_{10}H_{11}NO$
Molar mass	161.2 g/mol
CAS number	526-55-6
Physical state	White solid fine needless
Soluble in:	Soluble in ethanol, methanol, DMF or DMSO. Limited water solubility.
Chemical structure and characteristics of TRPT (Kosalec *et al.* 2008)	

Occurrence

The occurrence of TRPT is reported in feed and feed raw materials. TRPT was found in 75% (n=1,141) of animal feed collected worldwide, with maximum concentration of 10,270 µg/kg (Novak *et al.* 2019). Another study, carried out on Egyptian animal feed and maize, reported the presence of TRPT in 90% (n=77) and 16% (n=79) of the samples at concentrations of 10,878 µg/kg and 101 µg/kg respectively (Abdallah *et al.* 2017). In addition, occurrence of TRPT was reported in 71% (n=83, Europe) of feed and feed raw materials samples in mean concentration of 267 µg/kg (Streit *et al.* 2013).

Toxicity

TRPT was not cytotoxic *in vitro* for IPEC-J2 exposed to 150 µM for 48 h (Novak *et al.* 2019). Whereas, *in vivo* studies have reported that TRPT is a highly lipophilic compound that freely penetrates cell membranes. TRPT was injected into laboratory mammals and chicken and induced a sleep-like state and lethargy accompanied with alteration of body temperature. (Kosalec *et al.* 2011). When administered to laboratory animals, it was rapidly distributed to the brain and other tissues. TRPT also readily penetrates into lymphoid tissues and its immunosuppressive effects have been observed in mice (Ackerman and Seed 1976). TRPT induces apoptosis in human lung (lymphoblast) U937 cell line via activation of caspase-8 followed by caspase-3 (Inagaki *et al.* 2007). Despite interesting biological functions, the overall toxicity profile of TRPT has not yet been adequately explained. Only a few studies have dealt with mutagenicity and genotoxicity of TRPT (Kosalec *et al.* 2011).

1.4. Structure and function of the small intestine

The intestine constitutes the largest and most important barrier to prevent the passage of harmful intraluminal substances from the external environment into the organism, including foreign antigens, microorganisms, and their toxins (Pierron *et al.* 2016a). It is the first barrier to food contaminants. Following the ingestion of mycotoxin contaminated food or feed, intestinal epithelial cells can be exposed to high concentrations of toxicants, potentially affecting intestinal functions (Pinton and Oswald 2014; Bouhet and Oswald 2005). Indeed, the intestine is a privileged immune site, where immunoregulatory mechanisms simultaneously defend the body against pathogens, but also preserve tissue homeostasis to avoid immune mediated pathology in response to environmental challenges (Pinton and Oswald 2014).

The small intestine is a specialized abdominal tubular structure in length of 4 to 7 m (Thomson and Shaffer 1992) and its epithelium is a single-cell layer that constitutes the largest and most important barrier against the external environment. The intestinal epithelium has two critical functions: (i) it acts as a selective filter, allowing the translocation of essential dietary nutrients, electrolytes, and water from the intestinal lumen into the circulation; (ii) it acts as a barrier to prevent the passage of harmful intraluminal entities, including foreign antigens, microorganisms, and their toxins (Groschwitz and Hogan 2009). These functions are supported by the unique structure of layers of the gastrointestinal tract (GT). The GT is divided into several layers: the mucosa, the submucosa, the muscularis propria, and the serosa (Figure 2) (Rao and Wang 2010). The mucosa consists of an epithelial lining, a laminal propria of loose connective tissue, and the muscularis mucosae, which is composed of two layers of smooth muscle fibers, separating the mucosa from the submucosa. The submucosa is a layer of irregular loose connective tissue, containing large blood and lymphatic vessels, glands, lymphatic tissue, and a submucous (Meissner's) nerve plexus. The muscularis externa is formed of thick bundles of smooth muscle fibers arranged into two sublayers: circular in the internal sub layer and longitudinal in the external sublayer. Serosa is a thin layer of loose connective tissue. It is continuous with adjacent tissue in the esophagus. Serosa replace the adventitia in the gastrointestinal tract, which is composed of a layer of mesothelium and the underlying loose connective tissue, rich in blood and lymphatic vessels and adipose cells (Zhang 1999).

Mucosa is also referred to as a mucous membrane, as mucus production is a characteristic feature of gut epithelium. The membrane consists of epithelium, which is in direct contact with ingested food, and the lamina propria, a layer of connective tissue analogous to the dermis

(Betts *et al.* 2017). The small intestinal mucosa is constructed to act first as an absorptive surface and second as a barrier to potentially pathogenic substances and microorganisms. The intestinal epithelium is composed of absorptive cells such as enterocytes and protective cells, such as goblet cells, paneth cells and endocrine cells(Table 14) (Greaves 2011).

Table 14: The roles of the cells in the small intestinal mucosa (Betts *et al.* 2017).

Cell type	Location in the mucosa	Function
Absorptive	Epithelium/intestinal glands	Digestion and absorption of nutrients in chime
Goblet	Epithelium/intestinal glands	Secretion of mucus
Paneth cells	Intestinal glands	Secretion of the bactericidal enzyme lysozyme; phagocytosis
G cells	Intestinal glands of duodenum	Secretion of the hormone intestinal gastrin
I cells	Intestinal glands of duodenum	Secretion of the hormone glucoses-dependent insulinotropic peptide, which stimulate the release of insulin
K cells	Intestinal glands	Secretion of the hormone glucose-dependent insulinotropic peptide, which stimulates the release of insulin
M cells	Intestinal glands of duodenum and jejunum	Secretion of the hormone motilin, which accelerates gastric emptying, stimulates intestinal peristalsis, and stimulates the production of pepsin
S cells	Intestinal glands	Secretion of the hormone secretin

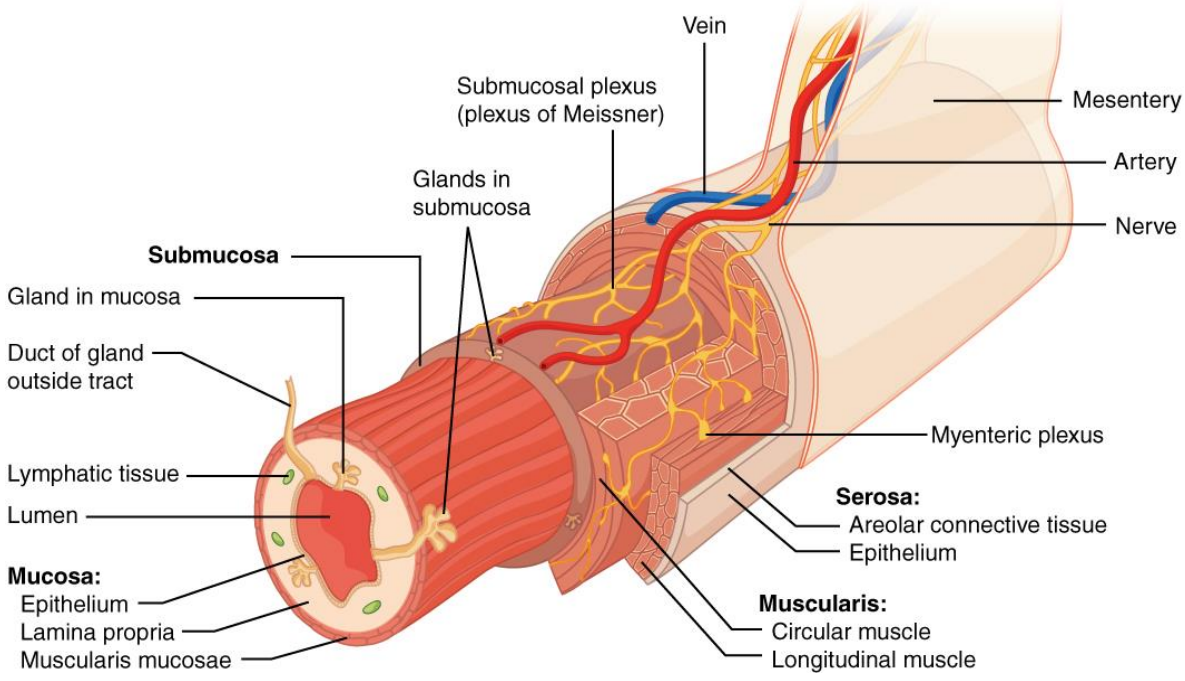


Figure 2: Layers of the alimentary canal. The wall of the alimentary canal has four basic tissue layers The mucosa, submucosa, muscularis, and serosa (Betts *et al.* 2017):

The normal gastrointestinal tract is a finely integrated system geared to carry out the assimilation of ingested foodstuffs. Assimilation (the process by which ingested foods reach

body fluids and cells) takes place in the small intestine (Thomson and Shaffer 1992). Thus, the intestine is potentially highly exposed to the food contaminants (Pinton and Oswald 2014).

In order to investigate the effect of food contaminants such as mycotoxins in the intestine, several *in vivo, in vitro* and *ex vivo* model are generally used so far. *In vitro* models of intestinal mucosa have been developed for studying enteritic diseases. *Ex vivo* tissue cultures represent a model between *in vitro* and *in vivo*, where whole tissue slices are cultured, such as organotypical slice cultures. In the *ex vivo* models, the cytoarchitecture is retained as well as many of the intercellular connections and interplays. Thus, metabolic processes more closely represent the *in vivo* situation. In the context of reducing the number of experimental animals, intestinal explants represent a powerful model (Kolf-Clauw *et al.* 2009; Fadeel *et al.* 2012; Pearce *et al.* 2018). Although, the *in vitro* studies can be criticized for being very different from the natural environment they have several advantages. First, they do not demand the technical skills in animal handling and have typically lower costs. Secondly, *in vitro* assays are more rapid and can be more easily performed. Furthermore, *in vitro* assays allow identification of direct effects on target cells. Finally, *in vitro* studies have the advantage of allowing genetic manipulation of cells as well as the utilization of cells and tissues from transgenic species (Goodwin 2007).

The toxic effect of DON and emerging mycotoxins on intestinal cells using *in vitro* model are summarized in Tables 15 and 16. This part is mainly focusing on *in vitro* studies, thus *in vivo* and *ex vivo* has not been included.

Table 15: Effect of DON on the intestine *in vitro*

Cells Type	Dose and time of exposure	Range or time point of toxicity	Endpoint	References
IPEC-1	10-50 µM (48 h) TEER 2-16 days	Toxicity >5 µM TEER >2 days	Decrease in claudin-3 & 4 tight junction protein Increase in 4 kDa dextran permeability Increase in E coli 28C translation Decrease in TEER	(Pinton *et al.* 2009)
	10-30 µM (24 – 48 h)	>10 µM	Decrease in TEER Decrease in claudin-3 & 4 tight junction protein Increase in 4 Dka dextran permeability	(Pinton *et al.* 2012)
	0. -13.5 µM (72 h)	>3 µM	Decrease in protein expression of ZO-1	(Diesing *et al.* 2011b)
IPEC-J2	0. -13.5 µM (72 h)	>6 µM	Affect the distribution pattern of ZO-1	(Diesing *et al.* 2011b)
	6.74 µM (1 - 48 h)	>1 h	Decrease in TEER values Decrease in protein expression of claudin-3, occludin and ZO-1 Affect the distribution pattern of ZO-1	(Gu *et al.* 2014)
	2.5-10 µM (24 – 48 h)	>2.5 µM 24 – 48 h	Inhibit proliferation Increase LDH release Decrease ATP contents	(Awad *et al.* 2012)
	20 µM (48 h)	-	Decreased proliferation	(Novak *et al.* 2019)
	20 µM (0 – 72 h) TEER (30 days)	>1 h TEER >7 days	Decrease in TEER Decrease in protein expression, claudin-3 & 4	(Springler *et al.* 2016a)

			through p44/42 phosphorylation	
	1.5-3 µM (24 – 72 h)	>1.5 µM	Inhibit proliferation Decrease in TEER	(Springler *et al.* 2016b)
	0.67–13 µM (24–72 h)	>0.67 µM >24 h	Decrease in TEER value Decrease in protein expression of claudin-3, occludin and ZO-1 Affect the distribution pattern of ZO-1	(Diesing *et al.* 2011a)
	0.03-3 µM (72 h)	>0.03 µM	Decrease in TEER values Increase in permeability of doxycycline and paromomycin	(Goossens *et al.* 2012)
	4 µM (12 h)	-	Decrease in TEER values Increase in permeability of 4 kDa FITC-dextran Increase in translocation of commensal *E coli* Increase in transcript level of claudin-1, claudin-4, occludin and ZO-1 Decrease in protein expression of claudin-3 and claudin-4	(Ling *et al.* 2016)
	0.001-100 µM (48 h)	>10 µM	Decrease in TEER	(Maresca *et al.* 2002)
	0.1–1 µg/mL (24 h)	>0.75 µg/mL	Increase in translocation of pathogenic *Salmonella typhimurium*	(Vandenbroucke *et al.* 2011)
HT-29	2-50 µM (24 h)	>5 µM	Inhibit proliferation Increase in total DNA damage Increase in p53 protein level and caspase-3 activity	(Bensassi *et al.* 2009)
	0.13-0.7 µM (6 -20 days)	>0.13 µM >15 days	Decrease in protein content Decrease in TEER	(Kasuga *et al.* 1998a)

			Increase in lucifer yellow permeability	
Caco-2	30 μM (48 h)	-	Decrease in TEER Decrease in claudin-4 tight junction proteins Increase in 4 kDa dextran permeability	(Pinton *et al.* 2009)
	10 μM (12 h)	-	Decrease in TEER values Increase in permeability of HRP and 4 kDa FITC dextran Increase in *E coli* K12 translocation	(Maresca *et al.* 2008)
	1.7-17 μM (24 h)	>1.7 μM	Decrease in TEER values Increase in permeability of mannitol Increase in transcript level of claudin-4 and occludin Decrease in protein expression of claudin-4	(De Walle *et al.* 2010)
	0.37-1.5 μM (6 – 120 h)	>0.75 μM >35 h	Decrease in horizontal impedance value of undifferentiated cells	(Manda *et al.* 2015)
	50–200 ng/mL (14 days)	>100 ng/mL	Decrease in TEER values	(Kasuga *et al.* 1998b)
	1.39 - 21.5 μM (24 h)	>1.39 μM	Decrease in TEER values Decrease in horizontal impedance value Increase in permeability of LY and 4 kDa FITC dextran Increase in transcript level of claudin-3, claudin-4, occludin and ZO-1 Decrease in tight junctional protein expression of claudin-1, claudin-3 and claudin-4 Affect the distribution pattern of claudin-1, claudin-3, claudin-4, occludin and ZO-1	(Akbari *et al.* 2014)

Table 16: Effect of emerging mycotoxins on the intestine *in vitro*

Cells Type	Toxins	Dose and time of exposure	Range or time point of toxicity	Endpoint	References
IPEC-J2	ENN-A	0 – 5 µM (72 h)	>2.5 µM	Decrease in TEER value Inhibit proliferation	(Springler *et al.* 2016b; Fraeyman *et al.* 2018; Novak *et al.* 2019)
		0 – 20 µM (48 h)	>5 µM	Inhibit proliferation	
		0 – 10 µM (24 h)	>5 µM	Early and late apoptosis Necrotic proliferation	
	ENN-A1	0 – 10 µM (72 h)	>5 µM	Decrease in TEER value Inhibit proliferation	
		0 – 20 µM (48 h)	>5 µM	Inhibit proliferation	
		0 – 10 µM (24 h)	>5 µM	Early and late apoptosis Necrotic proliferation	
	ENN-B	0 – 5 µM (72 h)	>1.5 µM	Decrease in TEER value Inhibit proliferation	
		0 – 20 µM (48 h)	>5 µM	Inhibit proliferation	
		0 – 100 µM (24 h)	>10 µM	Early and late apoptosis Necrotic proliferation	
	ENN-B1	0 – 5 µM (72 h)	>2.5 µM	Decrease in TEER value Inhibit proliferation	
		0 – 20 µM (48 h)	>5 µM	Inhibit proliferation	
		0 – 25 µM (24 h)	>10 µM	Early and late apoptosis Necrotic proliferation	
	BEA	0 – 10 µM	>3 µM	Decrease in TEER value	

		(72 h)		Inhibit proliferation	
		0 – 20 µM (48 h)	>2.5 µM	Inhibit proliferation	
		0 – 10 µM (24 h)	>5 µM	Early and late apoptosis Necrotic proliferation	
	API	0 - 2.5 µM (72 h)	>0.43 µM	Decrease in TEER value Inhibit proliferation	
		0 – 5 µM (48 h)	>0.2 µM	Inhibit proliferation	
	AFN	0 – 10 µM (72 h)	>1 µM	Decrease in TEER value Inhibit proliferation	
		0 – 20 µM (48 h)	>5 µM	Inhibit proliferation	
	EMO	0 – 150 µM (48 h)	>6.25 µM	Inhibit proliferation	
HT29	ENN-A	0.6 – 30 µM (24 – 48 h)	>6 µM	Decrease viability	(Meca *et al.* 2011)
	ENN-A1		>6 µM		
	ENN-B		>15 µM		
	ENN-B1		>15 µM		
Caco-2	ENN-A	0.6 – 30 µM (24 – 48 h)	>3 µM	Decrease viability	
	ENN-A1		>6 µM		
	ENN-B		>6 µM		
	ENN-B1		>6 µM		

1.5. Structure and function of the liver

The porcine liver consists of five lobes, the left lateral and medial, right lateral and medial, and caudate lobes (Figure 3) (Mulaikal and Emond 2018). The external anatomy is described from gross landmarks including the gallbladder, the vena cava and the hepatic ligaments. The internal anatomy is defined by the vascular structures and eight functionally independent segments, each segment with its individual blood supply and biliary drainage (Mulaikal and Emond, 2018; Sibulesky, 2013). Moreover, the liver is composed of a rich population of specialized cells that allow it to carry out complex functions. They can be grossly characterized as "parenchymal" cells (hepatocytes) and "nonparenchymal cells" (Mulaikal and Emond 2018). The parenchymal cells, make up 60 – 80% of liver cells and carry out the metabolic, detoxification, and synthetic functions of the liver, such as metabolism of amino acids, fats, carbohydrates, vitamins, byproduct wastes, and toxins (Table 17). Whereas the nonparenchymal cells makes 20 – 40% of the liver (Sheth and Bankey 2001; Racanelli and Rehermann 2006).

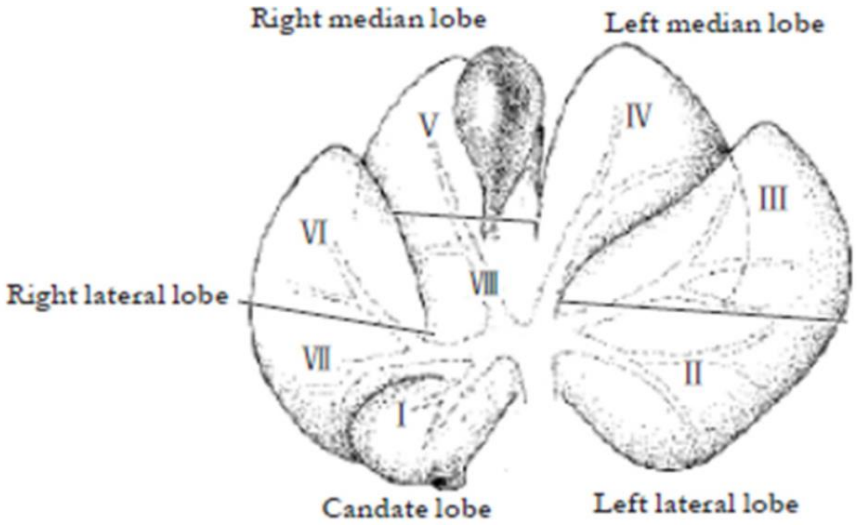


Figure 3: Anatomy and different lob of pig liver (Kim and Lee 2013).

The liver is a unique anatomical and immunological site in which antigen-rich blood from the gastrointestinal tract is pressed through a network of sinusoids and scanned by antigen presenting cells and lymphocytes (Racanelli and Rehermann 2006). Of the total hepatic blood flow (100–130 ml/min per 100 g of liver, 30 ml/min/kg/b.w), only one fifth to one third is supplied by the hepatic artery. The rest of blood about two thirds is supplied by portal vein. This blood contains oxygen and many nutrients brought to the liver from the intestines for processing (Lautt 2010). Any blood antigen or contaminant can target the liver (Sheth and Bankey 2001). Liver is also the main site of detoxification for xenobiotics, including

mycotoxins and represents a target organ for food contaminants. Liver detoxifies mycotoxins via biotransformation before reaching any tissue through the general bloodstream. The biotransformation process comprises a series of biochemical reactions leading to changes in the structure of contaminants facilitating their excretion from the body. A typical xenobiotic metabolism pathway contains a variety of enzymes that are involved in both phase I and phase II xenobiotics metabolism. The examples of phase I reactions are oxidation, reduction, dehalogenation, or hydrolysis and are catalyzed by several enzymes including cytochrome P450 (CYP1, CYP2 and CYP3). Phase II reactions are conjugation reactions, for example with glucuronic acid, sulfate, glutathione and/or amino acids (Slobodchikova *et al.* 2019). The majority of mycotoxin biomonitoring is performed using urine since it is non-invasive and accessible in relatively large volumes. These methods of analysis can be performed with and without B-glucuronidase treatment. B-glucuronidase catalyzes hydrolysis of conjugated mycotoxins, such as sulfate and glucuronide conjugates. Thus, the use of enzymatic hydrolysis can provide an appropriate alternative to direct metabolite monitoring for at least those mycotoxins which are predominately metabolized to phase II conjugated forms such as DON (Vidal *et al.* 2018).

The liver's lymphocyte population is selectively enriched in natural killer T cells which play critical roles in first line immune defense against invading pathogens, modulation of liver injury and recruitment of circulating lymphocytes (Racanelli and Rehermann 2006). The high local blood flow results in a high rate of interaction with foreign antigens, and confers immunologic responsibility on the liver. Because the liver acts as a detoxification center for the body, it can be regenerated if injured (Sheth and Bankey 2001). Lastly, the liver is the site of cholesterol synthesis and therefore crucial in the genesis of endogenous steroid hormones such as cortisol, aldosterone, and testosterone. While these hormones are synthesized in the adrenal gland, their precursors have an hepatic origin (Shen and Shi 2015).

Despite the similarity of digestive physiology between human and pig, their liver are different from the morphological point of view. Human liver consists of four lobes, the left, right, quadrate, and caudate lobes. Whereas, porcine liver consists of five lobes (Figure 3). Another difference is that inferior vena cava is divided into supra-and infra-hepatic in humans, whereas in porcine dissection between the liver and vena cava is very difficult (Kim and Lee 2013).

Table 17: Cellular microanatomy and their function

Cells type		Function
Hepatocytes		Hepatic regeneration Protein and lipids synthesis and metabolism Xenobiotic metabolisms
Stellate/Ito cells		Vitamin A and fat storage Scar tissue and wound healing
Liver Sinusoidal Endothelial cells		Regulate vascular resistance
Kupffer cells		Innate immunity Ischemia reperfusion injury
Dendritic cells		Innate immunity
Cholangiocyte		Bile duct cells
Lymphocytes	**NK**	Nonspecific targeting of tumor and viruses Innate immunity
	NKT	Target lipid antigens Innate and adaptive immunity
	T cells	Cell mediated, adaptive immunity
	B cells	Humoral mediated, adaptive immunity

(Mulaikal and Emond 2018)

We have reviewed the effects of DON and emerging mycotoxins on liver and hepatic cells extensively *in vitro* and *in vivo* (Tables 18, 19 and 20).

Table 18: Effect of DON on liver cells

In vitro				
Cells Type	**Dose and time of exposure**	**Range or time point of toxicity**	**Endpoint**	**References**
Rat's Clone9	0.1 – 25 μg/mL (24 h)	>0.1 μg/mL	Oxidative stress Decrease in the mitochondrial function	(Sahu *et al.* 2010)
	0 - 100 μg/mL (48 h)	>10 μg/mL	Cytotoxicity Double-stranded DNA (ds-DNA)content Oxidative stress	(Sahu *et al.* 2008)

			Decrease mitochondrial function	
Rat's MH1C1	0 – 25 μg/mL (24 h)	>0.1 μg/mL	Oxidative stress Decrease in the mitochondrial function	(Sahu *et al.* 2010)
Rat's NBL CL2		>0.1 μg/mL		
Rat's WRL68		>0.1 μg/mL		
HepG2	0 – 25 μg/mL (24 h)	>0.1 μg/mL	Oxidative stress Decrease in the mitochondrial function	(Sahu *et al.* 2010)
	1-10 μM (48 h)	>1 μM	Alteration of ATF3 expression	(Nielsen *et al.* 2009)
Human primary hepatocytes	0.1-100 μM (8 – 48 h)	>1 μM	Obvious cytotoxicity LDH release Activation of caspase-3	(Königs *et al.* 2008)
Rat's primary hepatocytes	0.01 – 100 μg/mL (3 h)	>10 μg/mL	Cytotoxicity Increases of the micronucleus (MN) frequencies	(Knasmüller *et al.* 1997)
	0.1-100 μg/mL (24 h)	>5 μg/mL	An increased percentage of large-sized nuclei	(Bradlaw *et al.* 1985)

Table 19: Effect of DON on the liver

In vivo				
Model	**Dose and time of exposure**	**Range or time point of toxicity**	**Endpoint**	**References**
Pigs	3 mg/kg feed (4 – 16 days)	>4 days	Histopathological lesions in the liver Disorganization of hepatic cords Hepatic cell vacuolization Apoptosis, megalocytosis, nuclear vacuolation	(Grenier *et al.* 2011)
	1.5 – 3 mg/kg feed (4 weeks)	1.5 µg/kg	Significant increase on histological changes in the liver	(Gerez *et al.* 2015)
	4 mg/kg feed (2 – 5 weeks)	2 – 5 weeks	Oxidative stress and lipid peroxidation	(Wu *et al.* 2014a)
	4 mg/kg feed (15 – 30 days)	15 – 30 days	Increased serum concentrations of ALP, ALT and AST	(Xiao *et al.* 2013)
	6 mg/kg feed (3 weeks)	-	Relative liver weights Increase of ALP, ALT and AST	(Wu *et al.* 2013)
	1100 µg/kg feed (6 weeks)	-	Damage in liver tissue Decrease of average daily gain (ADG) Alteration of immune system through a tendency to increase monocytes and immunoglobulins.	(Weaver *et al.* 2013)
	300–900 µg/kg feed (5 weeks)	>300 µg/kg	Fibrosis in liver tissues	(Chaytor *et al.* 2011)
	1 mg/kg b.w *injection* (6 – 24 h)	>6 h	Cleaving of caspase-3 Dilation of sinusoids Apoptotic hepatocytes, Apoptotic bodies of hepatocytes, (acidophilic bodies)	(Mikami *et al.* 2010)

	0.2–0.4 mg/kg feed (12 days)	0.2–0.4 mg/kg	Regressive lesions in liver Hyperaemia of the blood vessels Hepatocytes and necrosis of the individual hepatic cells	(Zielonka *et al.* 2009)
	1 mg/kg feed (6 weeks)	-	Decrease in total protein, albumin, and globulin Increase of ALT, AST and γ-glutamyltransferase Increase of mRNA expression levels of IFN-γ, TNF-α, IL-2	(Chen *et al.* 2008)
	0.2 – 9.57 mg/kg feed (5 weeks)	>0.2 mg/kg	Glycogen reduction Increase of hemosiderin particles Thickness of interlobular connective tissue septum High contents of smooth endoplasmic reticulum (ER) Loss of ribosomes Increased number of fatty and autophagic vacuoles	(Tiemann *et al.* 2006)
	8.6 mg/kg feed (16 h)	-	Decrease of serum activity of the liver enzyme glutamate dehydrogenase	(Döll *et al.* 2003)
	5.5 g/kg feed (3 weeks)	-	Decrease in liver weight	(Swamy *et al.* 2002)
Rats	5 mg/kg feed (3 weeks)	-	Lipid peroxidation DNA fragmentation Decreased hepatic glutathione content Upregulating mRNA Fas and TNF-α gene expression	(Abdel-Wahhab *et al.* 2015)
	8 µg/g feed (2 – 4 weeks)	>2 weeks	Increase of ALP and ALT	(Qiang *et al.* 2011)
	0.5 – 5 mg/kg feed	>2.5 mg/kg	Increase in maternal liver weight/ body weight ratios	(Collins *et al.* 2006)

	(3 weeks)		Cytoplasmic alterations of hepatocytes	
Mice	1000 mg/kg feed (2 weeks)	-	Cellular apoptosis DNA damage Positive MN[1]	(Singh *et al.* 2015)
	1- 25 µg/kg.b.w (10 – 30 days)	25 µg/kg in 10 days >2.5 µg/kg in 30 days	IL-1β increase and a modulation of proinflammatory gene expression in liver	(Tardivel *et al.* 2015)
	5 mg/kg feed (2 weeks)	-	Activities of ALT and/or AST Decreased albumin and/or total protein concentration in the serum Oxidative stress Upregulation of the apoptotic genes caspase-3	(Sun *et al.* 2014)
	3 mg/kg feed (7 weeks)	-	Changes in liver morphology Oxidative stress	(Hou *et al.* 2013)
	12 mg/kg feed (1 – 5 h)	>1h	Hepatic suppressors of cytokine signaling mRNA	(Amuzie *et al.* 2009)
	1.78 mg/kg feed (4 weeks)	-	Increase of liver microsomal pentoxyresorufin depentylase Increase of cytosolic glutathione transferase activities	(Gouze *et al.* 2006)
	3 mg/kg.b.w (8 weeks)	-	Fibrosis of liver portal and periportal veins	(Bilgrami 1991)
	10 g/kg feed (6 weeks)	-	Decrease of manganese and molybdenum in liver	(Hunder *et al.* 1991)
	25 mg /kg b.w (2- 4 h)	2 -4h	Elevation of TNF-α and TGF-β in liver	(Azconaolivera *et al.* 1995)

[1] Micronucleus

Table 20: Effect of emerging mycotoxins on liver cells

Cells Type	Toxins	Dose and time of exposure	Range or time point of toxicity	Endpoint	References
HepG2	ENN-A	0- 100 µM (48h)	>3 µM	Inhibit proliferation	(Meca *et al.* 2011; Juan-García *et al.* 2013, 2015; Jonsson *et al.* 2016; Svingen *et al.* 2017; Olleik *et al.* 2019)
		1.5 - 3 µM (24 – 72h)	≥ 1.5 µM ≥ 24h	Inhibit proliferation Apoptosis Necrosis	
		0.6 - 30 µM (24 – 48h)	>6 µM > 24h	Decrease viability	
	ENN-A1	0- 100 µM (48h)	>5 µM	Inhibit proliferation	
		1.5 - 3 µM (24 – 72h)	≥ 1.5 µM ≥ 24h	Apoptosis Necrosis	
		0.6 - 30 µM (24 – 48h)	>6 µM > 24h	Decrease viability	
	ENN-B	0- 100 µM (48h)	>5 µM	Inhibit proliferation	
		1.5 - 3 µM (24 – 72h)	≥ 1.5 µM ≥ 24h	Inhibit proliferation Apoptosis Necrosis	
		0.6 - 30 µM (24 – 48h)	>6 µM > 24h	Decrease viability	
		10 45 µM (24h)	≥ 1.5 µM ≥ 24h	Decrease viability Apoptosis Gene modulation	
		0.009-100 µM (24 – 72h)	>0.9 µM ≥ 24h	Decrease lysosome activity Decrease plasma membrane integrity Concomitant effects on mitochondrial area Decrease nuclear count	
	ENN-B1	0- 100 µM (48h)	>5 µM	Inhibit proliferation	

		1.5 - 3 µM (24 – 72h)	≥ 1.5 µM ≥ 24h	Apoptosis Necrosis	
		0.6 - 30 µM (24 – 48h)	>6 µM > 24h	Decrease viability	
	BEA	0- 100 µM (48h)	>3 µM	Inhibit proliferation	
		1.5 - 3 µM (24 – 72h)	≥ 1.5 µM ≥ 24h	Apoptosis Necrosis	
		0.009-100 µM (24 – 72h)	>0.9 µM ≥ 24h	Decrease lysosome activity Decrease plasma membrane integrity Concomitant effects on mitochondrial area Decrease nuclear count	

2. Objectives of the study

Mycotoxins are common natural contaminants present in food and feed samples (FAO 2004), and they occur throughout the world. More than 50% of the world's cereal grains are contaminated with mycotoxins. Despite of many years of research and the introduction of good agricultural and manufacturing practices, mycotoxins occurrence remains a global problem. Their acute and chronic dietary exposures can induce a variety of adverse health effects in humans and animals (Eskola *et al.* 2019). International enquiry's on existing mycotoxins legislation in foodstuffs and animal feedstuffs have been carried out several times (FAO 2004). Nevertheless, so far, only few mycotoxins are regulated worldwide. In European Union, only DON, ZEN, ochratoxin A, patulin, FUM, T-2 and HT-2 toxins are regulated (Lerda 2011). In addition to those regulated mycotoxins, other fungal secondary metabolites have been discovered which are neither routinely determined, nor legislatively regulated and have been defined as emerging mycotoxins (Vaclavikova *et al.* 2013). They are usually co-produced with other well-known toxins (Eskola *et al.* 2019; Hussein and Brasel 2001).

The toxicity of mycotoxins when present together, cannot always be predicted based upon their individual toxicities. Multi-exposure may lead to additive, synergistic or antagonistic toxic effects (Alassane-Kpembi *et al.* 2015). Therefore, analysis of the combined toxicity of mycotoxins is highly important. However, at the present very little is known about the health risk from combined exposure to mycotoxins.

In this study, we aimed to provide additional information about the occurrence of emerging mycotoxins and their combined effects when present in mixture with DON. DON is one of the most widely distributed type B trichothecene contaminants found in food and animal feed (Sprando *et al.* 2005), whereas emerging mycotoxins have been identified but less investigated so far (Vaclavikova *et al.* 2013). In order to do so, pig finished feed samples were collected worldwide market. Then combined toxicity of DON and the 10 most prevalent emerging mycotoxins was assessed on porcine intestinal cell line (IPEC-1), using their concentration observed in the feed.

The second objective of this thesis is driven by the fact that the intestine is not the only target for food contaminants. Indeed, liver which is the main site of metabolism and detoxification of xenobiotics is also a target organ for mycotoxins (Zain 2011). Therefore, we developed a tool, called precision cut liver slices (PCLS), in order to assess the toxicity of mycotoxins on this *ex vivo* model and the tests performed during this thesis were focused on DON.

2.1. Contribution of this doctoral thesis

Among food/feed contaminants, mycotoxins have been regularly classified in the top ten and top one causes of alert notification for RASFF in EU members and non-members countries respectively (RASFF 2018). So far, numerous studies have been carried out on the toxicity of well-known mycotoxins such as DON, ZEN, FUM, AFs whereas, emerging mycotoxins are still poorly investigated.

So far, most of the studies on the occurrence of mycotoxins are performed in cereal grains and raw materials. However, presence and concentration of mycotoxins in compound feed is not always similar as in raw materials (Streit *et al.* 2012). It is important to investigate mycotoxins in compound and finished feed. Furthermore, most of the studies and surveys on the occurrence of mycotoxins are limited to one region or one country (Streit *et al.* 2013; Abdallah *et al.* 2017). Occurrence of mycotoxins and contamination levels across the globe is different and it is important to have a global view on the worldwide occurrence of mycotoxins in feed samples. The aim of this study is to investigate the occurrence of emerging and regulated mycotoxins in finished pig feed worldwide.

Among farm animals, pig is one of the most sensitive species to the deleterious effects of mycotoxins. It can be exposed to high concentration of mycotoxins due to its rich cereal diets (Pinton *et al.* 2010). Following ingestion of contaminated feed, the gastrointestinal tract is the first physiological barrier against food contaminants, as well as the first target for these toxicants (Pinton and Oswald 2014).

The toxicity of known mycotoxins are quite well investigated, the knowledge on the potential toxicity of emerging mycotoxins is still scarce. Therefore, this study aimed at investigating and characterizing the potential toxicity of the 10 most prevalent and DON co-occurring emerging mycotoxins using a model of porcine intestinal epithelial cells. The risk for human and animal health related to the presence of combinations of mycotoxins should not be neglected. In this context, most of the published studies on mixtures have focused on iso-toxicity or combination of identical ratios (1:1), regardless of their ratios in food or feed (Ficheux *et al.* 2012; Prosperini *et al.* 2014b; Fernández-Blanco *et al.* 2016) . To the best of our knowledge, there is no study investigating the combined toxicity based on the ratios concerning the occurrence in the food or feed. Herein, this study will investigate combined toxicity of emerging mycotoxins and DON based on the actual ratios determined in pig finished feed worldwide.

Some of the mycotoxins such as DON can cross the intestinal barrier, reach into the blood circulation and finally reach to the liver. Of the total hepatic blood flow (100–130 ml/min per 100 g of liver, 30 ml/min/kg/b.w), only one fifth to one third is supplied by the hepatic artery. The rest of blood (about two thirds) is supplied by portal vein. This blood contains oxygen and many nutrients brought to the liver from the intestines for processing (Lautt 2010). Therefore, not only the intestine can be a target of mycotoxins, the liver can be also a target organ to mycotoxins. Most of the published studies on the effect of DON on liver are performed either *in vivo* or *in vitro* (Nielsen *et al.* 2009; Sahu *et al.* 2010; Wu *et al.* 2014a; Gerez *et al.* 2015). Although, both models are used in experimentation, but none of them are considered an ideal model. Since *in vitro* assays do not allow assessing more than one cell at a time and *in vivo* assays require large number of animals and is quite costly. In the context of reducing the number of experimental animals, *ex vivo* models represent a powerful tool as many samples can be obtained from one animal (Kolf-Clauw *et al.* 2013). In the *ex vivo* models, the cytoarchitecture should be retained as well as many of the intercellular connections and interplays.

In order to investigate the toxicity of mycotoxins in liver, the precision cut liver slices (PCLS), *ex vivo* explants with well-defined thickness represent a promising model (Graaf *et al.* 2010). They contain all cell types of the tissue in their natural environment and natural architecture, with intercellular and cell-matrix intact interactions (Zimmermann *et al.* 2009). To the best of our knowledge, PCLS have never been used to investigate the toxicity of mycotoxins (Graaf *et al.* 2010). This study will aim developing this new tool in order to assess the toxicity of mycotoxins using an *ex vivo* model of liver explants.

3. Article

Co-Occurrence of DON and Emerging Mycotoxins in Worldwide Finished Pig Feed and Their Combined Toxicity in Intestinal Cells

toxins

MDPI

Article

Co-Occurrence of DON and Emerging Mycotoxins in Worldwide Finished Pig Feed and Their Combined Toxicity in Intestinal Cells

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Abstract: Food and feed can be naturally contaminated by several mycotoxins, and concern about the hazard of exposure to mycotoxin mixtures is increasing. In this study, more than 800 metabolites were analyzed in 524 finished pig feed samples collected worldwide. Eighty-eight percent of the samples were co-contaminated with deoxynivalenol (DON) and other regulated/emerging mycotoxins. The Top 60 emerging/regulated mycotoxins co-occurring with DON in pig feed shows that 48%, 13%, 8% and 12% are produced by *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* species, respectively. Then, the individual and combined toxicity of DON and the 10 most prevalent emerging mycotoxins (brevianamide F, cyclo-(L-Pro-L-Tyr), tryptophol, enniatins A1, B, B1, emodin, aurofusarin, beauvericin and apicidin) was measured at three ratios corresponding to pig feed contamination. Toxicity was assessed by measuring the viability of intestinal porcine epithelial cells, IPEC-1, at 48-h. BRV-F, Cyclo and TRPT did not alter cell viability. The other metabolites were ranked in the following order of toxicity: apicidin > enniatin A1 > DON > beauvericin > enniatin B > enniatin B1 > emodin > aurofusarin. In most of the mixtures, combined toxicity was similar to the toxicity of DON alone. In terms of pig health, these results demonstrate that the co-occurrence of emerging mycotoxins that we tested with DON does not exacerbate toxicity.

Keywords: global survey; finished pig feed; co-occurrence; emerging mycotoxins; DON; toxicity; combined toxicity; IPEC-1

Key Contribution: A worldwide survey of finished pig feed demonstrates the co-occurrence of DON and emerging mycotoxins. Assessment of their combined toxicity with DON at realistic ratios revealed that their toxicity was similar to that of DON alone.

1. Introduction

Mycotoxins are low molecular weight fungal secondary metabolites that trigger a detrimental response when ingested by humans and animals. They are mainly produced by filamentous fungi belonging to *Aspergillus*, *Fusarium* and *Penicillium* species [1]. Mycotoxin contamination can occur all along the food chain from field to storage, including the food process. This depends upon the requirements of fungi, and *Fusarium* mostly occurs in the field, whereas *Aspergillus* and *Penicillium* mostly occurs during storage.

Because of their toxicity and occurrence, deoxynivalenol (DON), zearalenone, aflatoxins, ochratoxin A, patulin, fumonisins and T-2/HT-2 toxins are regulated in Europe. For example, the maximum recommendations limits that are set up for complete piglet feed are 0.9, 0.1, 0.05, 5 and 0.25 mg/kg feed for DON, zearalenone, ochratoxin, fumonisins and T2 + HT2, respectively [2,3]. However, in addition to regulated mycotoxins, many other fungal secondary metabolites are being identified in food and feed [4,5]. Metabolites that are neither routinely determined, nor legislatively regulated, have been defined as 'emerging mycotoxins' [6], while the derivatives of regulated mycotoxins that are undetectable using conventional analytical techniques due to their modified structure, are defined as 'modified/masked mycotoxins' [4,7]. Recent findings showed that more than 70% of the world's cereal grains are contaminated by mycotoxins [8,9], often in a mixture [10].

Among regulated mycotoxins, DON very frequently contaminates cereals (wheat, barley, oats, rye and maize, and less frequently rice, sorghum and triticale) and cereal-based food and feed. DON belongs to the group of B-trichothecenes, and is one of the most widely distributed contaminants in human food and animal feed. In a total of more than 25,000 samples collected from 28 European countries between 2007 and 2014, DON was found in 47% of 4000 feed samples and 45% of 1621 unprocessed grains with no defined end use, respectively [11]. Even though DON is considered as a non-carcinogenic compound [12], the maximum level of this toxin in food and feed have been set up in different countries. For example, in complete piglet feed, the maximum limits are 0.9, 1 and 5 mg/kg feed in Europe, Canada and the USA, respectively [2,13]. Exposure to high concentrations of DON is associated with diarrhea, vomiting (emesis), leukocytosis and gastrointestinal bleeding. Chronic exposure affects growth, immunity and intestinal barrier function in animals [14–16]. This toxin interacts with the peptidyl transferase region of the 60S ribosomal subunit, inducing 'ribotoxic stress', resulting in the activation of mitogen-activated protein kinases (MAPKs) and their downstream pathways [14,17].

Among emerging mycotoxins, those that occur most frequently are enniatins (ENNs), beauvericin (BEA), apicidin (API), aurofusarin (AFN), culmorin, butenolide, fusaric acid, moniliformin, fusaproliferin and emodin (EMO). They are produced by *Fusarium* species except EMO, which is produced by *Aspergillus* species [6,18]. ENNs and BEA were detected in food (63% and 80%), feed (32% and 79%), and unprocessed grains (24% and 46%) collected between 2010 and 2014 in 12 European countries [19]. AFN, API, brevianamide-F (BRV-F), EMO and tryptophol (TRPT) were also found in pig feed (80%, 52%, 65%, 63% and 75%) [20], Egyptian animal feed (73%, 17%, 86%, 98% and 90%) [21] and feed raw materials (84%, 55%, 5%, 74%, 59%) [22].

Multiple mycotoxins are frequently present in food and feed [10]. The co-occurrence of DON, aflatoxins, fumonisins, zearalenone and other fungal secondary metabolites in maize seeds and grains, as well as in animal feed, has been reported [21–23]. The presence of different fungi on the same raw material, the ability of fungal species to produce several toxins, as well as the various commodities present in completed feed, can explain this multiple contamination [24,25]. Compound feed is particularly prone to multiple contaminations, as it typically contains a mixture of several raw materials.

The co-occurrence of mycotoxins is challenging for at least two reasons: (i) The toxicity of mycotoxins when present together cannot always be predicted based upon their individual toxicity and (ii) the risk assessment is performed on a chemical-by-chemical basis [24,25]. Scientific interest in the toxicity of these mixtures of mycotoxins is currently increasing rapidly [26–29]. Several studies have investigated the combined toxicity of regulated mycotoxins on the intestine [30–33], but the combined effect of regulated and other mycotoxins is poorly documented [34,35].

Among farm animals, pig is one of the most sensitive species to mycotoxins [36]. As feed raw materials are potentially contaminated by several fungi at a time, and completed feed is made from various commodities, pig can be exposed, through its rich cereal diet, to high concentrations of mixtures of mycotoxins [10,37]. The sanitary and economic losses due to mycotoxin contamination are important in the pig industry, even if they are hard to estimate precisely [38].

The aims of this study were thus to (i) determine the prevalence and concentration of mycotoxins present in finished pig feed and (ii) assess the intestinal combined toxicity of DON in mixture with the 10 most prevalent emerging mycotoxins present in pig feed using realistic ratios.

2. Results

2.1. Occcurrence and Abundance of Emerging Mycotoxins and DON in Finished Pig Feed

A total of 524 finished pig feed samples collected worldwide were analyzed, and more than 235 different metabolites were detected, including regulated mycotoxins, emerging mycotoxins and modified/masked mycotoxins. Table 1 lists the 60 most prevalent fungal metabolites that contaminated more than 20% of the finished pig feed samples. Among regulated mycotoxins, DON was detected in 463 samples (88%), mostly in the Northern Hemisphere and in relatively similar concentrations in samples from all countries (median concentration 206 µg/kg) (Figure 1A,B).

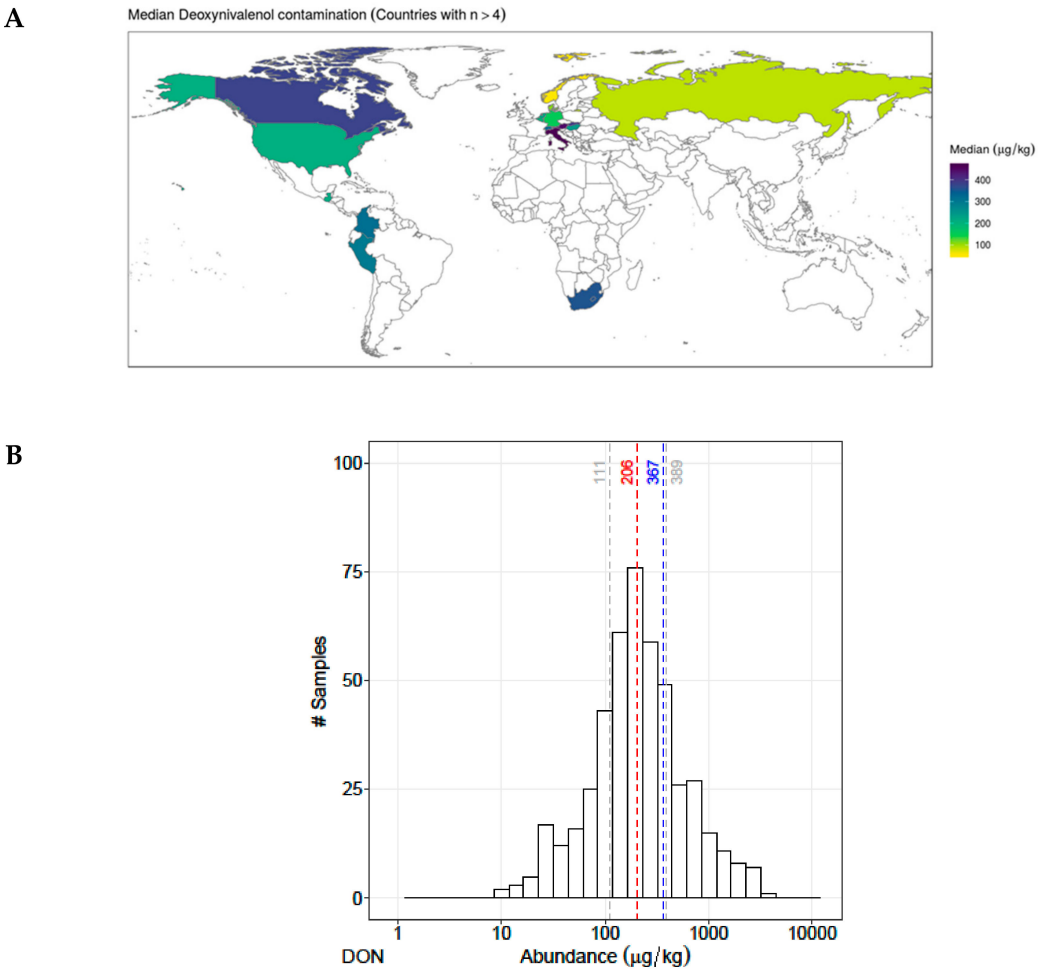

Figure 1. (**A**) Worldwide contamination of deoxynivalenol (DON). Concentration of DON is highlighted by colors, where yellow indicates > 100 µg/kg, green > 200 µg/kg, dark green > 300 and dark blue indicates > 400 µg/kg. (**B**) Abundance of DON in pig finished feed (P25, median, mean, P75). X axis represents the distribution of the concentration, Y axis describes the number of contaminated samples.

All DON-contaminated samples were co-contaminated by other mycotoxins. The distribution of the samples was checked. Table 1 gives the descriptive statistics for DON and the most abundant metabolites that co-occur with DON.

Table 1. Top 60 emerging and regulated mycotoxins co-occurring with DON in finished pig feed.

	Metabolites	Occurrence *n* (%)	Co-occurrence with DON *n* (%)	Contamination Level (µg/kg Feed)			Correlation (DON and Other Mycotoxins)	
				P25	P50	P75	Coefficient	*p*-Value
1	Deoxynivalenol	463 (88%)	463 (100%)	111	206	389	1.00	NA
2	Culmorin	492 (94%)	458 (99%)	38	107	247	0.50	0.00
3	Zearalenone	502 (96%)	449 (97%)	9	18	46	0.64	0.00
4	Brevianamide F	500 (95%)	446 (96%)	17	28	45	0.17	0.00
5	Cyclo-(L-Pro-L-Tyr)	494 (94%)	434 (94%)	117	196	371	0.14	0.00
6	Enniatin B	479 (91%)	430 (93%)	9	32	83	0.02	0.61
7	Enniatin B1	481 (92%)	430 (93%)	10	37	87	0.04	0.39
8	Asperglaucide	470 (90%)	419 (90%)	18	38	94	-0.11	0.02
9	Emodin	472 (90%)	418 (90%)	3	5	10	-0.01	0.91
10	Aurofusarin	464 (89%)	417 (90%)	87	211	548	0.59	0.00
11	Moniliformin	469 (90%)	416 (90%)	7	17	45	0.11	0.03
12	Beauvericin	464 (89%)	411 (89%)	4	7	13	0.32	0.00
13	Enniatin A1	459 (88%)	411 (89%)	5	16	33	0.09	0.08
14	3-Nitropropion acid	455 (87%)	407 (88%)	3	6	10	-0.03	0.57
15	Tryptophol	454 (87%)	407 (88%)	119	197	319	0.10	0.04
16	15-Hydroxyculmorin	429 (82%)	391 (84%)	76	142	277	0.79	0.00
17	Equisetin	424 (81%)	386 (83%)	5	10	23	0.01	0.80
18	Infectopyron	409 (78%)	366 (79%)	108	263	449	-0.16	0.00
19	DON-3 Glucoside	380 (73%)	362 (78%)	10	21	47	0.79	0.00
20	Neoechinulin A	407 (78%)	357 (77%)	10	19	42	0.06	0.22
21	Tenuazonic-acid	384 (73%)	347 (75%)	53	90	182	0.04	0.49
22	Alternariol	366 (70%)	333 (72%)	2	4	9	0.01	0.79
23	Rugulusovin	373 (71%)	332 (72%)	4	7	14	0.15	0.01
24	Tentoxin	342 (65%)	319 (69%)	2	3	6	-0.03	0.56
25	Apicidin	341 (65%)	310 (67%)	3	7	11	-0.13	0.02
26	Fumonisin B1	332 (63%)	304 (66%)	26	70	254	0.14	0.02
27	Nivalenol	315 (60%)	296 (64%)	10	24	57	0.14	0.02
28	Cyclo-(L-Pro-L-Val)	337 (64%)	286 (62%)	85	137	246	0.15	0.01
29	Epiequisetin	307 (59%)	285 (62%)	2	4	7	0.05	0.42
30	Citreorosein	317 (60%)	283 (61%)	3	5	8	0.12	0.05
31	Enniatin A	306 (58%)	282 (61%)	2	3	5	-0.01	0.81
32	Alternariolmethylether	307 (59%)	275 (59%)	2	3	5	0.09	0.14
33	Altersetin	301 (57%)	275 (59%)	13	29	76	0.18	0.00
34	Asperphenamate	311 (59%)	269 (58%)	5	11	27	-0.02	0.75
35	Lotaustralin	288 (55%)	257 (56%)	15	30	85	-0.10	0.13
36	Butenolid	253 (48%)	242 (52%)	22	37	70	0.32	0.00
37	Kojic acid	262 (50%)	241 (52%)	43	74	148	-0.06	0.39
38	Enniatin B2	258 (49%)	238 (51%)	2	3	5	-0.01	0.84
39	Fumonisin B2	258 (49%)	237 (51%)	19	50	143	0.16	0.01
40	Zearalenone Sulfate	236 (45%)	222 (48%)	10	25	53	0.25	0.00
41	Antibiotic Y	233 (44%)	215 (46%)	40	111	402	-0.02	0.75
42	T2 Toxin	235 (45%)	209 (45%)	2	4	9	0.12	0.07
43	Macrosporin	219 (42%)	202 (44%)	2	3	8	0.02	0.76
44	N-Benzoyl-Phenylalanine	220 (42%)	191 (41%)	3	5	11	-0.02	0.82
45	Flavoglaucin	206 (39%)	175 (38%)	7	16	34	0.05	0.51
46	Curvularin	196 (37%)	171 (37%)	2	4	8	-0.09	0.27
47	Questiomycin A	178 (34%)	162 (35%)	4	10	20	0.22	0.01
48	Rubellin D	179 (34%)	161 (35%)	4	8	18	0.10	0.21
50	Bikaverin	171 (33%)	153 (33%)	10	25	56	0.27	0.00
50	Fusarinolic-acid	157 (30%)	153 (33%)	47	130	320	0.3	0.00
51	Fumonisin B4	165 (31%)	149 (32%)	11	23	68	0.2	0.03
52	Cytochalasin J	170 (32%)	146 (32%)	13	29	63	0.1	0.46
53	Ergometrine	152 (29%)	145 (31%)	6	11	24	0.0	0.57
54	Ergocristine	151 (29%)	143 (31%)	2	5	13	0.2	0.02
55	Fumonisin B3	154 (29%)	136 (29%)	24	48	103	0.1	0.15
56	HT2-toxin	149 (28%)	134 (29%)	13	20	30	0.2	0.01
57	Monocerin	144 (27%)	133 (29%)	1	2	3	0.2	0.02
58	Chrysogin	136 (26%)	126 (27%)	7	12	17	0.4	0.00
59	Ergosin	128 (24%)	123 (27%)	3	6	13	-0.1	0.39
60	5-Hydroxyculmorin	121 (23%)	117 (25%)	107	170	304	0.7	0.00

The 60 mycotoxins found in more than 20% of the 524 samples of finished pig feed. Their concentrations in the three quartiles (P25, P50 and P75) are expressed in µg/kg of feed. The correlation of their concentration with DON and the associated P-value was calculated using Spearman's rank correlation coefficient.

From this list, the most prevalent emerging mycotoxins co-occurring with DON and which were commercially available were selected for the toxicological studies. Because of its high toxicity [39],

apicidin (API) was included in the list. The worldwide distributions of these compounds are presented in Supplementary Figure S1. Except for API, which was detected in only 67% of DON-contaminated samples, the selected emerging mycotoxins were present in more than 87% of them (Table 1). Three compounds API, emodin (EMO) and beauvericin (BEA) were detected in a median concentration range of 5 to 10 μg/kg feed. The median concentration of four metabolites, enniatins A1, B, B1 (ENN-A1, B, B1) and brevianamide F (BRV-F), was in the range of 15-40 μg/kg feed, and the last three compounds aurofusarin (AFN), cyclo-(L-Pro-L-Tyr) (Cyclo) and tryptophol (TRPT) had median concentrations close to 200 μg/kg feed, like DON (Supplementary Figure S2). Despite their high co-occurrence with DON, with the exception of AFN, and to a lesser extent BEA, the concentration of these mycotoxins showed limited correlation with DON concentration (Table 1 and Supplementary Figure S3).

2.2. Intestinal Toxicity of Emerging Mycotoxins Found in Pig Feed, alone or Combined with DON

2.2.1. Individual Toxicity of DON and Emerging Mycotoxins

The individual intestinal toxicity of 10 selected emerging mycotoxins, as well as that of DON, was first analyzed at a wide range of concentrations (Supplementary Figure S4). As shown in Supplementary Figure S4, all tested metabolites exhibited dose-dependent toxicity toward intestinal epithelial cells, except BRV-F, Cyclo and TRPT, that were not toxic for this porcine cell line. AFN, EMO and ENN-B1 reduced the viability of IPEC-1, but their toxicity was less than that of DON. The toxicity of BEA and ENN-B was close to that of DON, whereas API and ENN-A1 were more toxic than DON. As shown in Figure 2, low doses of API (0.01–0.3 μM) significantly stimulated the proliferation of IPEC-1. When the doses leading to a 50% reduction in the cell viability (IC_{50}) of these emerging mycotoxins were compared with the dose of DON, then BRV-F, Cyclo and TRPT were classified as non-toxic metabolites, while EMO, AFN and ENN-B1 were given as moderately toxic metabolites, and finally API, ENN-A1, ENN-B and BEA as highly toxic metabolites (Table 2).

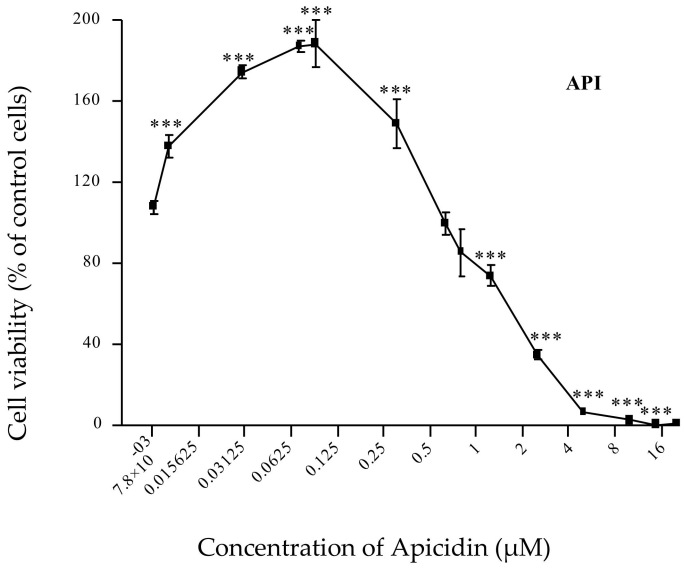

Figure 2. Dose effect curve of individual toxicity of apicidin (API). Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference between control and different doses of API *** $p < 0.001$.

Table 2. IC_{50} values of the selected emerging mycotoxins on IPEC-1 cells.

Metabolites	Abbreviation	IC_{50} (μM)	Toxicity
Brevianamide F	BRV-F	Non-Toxic	Non-toxic
Cyclo-(L-Pro-L-Tyr)	Cyclo	Non-Toxic	
Tryptophol	TRPT	Non-Toxic	
Aurofusarin	AFN	19.1 ± 3.4	Moderately toxic
Emodin	EMO	19.0 ± 0.7	
Enniatin B1	ENN-B1	13.5 ± 2.5	
Enniatin B	ENN-B	4.4 ± 0.9	Highly toxic
Beauvericin	BEA	4.3 ± 1.8	
Deoxynivalenol	DON	3.2 ± 0.7	
Enniatin A1	ENN-A1	1.6 ± 0.3	
Apicidin	API	1.5 ± 0.5	

Data are the mean ± the standard error of the mean (SEM) of three biological replicates.

2.2.2. Combined Toxicity of DON and Emerging Mycotoxins

Next, the combined toxicity of DON and the selected emerging mycotoxins was assessed. As mentioned above, the concentration of these secondary metabolites was not correlated with the concentration of DON (Table 1 and Supplementary Figure S3). Thus, to account for the different situations to which pigs can be exposed, three ratios were tested (Supplementary Table S1). Ratio 1 was calculated using the P25 (1st quartile) concentration of the emerging mycotoxin and P75 (3rd quartile) concentration of DON. Ratio 2 was calculated using the median (2nd quartile) concentration of DON and each emerging mycotoxin. Ratio 3 was calculated using the P75 concentration of the emerging mycotoxin and P25 concentration of DON. For each ratio, serial dilutions were tested to obtain a dose-effect curve that encompassed the realistic concentrations of the mixture of DON and the tested metabolites.

2.2.3. Combined Toxicity of DON and the Non-Toxic Secondary Metabolites (BRV-F, Cyclo and TRPT)

First, the combined toxicity of DON and the 'non-toxic' secondary metabolites BRV-B, Cyclo and TRPT were analyzed. As shown in Figure 3, whatever the ratios tested, the toxicity of the combination of DON and the compound being tested was similar to the toxicity of DON alone.

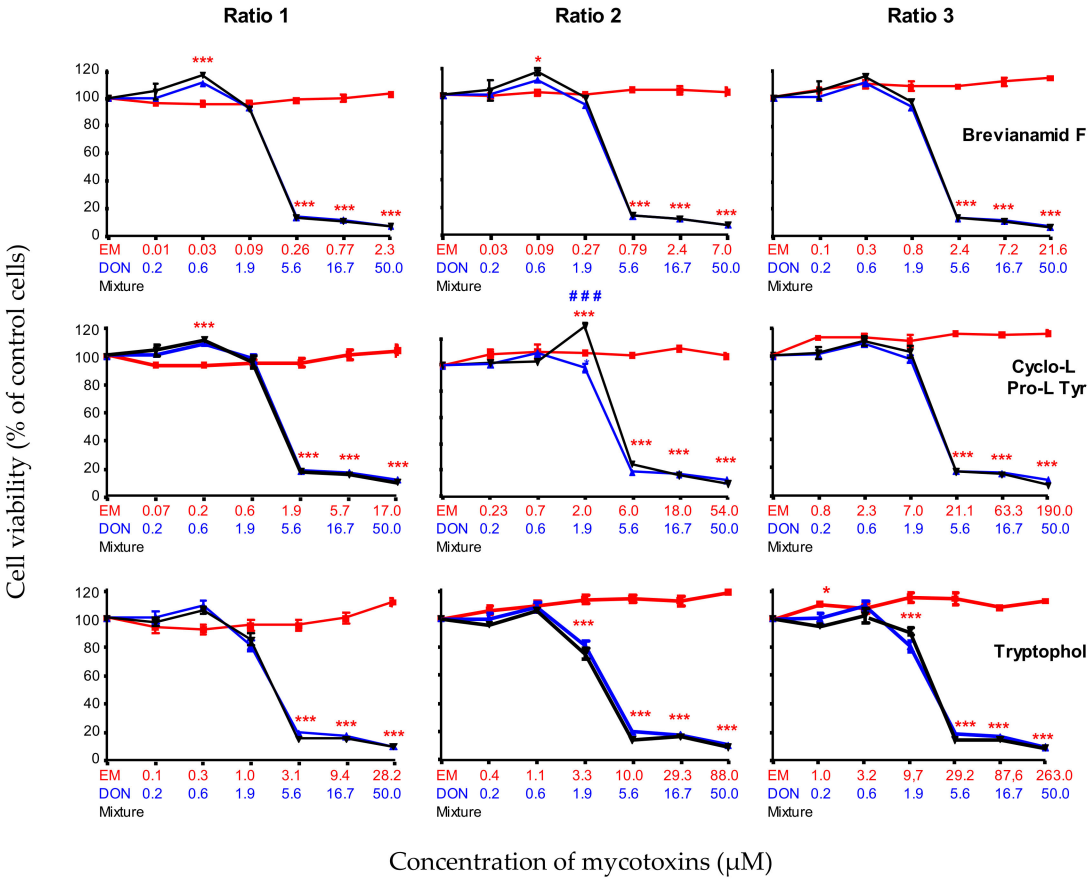

Figure 3. Dose-effect curves of deoxynivalenol (DON) (blue lines and symbols), emerging mycotoxins (brevianamide-F (BRV-F), cyclo-(L-Pro-L-Tyr) (Cyclo) and tryptophol (TRPT)) alone (red lines and symbols), or in combination with DON (black lines and symbols) at different ratios: ratio 1 was calculated from the P25 concentration of emerging mycotoxin and P75 concentration of DON; ratio 2 was calculated from the median concentration of emerging mycotoxin and DON; ratio 3 was calculated from the P75 concentration of emerging mycotoxin and the P25 concentration of DON. Six serial dilutions of each ratio were tested (Emerging mycotoxin alone, DON alone, mixture). Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference between emerging mycotoxins alone and the mixtures *** $p < 0.001$. Significant difference between DON alone and the mixtures # # # $p < 0.001$.

2.2.4. Combined Toxicity of DON and the Moderately Toxic Secondary Metabolites (AFN, EMO and ENN-B1)

Next, the combined toxicity of DON and the moderately toxic metabolites AFN, EMO and ENN-B1 was assessed (Figure 4). The toxicity of AFN, EMO and ENN-B1 was minimal when used at ratio 1. In these conditions, the toxicity of the combination of DON and emerging toxins was similar to the toxicity of DON alone. Ratio 2 reached toxic concentrations of AFN and ENN-B1, but the toxicity of the mixture was similar to the toxicity of DON alone, except at the highest concentration of ENN-B1 (4.1 µM), where the toxicity of the mixture (ENN-B1 4.1 µM + DON 50 µM) was higher than the toxicity of ENN-B1 alone, but lower than the toxicity of DON alone.

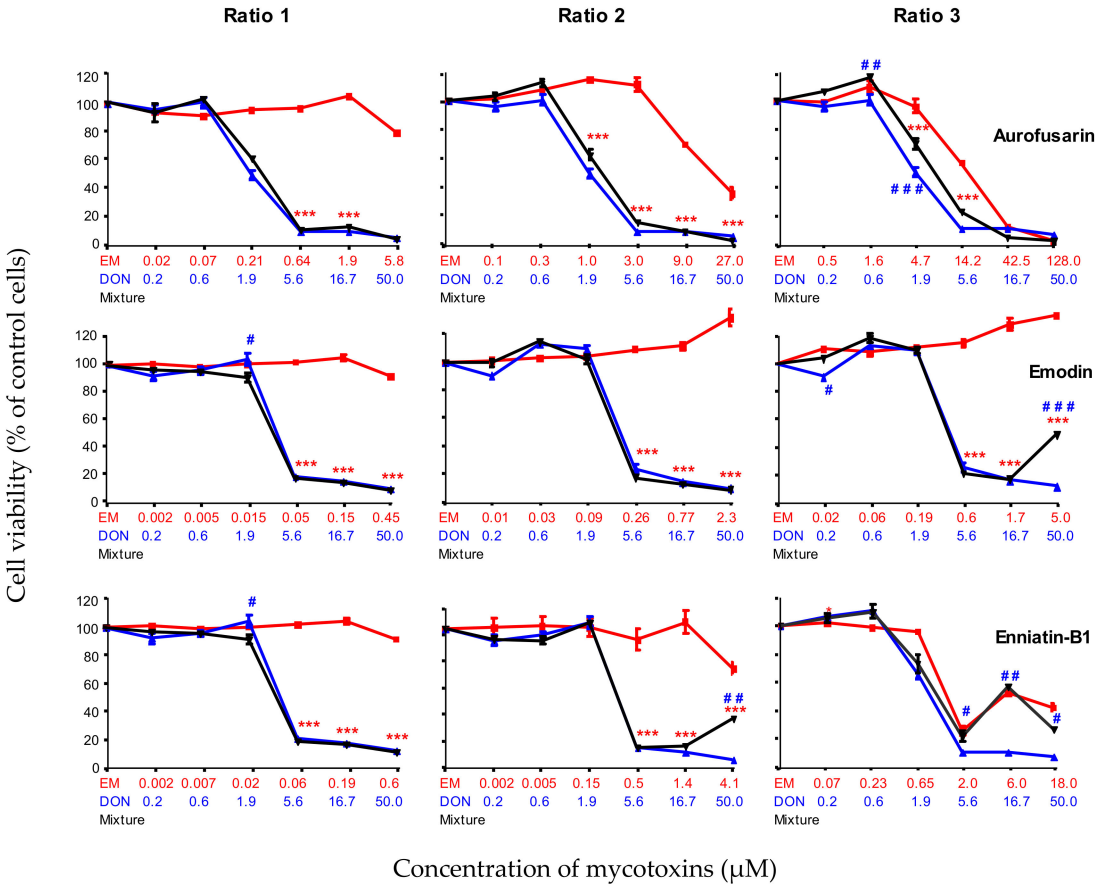

Figure 4. Dose-effect curves of DON (blue lines and symbols), emerging mycotoxins (AFN, EMO and ENN-B1) alone (red lines and symbols) or in combination with DON (black lines and symbols) at different ratios: ratio 1 was calculated from the P25 concentration of emerging mycotoxin and P75 concentration of DON; ratio 2 was calculated from the median concentration of emerging mycotoxin and DON; ratio 3 was calculated from the P75 concentration of emerging mycotoxin and P25 concentration of DON. Six serial dilutions of each ratio were tested (Emerging mycotoxin alone, DON alone, mixture). Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference between emerging mycotoxins alone and the mixtures *** $p < 0.001$. Significant difference between DON alone and the mixtures # # # $p < 0.001$.

When cytotoxicity was tested at ratio 3, the toxicity of AFN + DON was identical to the one of DON alone except at the concentrations DON 0.6 µM + AFN 1.6 µM and DON 1.9 µM + AFN 4.7 µM, when the toxicity of the mixture was slightly lower than the toxicity of DON alone. At this ratio, EMO alone was still not toxic, and even induced proliferation (up to 130% of treated cells).

The combined toxicity of DON and EMO was similar to the toxicity of DON alone except at the highest concentration of EMO, when the mixture of DON (50 µM) and EMO (5 µM) was still less toxic than DON alone. The combined toxicity of ENN-B1 + DON was the same as the toxicity of DON alone, except at the highest concentrations of ENN-B1 (6 and 18 µM), when the toxicity of the mixture was the same as the toxicity of ENN-B1 alone, but lower than the toxicity of DON alone.

In conclusion, our data showed that the toxicity of the combination of DON and emerging mycotoxins such as AFN, EMO and ENN-B1 was similar to or lower than the toxicity of DON alone, whatever the ratio used.

2.2.5. Combined Toxicity of DON and The Highly Toxic Secondary Metabolites (ENN-B, BEA, ENN-A1and API)

The combined toxicity of DON and highly toxic compounds (ENN-B, BEA, ENN-A1 and API) was also analyzed at different ratios (Figure 5). At the first tested ratio (ratio 1), ENN-B, BEA, ENN-A1 and API were not toxic, and their combined toxicity in the presence of DON was similar to the toxicity of DON alone. Ratio 2 reached toxic concentrations of ENN-B, ENN-A1 and API.

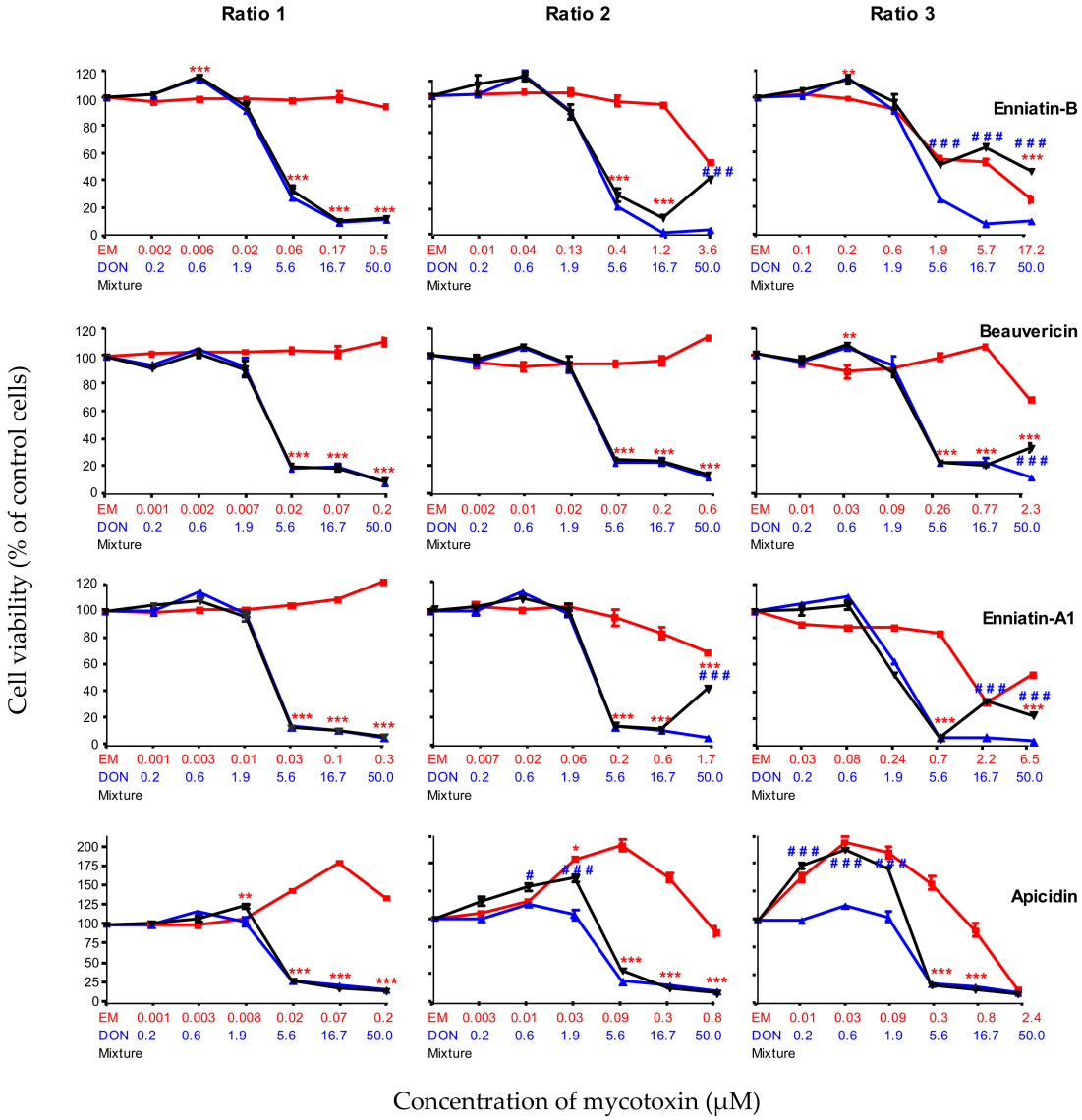

Figure 5. Dose-effect curves of DON (blue lines and symbols), emerging mycotoxins (ENN-B, BEA, ENN-A1 and API) alone (red lines and symbols) or in combination with DON (black lines and symbols) at different ratios: ratio 1 was calculated from the P25 concentration of the emerging mycotoxin and the P75 concentration of DON; ratio 2 was calculated from the median concentration of emerging mycotoxin and DON; ratio 3 was calculated from the P75 concentration of emerging mycotoxin and the P25 concentration of DON. Six serial dilutions of each ratio were tested (Emerging mycotoxin alone, DON alone, mixture). Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference between emerging mycotoxins alone and the mixtures *** $p <$ 0.001. Significant difference between DON alone and the mixtures # # # $p < 0.001$.

For ENN-B and ENN-A1, the toxicity of the mixture was similar to the toxicity of DON alone, except at the highest concentrations of ENN-B (3.6 μM) and ENN-A1 (1.7 μM). The combined toxicity

of DON (50 μM) + ENN-B (3.6 μM) was similar to the toxicity of ENN-B alone, but lower than the toxicity of DON alone, whereas the combined toxicity of DON (50 μM) + ENN-A1 (1.7 μM) was higher than the toxicity of ENN-A1 alone, but still lower than the toxicity of DON alone. The combined toxicity of API and DON was similar to the toxicity of DON only for the higher doses. For the doses lower than DON (5.6 μM) + API (0.09 μM) the combined toxicity was lower than the toxicity of DON alone, but higher than API alone.

At ratio 3, the combined toxicity of DON and ENN-B displayed a different characteristic. At a high concentration, the combined toxicity of DON (50 μM) + ENN-B (17.2 μM) was lower than the toxicity of either DON alone or ENN-B alone. At this ratio, the toxicity of the mixture of DON and BEA was similar to the toxicity of DON alone, except at the highest concentration of BEA (2.3 μM) when mixed with DON (50 μM), where the toxicity was higher than that of BEA alone, but lower than that of DON alone. The combined toxicity of DON (16.7μM) + ENN-A1 (2.2 μM) at ratio 3 was similar to the toxicity of ENN-A1 alone, but lower than the toxicity of DON alone; the toxicity of DON (50 μM) + ENN-A1 (6.5 μM) was higher than the toxicity of ENN-A1 alone, but lower than the toxicity of DON alone. Finally, the combined toxicity of DON and API, at concentrations lower than DON (5.6 μM) + API (0.3 μM), was always similar to API alone, but lower than DON alone, whereas at higher concentrations, it was similar to the one of DON alone and higher than the one of API alone. As mentioned above, a proliferation of IPEC-1 cells was observed at low concentrations of API.

In conclusion, our data showed that the toxicity of the combination of DON and highly toxic emerging mycotoxins such as ENN-B, BEA, ENN-A1 and API, whatever the ratio used, was similar to or lower than the toxicity of DON alone.

3. Discussion

Progress in analytical methods enabled the discovery of numerous fungal secondary metabolites that are the subject of increasing attention today due to their prevalence in human food and animal feed [19,20]. In the present study, 524 samples of finished pig feed were analyzed. In addition to regulated mycotoxins such as DON, zearalenone and fumonisin B1, less known secondary metabolites were detected.

As already described in other surveys [20–22], BRV-F, Cyclo, TRPT, ENNs, EMO, BEA and AFN, culmorin, and moniliformin were highly prevalent emerging mycotoxins detected in more than 85% of pig feed samples. The diversity of the metabolites detected is very likely related to the wide range of fungal species that contaminate the raw materials used to make pig feed. Indeed, *Fusarium* species produce ENNs (A1, B and B1), BEA, AFN, API, culmorin and moniliformin, while *Penicillium* species produce BRV-F and Cyclo, *Aspergillus* EMO and *Acremonium* TRPT [22,40].

The toxicity of these new poorly documented metabolites was also investigated in the present study. The results of our analyses showed that, even at high concentrations of up to 300 μM, BRV, Cyclo and TRPT are not toxic to intestinal cells. Similar results were recently obtained using another porcine intestinal cell line, IPEC-J2, and a different readout, cellular protein content [20]. Interestingly, at a much higher concentration (2 mM), TRPT induced DNA damage in HepG2, A549 and THP-1 cells [41]. AFN and EMO were identified as moderately toxic compounds at relative IC_{50} values of 19.1 μM and 19 μM, respectively. These emerging mycotoxins were found to be more toxic for IPEC-J2 with relative IC_{50} of 9.3 μM and 13.1 μM, respectively [20]. On the other hand, human multiple myeloma blood cells were less sensitive to EMO (IC_{50} 38 μM) [42]. According to their IC_{50}, ENNs were ranked in the following order of toxic potency ENN-A1 > ENN-B > ENN-B1. Similar ranking was reported for HT-29 [43] and IPEC-J2 [44]. ENN-A1 is also more toxic than ENN-B1 for Caco-2 and HepG2, but ENN-B displayed no toxicity at all [43]. The mechanism of toxicity of ENNs is related to their ionophoric properties [19] that facilitate the transport of mono- or divalent cations such as K^+ or Ca^{2+} across membranes, but the relative sensitivity of the different cell lines to the different ENNs is still not understood. The high toxicity of BEA has already been reported in other cell lines of human and porcine origin, including Caco-2, HT-29 and IPEC-J2 [20,43]. BEA also has ionophoric properties

and increase ions permeability (Na^+ K^+ and Ca^{2+}) in biological membrane, this mechanism of action participates to the toxicity of this mycotoxin [6,19,44].

Data on the toxicity of API are very limited. We confirmed the toxicity of this metabolite when used at high concentration (> 0.5 µM) [20,39]; we also observed that low concentrations of API (<0.1 µM) stimulate the proliferation of porcine intestinal cells. The proliferative effect of low doses of some mycotoxins has already been described. For example, up to 200% proliferation has been observed in lymphocytes and splenocytes exposed to low doses (> 0.1µM) of DON, nivalenol, aflatoxin B1 or fumonisin B1 [45]. Because of its high prevalence and its low IC_{50}, it would be of great interest to deepen our knowledge of API. The toxicity of other very prevalent metabolites, such as culmorin and moniliformin, could not be evaluated in this study because they were not commercially available in the quantities required for cellular experiments.

Different studies have described the co-occurrence of mycotoxins in cereals and other raw materials used for animal feed in several regions of the globe [21,46,47]. In the present study, we investigated the co-occurrence of mycotoxins in pig feed. We identified 60 metabolites that co-occur with DON in more than 20% of samples, confirming the prevalence of co-contamination, and as a result, the exposure of animals to a mixture of mycotoxins. Our results are in accordance with those of previous surveys of raw materials and finished feed [20,46]. Although the exact composition of the analyzed pig feed is not known, the main component of the majority of the samples is maize. The extraction efficiencies are between 85%-95% determined in seven different matrices [48]. However, the focus of our study is to provide a general picture of the worldwide contamination of pig feed and to test realistic ratios of fungal compounds in vitro.

Despite the very high frequency of co-contamination by DON and emerging mycotoxins, the correlation between the concentrations of DON and emerging mycotoxins was very low. The correlation between DON and emerging mycotoxins is poorly documented. In winter wheat, Blandino and co-workers [49] addressed the correlation between DON and other mycotoxins produced by *Fusarium graminearum* and *F. culmorum*, and showed that correlations between DON and either culmorin or moniliformin were significant (0.94 and 0.42, respectively). By contrast, the correlations between DON and AFN or BEA were not significant (0.2 and -0.14, respectively). Correlations between the concentration of DON and its modified forms enabled EFSA to calculate ratios between these different toxins [11].

In the present study, the absence of correlation between the concentrations of the emerging mycotoxins and DON could be explained by the diversity of fungi that produce the various metabolites via different biosynthetic pathways. Furthermore, pigs feed is made of different raw materials, which also explains the lack of correlation between the amounts of the different fungal metabolites involved.

The main objective of the present study was to assess the combined toxicity of DON and emerging mycotoxins. As no correlation was found, to encompass the situations to which animals may be exposed, different plausible ratios were tested. These ratios were based on the P25, the median and the P75 concentrations of DON and emerging mycotoxins observed in pig feed, because these summary statistics are robust to extreme outlier values. In most cases, we observed that when the non-toxic metabolites (BRV, Cyclo and TRPT) were present in mixture with DON, whatever the doses or the ratio, the effect was driven by DON. We observed a similar trend for the combined toxicity of DON with moderate and highly toxic metabolites ENNs, BEA, API, AFN and EMO. The effect of the mixtures was mostly similar to the effect of DON alone. The only exception was when very high concentrations of DON were used, in which cases surprisingly, the toxicity of the mixture was lower than the toxicity of DON alone.

To the best of our knowledge, this is the first study to investigate the interactions of different mycotoxins using plausible ratios. Most published studies used toxins of equal toxicity, regardless of their concentration in food or feed. For example, exposure of Caco-2 or IPEC-1 cells to low doses of DON, combined with isotoxic concentrations of nivalenol and/or their acetylated derivatives, led to a synergistic effect [31,32]. Similarly, mixtures of ENNs as well as mixtures of DON, ENNs and alternariol

managed to induce synergistic cytotoxicity in Caco-2 cells [50,51]. A synergistic inflammatory effect was also observed in porcine intestinal explants co-exposed to DON and nivalenol [33].

In the future, these original data on the intestinal toxicity of realistic mixtures of DON and emerging mycotoxins should be completed by toxicity studies on other types of cells to account for all target organs. In vivo experiments are also needed to confirm the in vitro data. More widely, the effects of other mixtures of mycotoxins or of mycotoxins with other food contaminants should be investigated at realistic doses. As the pig is a good model for human toxicity studies of food contaminants [52], the results would be useful to estimate the effects of similar mixtures of toxins on human health. Better knowledge of the occurrence and toxicity of the real mixtures present in food is a precondition for the assessment of health risk [29].

4. Conclusions

This global survey of finished pig feed confirmed that such feed is co-contaminated by DON and emerging mycotoxins. However, despite the high percentages of co-occurrence, no correlation was found between the concentration of DON and most of these emerging mycotoxins. Using ratios based on the concentration of DON and emerging mycotoxins in feed, we observed that the toxicity of most of the mixtures was similar to the toxicity of DON alone. This demonstrates that, when these emerging mycotoxins are present together with DON, the toxicity of the mixture is not exacerbated.

5. Materials and Methods

5.1. Extraction and Analysis of Metabolites

A total of 524 finished pig feed samples were collected from 2014 to 2018 on the world market, but most in Europe (76.5%) and North America (15.8%) and fewer in Asia (3.2%), South Africa (1.5%), Australia (2 samples) and some of unknown origin (2.5%) (Figure 1 and Supplementary Figure S1), and more than 800 analytes, including fungal and bacterial secondary metabolites, were sought. Samples were provided by the BIOMIN Mycotoxin Survey, and the analyses were performed using the Liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS) multi-mycotoxin method described by Malachova [5].

A QTrap5500 LC-MS/MS System (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 Series ultra high performance liquid chromatography (UHPLC) System (Agilent Technologies, Waldbronn, Germany) was used for detection and quantification of the analytes (Supplementary Figures S5 and S6). Chromatographic separation was performed on a Gemini® C18-column (150 × 4.6 mm i.d., 5 µm particle size) equipped with a C18 security guard cartridge (4 × 3 mm i.d.) (all from Phenomenex, Torrance, CA, USA) at 25 °C. Elution was carried out in binary gradient mode. Both mobile phases contained 5 mm ammonium acetate ($NH_4CH_3CO_2$) and were composed of methanol/water/acetic acid in a ratio of 10:89:1 (*v*/*v*/*v*; eluent A) or 97:2:1 (*v*/*v*/*v*; eluent B). After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 50% within 3 min. Further linear increase of B to 100% within 9 min was followed by a hold-time of 4 min at 100% B and 2.5 min column re-equilibration at 100% A. The flow rate was 1000 µL/min. ESI-MS/MS was performed in the scheduled multiple reaction monitoring (sMRM) mode both in positive and negative polarities in two separate chromatographic runs. The sMRM detection window of each analyte was set to the respective retention time ± 27 s and ± 42 s in positive and in negative mode, respectively. The target scan time was set to 1 s. Confirmation of positive analyte identification is obtained by the acquisition of two sMRMs per analyte (with the exception of moniliformin and 3-nitropropionic acid, that each exhibit only one fragment ion), which yields 4.0 identification points according to commission decision 2002/657/EC (EU, 2002).

A total of 235 mycotoxins and other fungal secondary metabolites were detected and quantified in the samples of finished pig feed analyzed. The threshold of relevant concentrations was set at > 1.0 µg/kg or the limit of detection, whichever was higher. Samples were collected only by trained staff,

or after the instruction of untrained staff according to a protocol. A minimum of 500 g homogenized sample was sent to the laboratory of the Institute of Bioanalytics and Agro-Metabolomics at the University of Natural Resources and Life Sciences Vienna (BOKU) in Tulln, Austria. Samples were milled and extracted with a mixture of acetonitrile, water and acetic acid (79:20:1, per volume) on a shaker for 90 min. The solution was centrifuged, after which the supernatant was diluted and injected into an LC-MS/MS system (electrospray ionization and mass spectrometric detection using a quadrupole mass filter). Quantification was done by comparing an external calibration using a multi-analyte stock solution. During the period 2014 to 2018, the number of substances measured using this method increased each year, and more substances were included in the survey. Nevertheless, the list of compounds investigated in this manuscript were those measured in 2014. All concentration data were collected in a single file, and sample information such as sampling year and month, country and region of origin and sample matrix were added for subsetting. Data were imported and analyzed in R v 3.5.1 mainly using tidy-verse packages (www.tidyverse.org). Spearman correlation coefficients and associated *p*-values were calculated with the corr.test function from the psych R package. Data were plotted (including maps) using ggplot2.

5.2. Toxins

For the cytotoxicity test, toxins were purchased from Sigma (St Quentin Fallavier, France): deoxynivalenol (DON) (purity > 98%), tryptophol (TRPT) (purity > 97%), apicidin (API) (purity > 98%) and emodin (EMO) (purity > 90%). Enniatins (ENNs) (A1, B, B1, purity > 99%), cyclo-(L-Pro-L-Tyr) (Cyclo) (purity > 98%), and brevianamide-F (BRV-F) (purity > 95%) were purchased from BioAustralis (Smithfield, Australia), beauvericin (BEA) (purity > 95%) and aurofusarin (AFN), (purity > 97%) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). All mycotoxins were dissolved in dimethyl sulfoxide (DMSO) (Sigma) to prepare stock solutions stored at −20 °C. Working dilutions were freshly prepared in cell culture medium for each experiment.

To convert the concentration in the pig feed into the concentration to which intestinal cells might be exposed, we assumed, as already done in previous studies [53], that mycotoxins were ingested in one meal, diluted in 1 L of gastrointestinal fluid and were entirely bioaccessible. Next, the ratio of DON to emerging mycotoxins was calculated based on three plausible scenarios according to the concentration of DON and emerging mycotoxins in the feed (Supplementary Table S1).

Several 3-fold dilutions of each individual toxin and mixtures at different ratios were performed to account for the concentrations present in feed.

5.3. Cell Culture and Cytotxicity Assay

IPEC-1, derived from the small intestine of a newborn unsuckled piglet were maintained in complete medium (Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 medium (Sigma) plus 1% Penicillin Streptomycin (Eurobio, Courtaboeuf, France), 5% fetal bovine serum (FBS) (Eurobio), 1% L-glutamine (Eurobio), 5 µg/L epidermal growth factor (EGF) (Becton–Dickinson, Le Pont de Claix, France), and 1% ITS solution (insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 ng/mL), (Sigma Aldrich)) at 39 °C under 5% CO_2, as previously described [54].

For the cytotoxicity experiments, cells were seeded in 96-white-well flat-bottom cell culture plates (Costar, Cambridge, MA, USA) at a rate of 10^4 cells per well in 100 µL culture medium. After 24 h, the medium was replaced by complete medium without FBS containing the mycotoxins and incubated for a further 48 h. Toxicity was then assessed using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Charbonnières-les-Bains, France) that determine the number of viable cells based on the quantitation of adenosine triphosphate (ATP). The luminescent signal produced by the luciferase reaction, reflecting the presence of metabolically active cells, was read using a multiplate reader (TECAN, Lyon, France). The results were obtained by calculating the percentage of viability obtained by calculating the ratio of the luminescence in treated samples and the luminescence in non-treated samples.

5.4. Statistical Analysis

The reported values on viability are expressed as the means ± the standard error of the mean (SEM) of at least three biological replicates, with duplicate wells for each dose. The IC_{50} value, the dose of each toxin leading to 50% viability, was determined using CompuSyn statistical software (CompuSyn Version-1 Inc. Paramus, NJ, USA). Significant differences between groups were analyzed using the Bonferroni multiple comparison test in GraphPad (GraphPad Prism 4 La Jolla, CA, USA).

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/11/12/727/s1, Figure S1: Worldwide contamination of emerging mycotoxins. Figure S2: Abundance of emerging mycotoxins in finished pig feed. Figure S3: Scatter plot of the correlation between DON and 10 selected emerging mycotoxins. Figure S4: Dose-effect curves of the individual toxicity of DON and 9 selected fungal emerging mycotoxins Table S1: Concentration of the metabolites in 3 quartiles (P25, P50 and P75) and ratios between the concentration of DON and 10 selected emerging mycotoxins. Figure S5: LC-ESI(+)-MRM chromatograms of one pig feed sample. Figure S6: Overlay of the extracted ion chromatograms of both quantifier as well as qualifier of the 11 investigated compounds in one pig feed sample.

Author Contributions: A.K.K., I.P.O. and P.P. designed the experiment, A.K.K. and M.N. performed the experiments, B.N. and G.S. contributed to provide raw data on finished pig feed, T.J. analyzed raw data on finished pig feed, P.G.P.M. performed statistical analysis on correlation, A.K.K. analyzed the cytotoxicity results, A.K.K., P.P. and I.P.O. wrote the paper, B.N. and P.G.P.M. reviewed the paper.

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Conflicts of Interest: B.N., T.J. and G.S. are employed by BIOMIN. However, this circumstance did not influence the design of the experimental studies or bias the presentation and interpretation of results. The other authors, A.K.K., P.G.P.P., M.N., P.P. and I.P.O. declare no conflict of interest.

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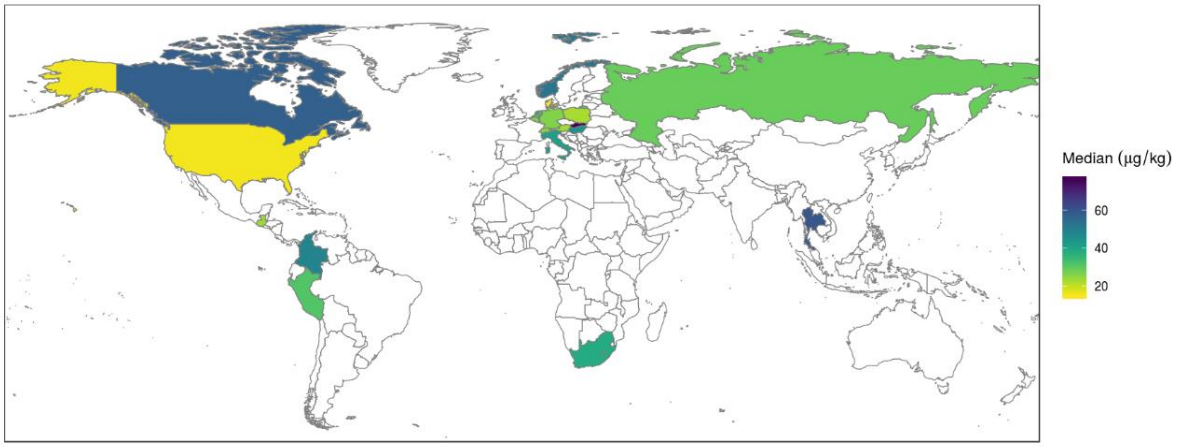
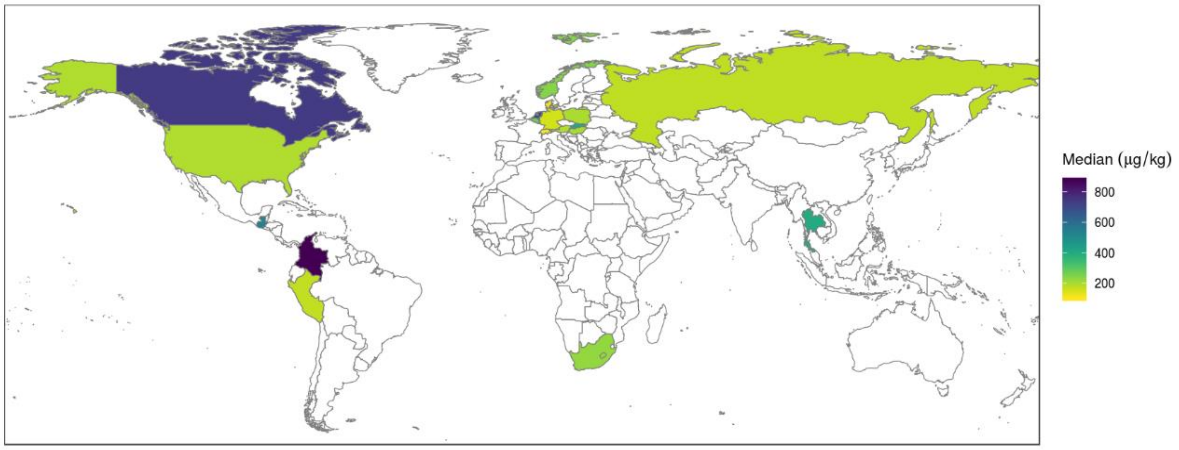
4. Supplementary Data of Article

Median Brevianamid F contamination (Countries with n > 4)



Median cyclo L-Pro-L-Tyr contamination (Countries with n > 4)



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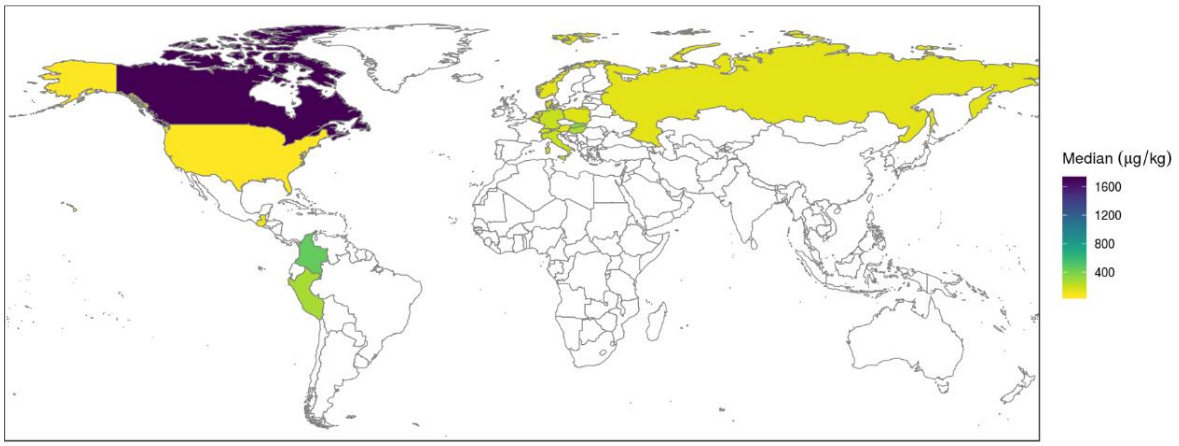
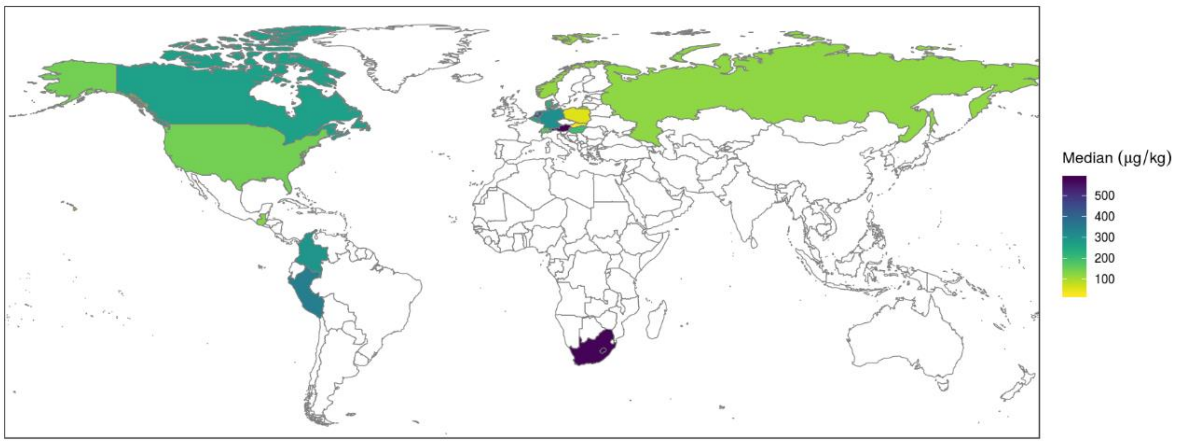
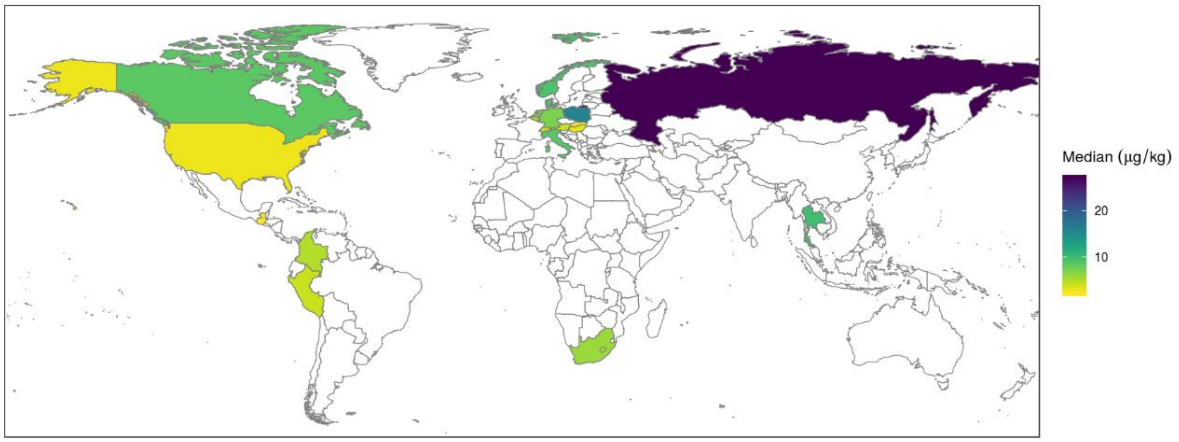


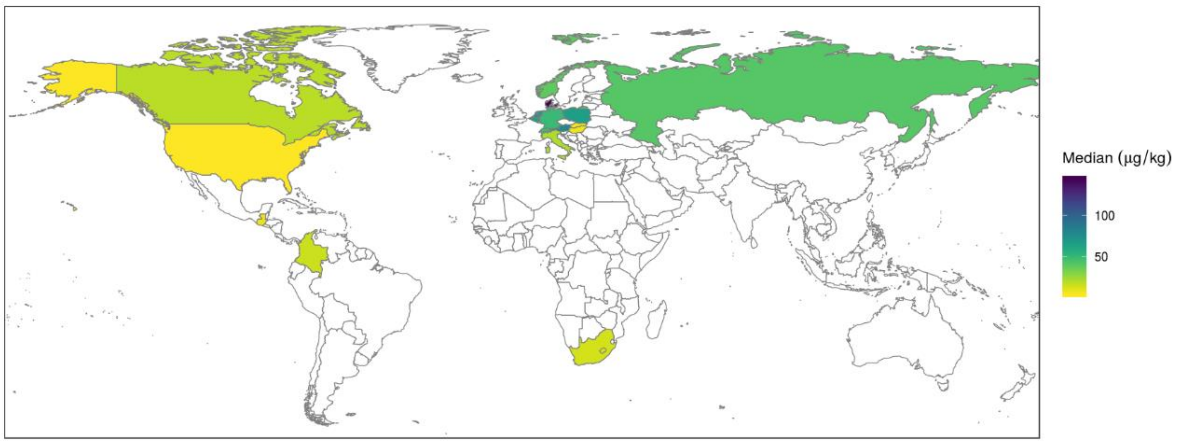
Median Aurofusarin contamination (Countries with n > 4)



Median Emodin contamination (Countries with n > 4)



Median Enniatin B1 contamination (Countries with n > 4)



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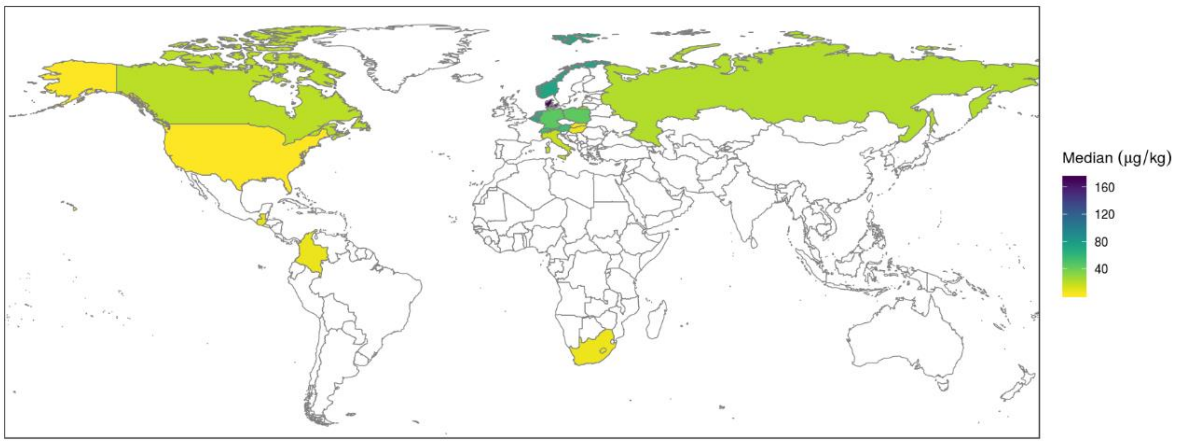
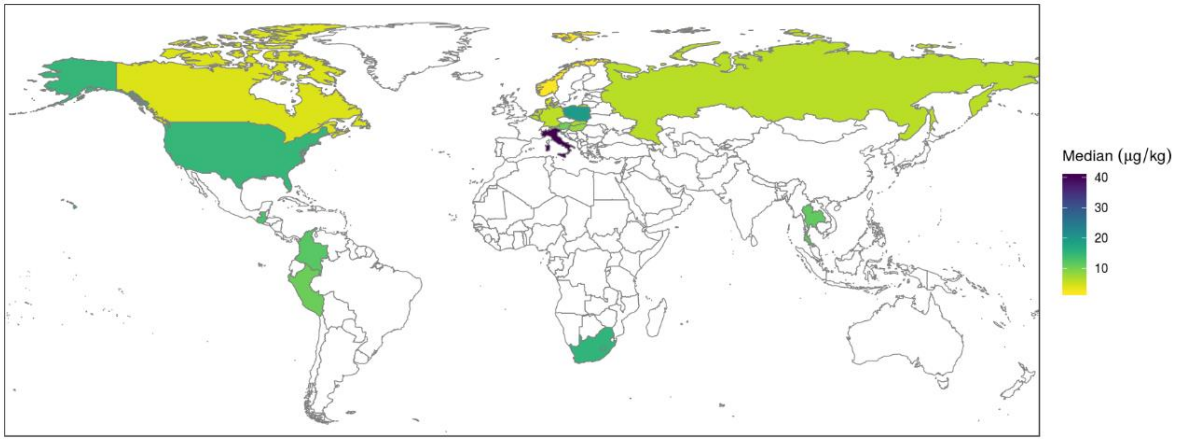


Median Beauvericin contamination (Countries with n > 4)



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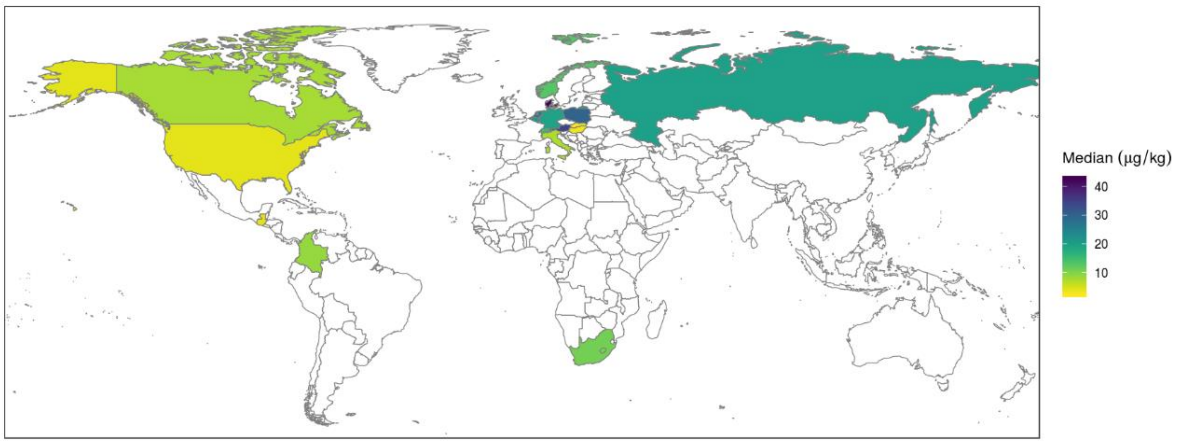
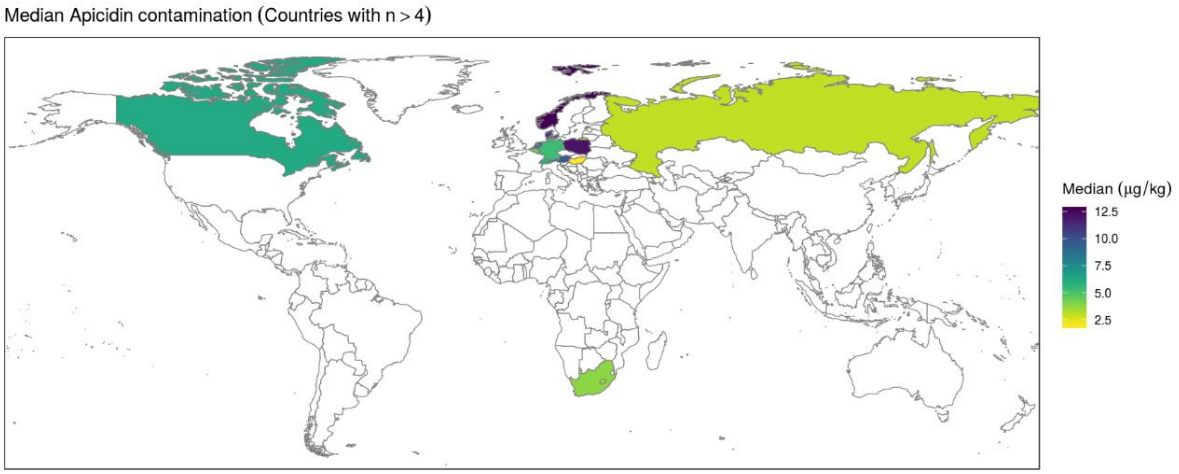
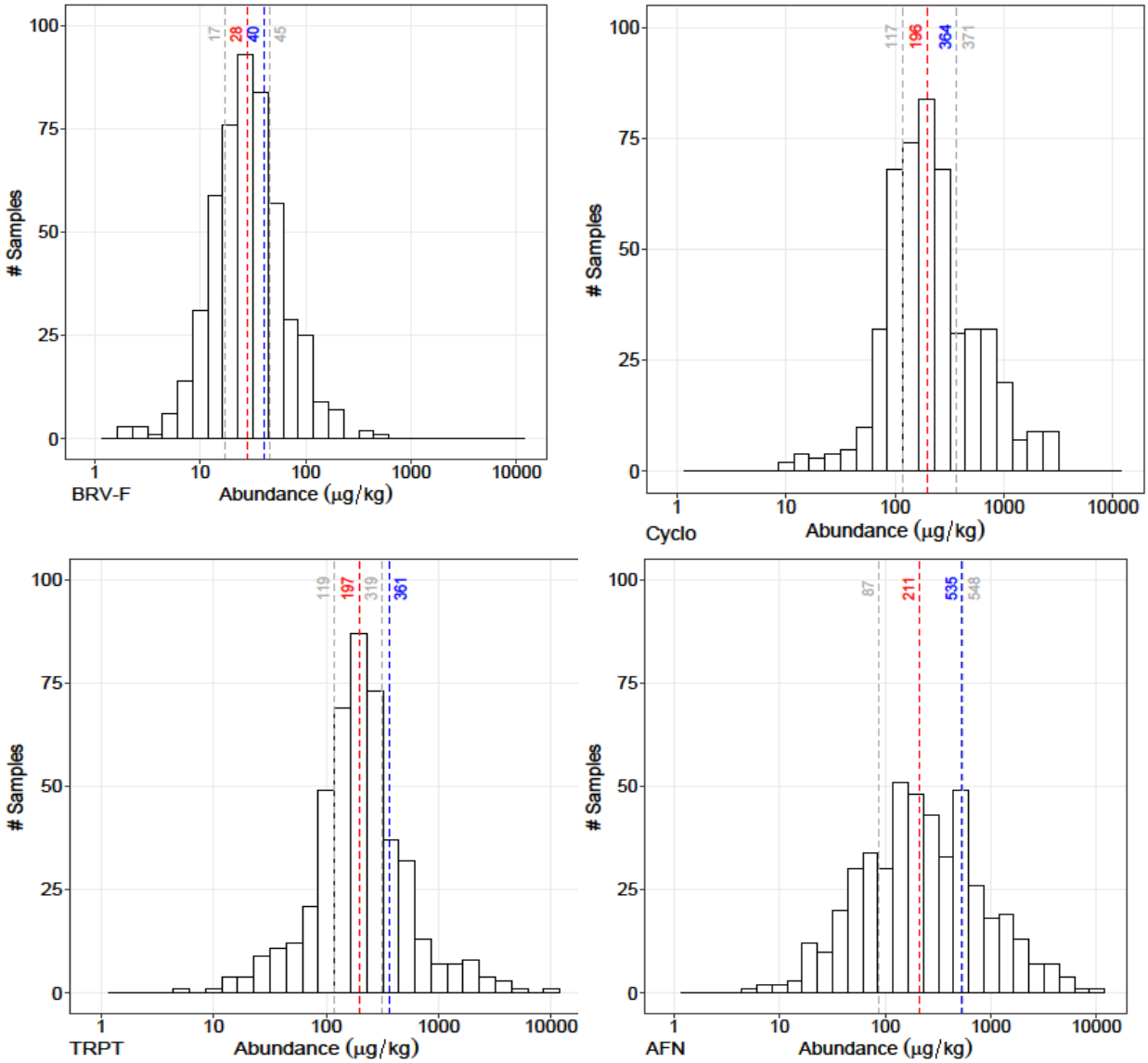
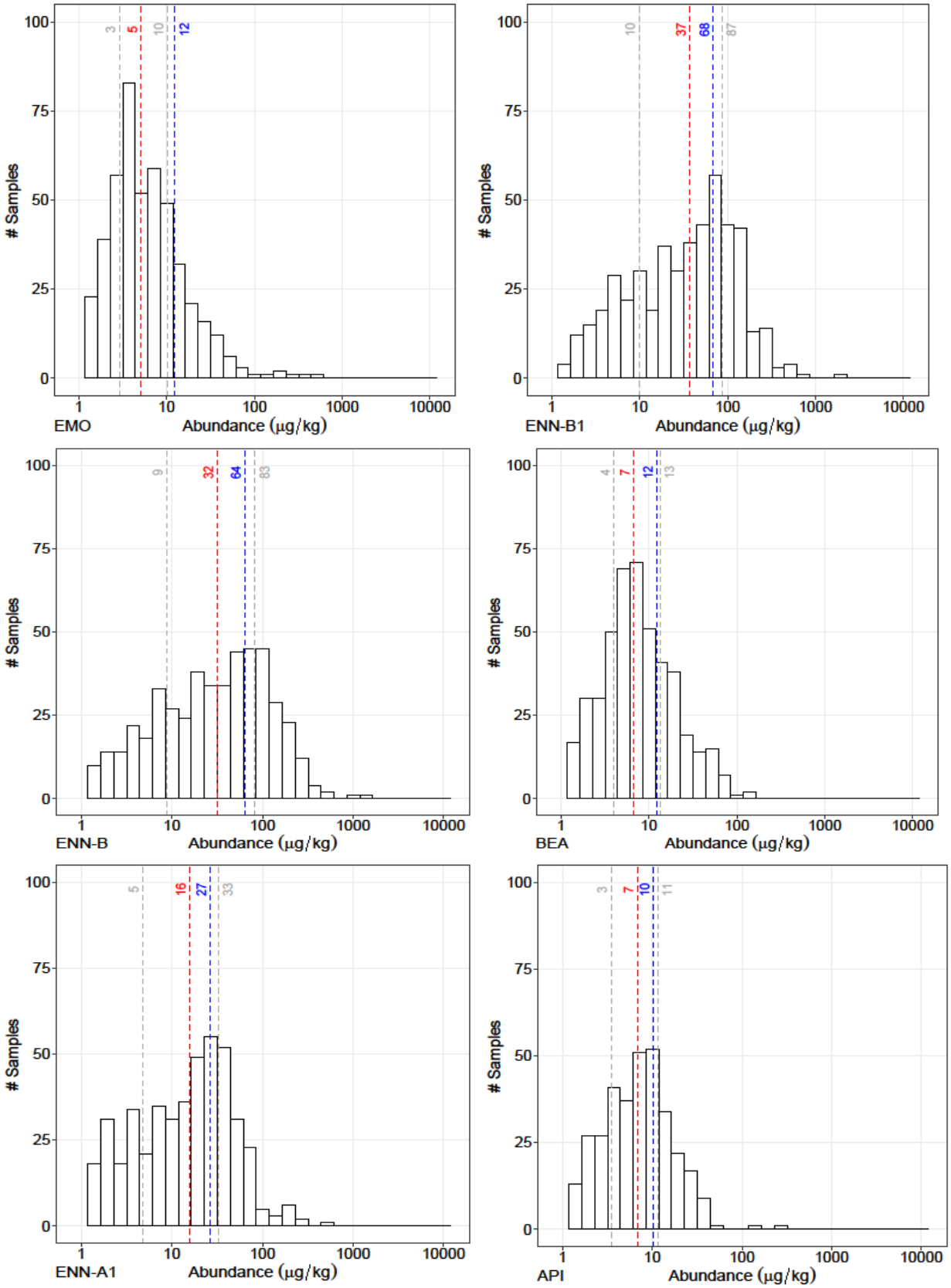


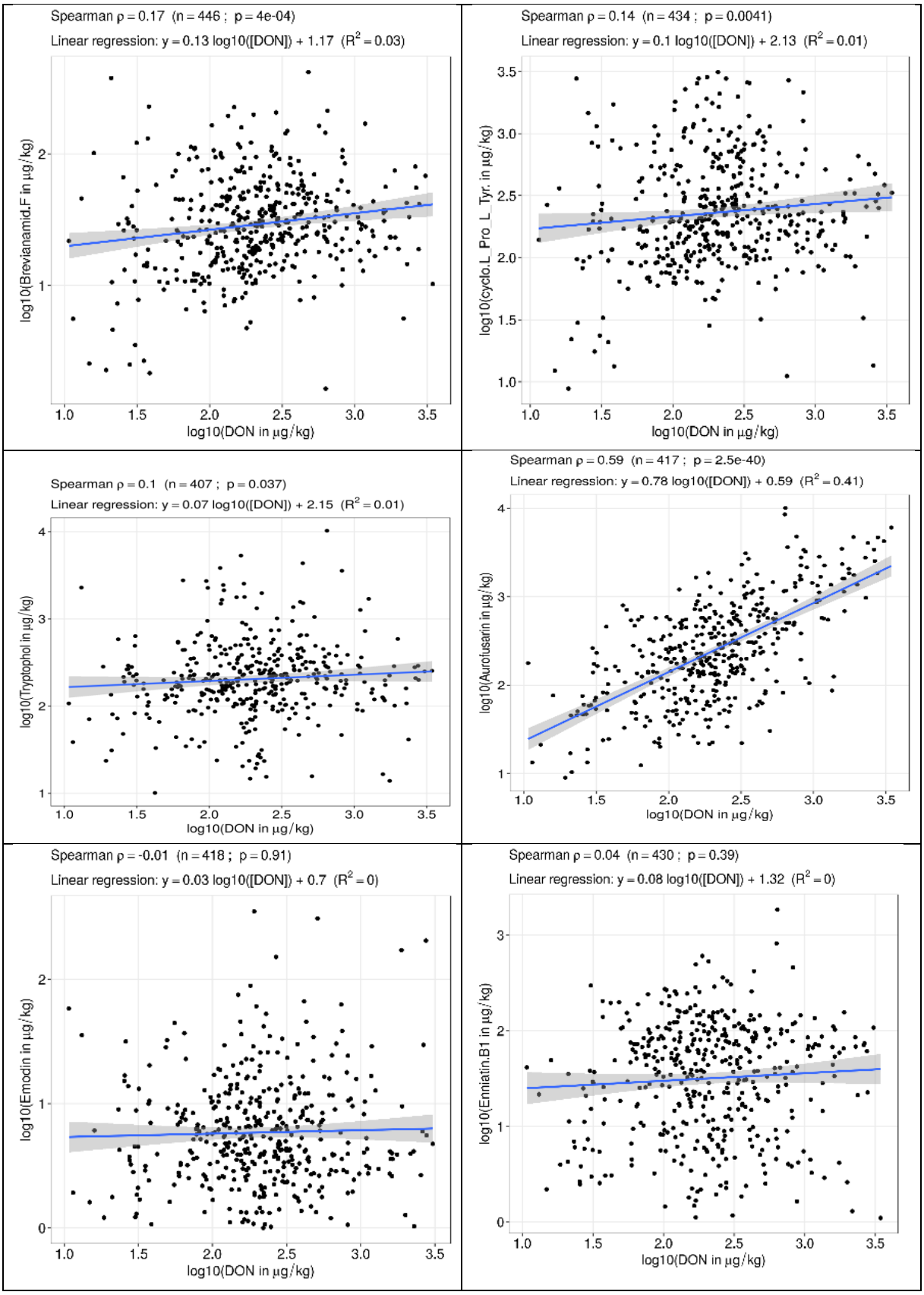


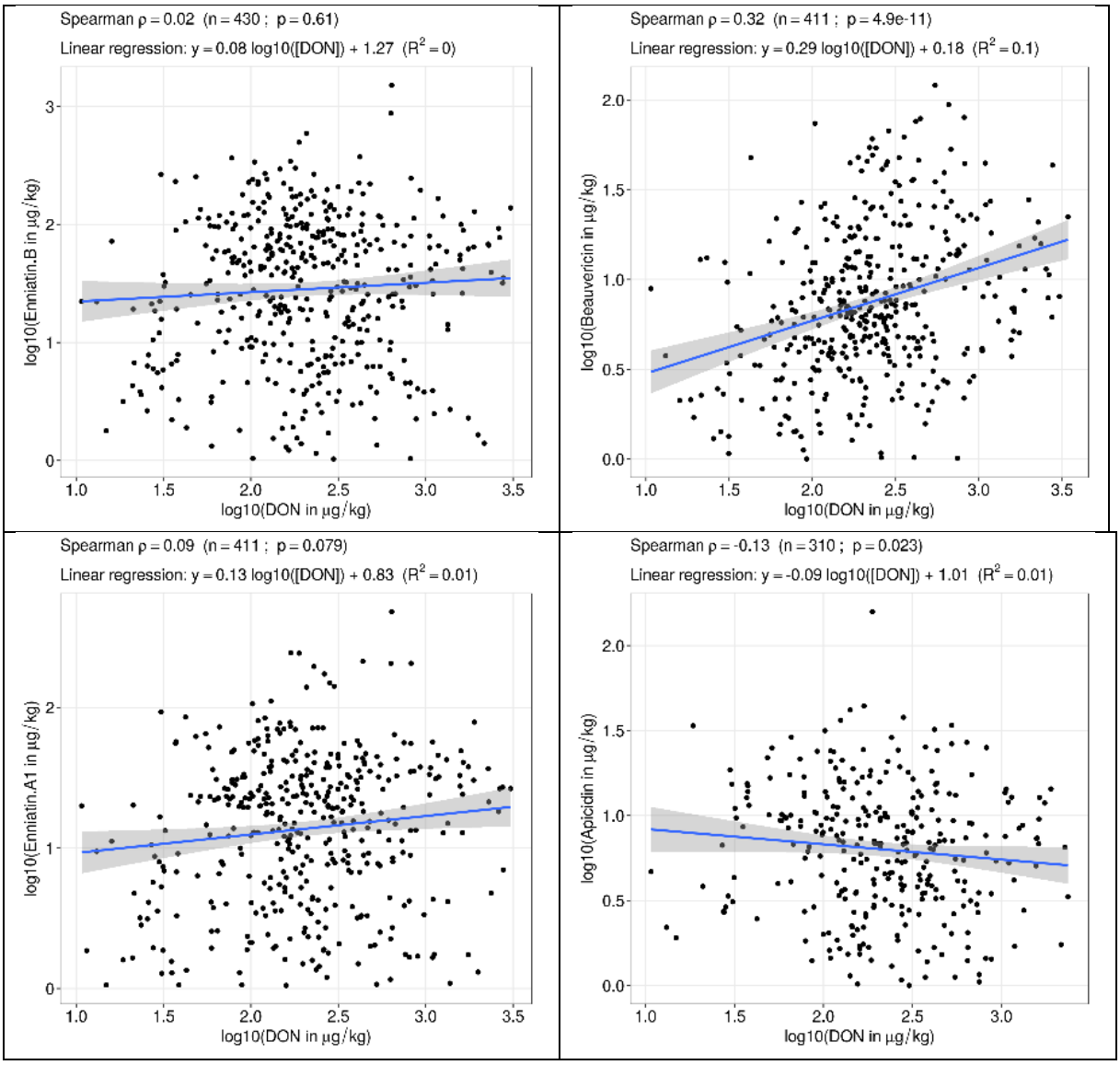
Supplementary Figure S1. Worldwide contamination of BRV-F, Cyclo, TRPT, EMO, ENN-B1, AFN, ENN-A1, ENN-B, BEA and API.



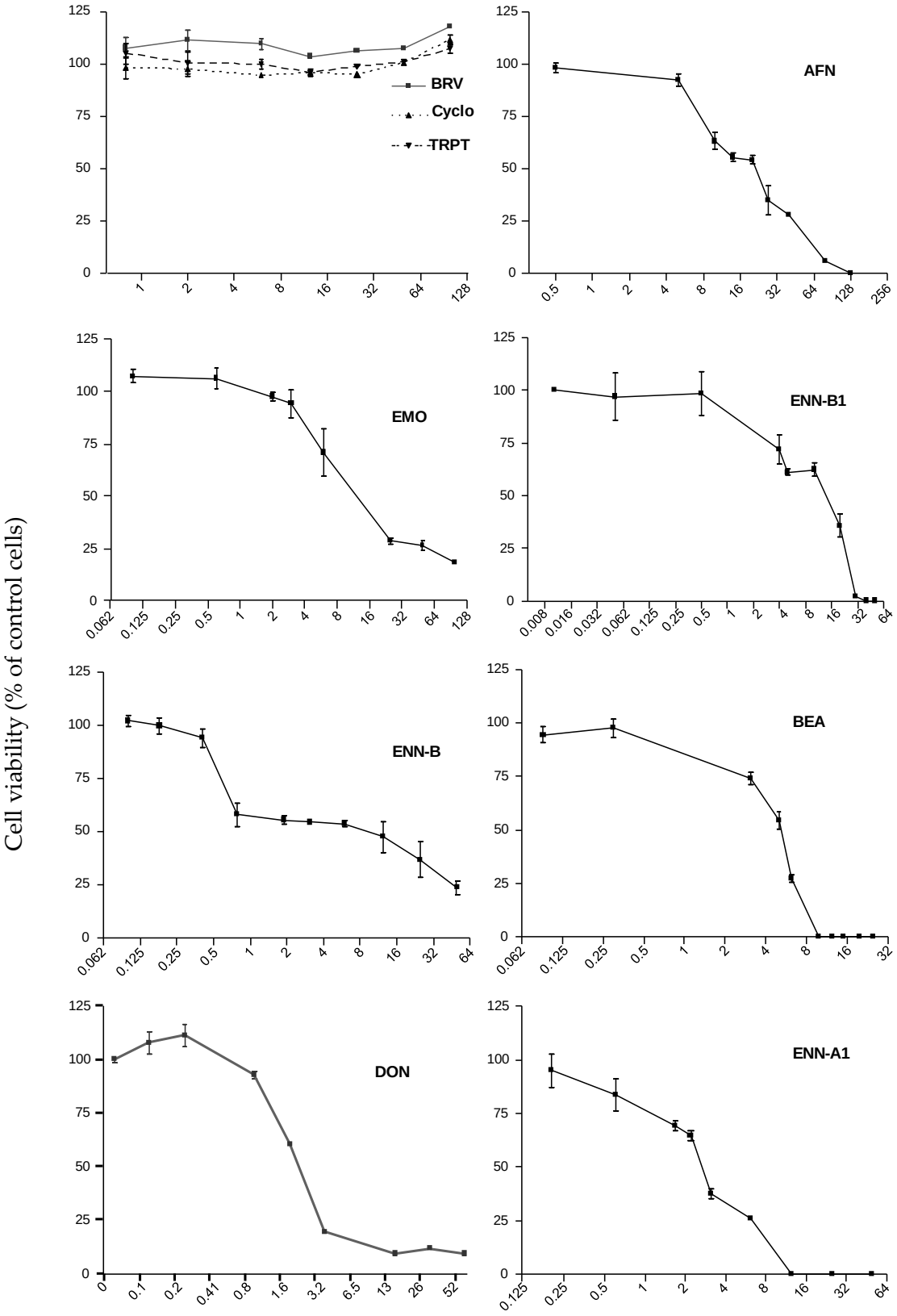


Supplementary Figure S2. Abundance of emerging mycotoxins in pig feed (P25, median, mean, P75). X axis represents the distribution of the concentrations of fungal secondary metabolites (ppb). Y-axis describes the number of co-contaminated samples by DON and other emerging mycotoxins. Median concentrations of AFN, TRPT and Cyclo were ≈ 200 ppb, median concentrations of ENN-B1, ENN-B, BRV-F and ENN-A1 were 15 -40 ppb, and median concentrations of API, BEA and EMO were 5-10 ppb


Spearman ρ = 0.17 (n = 446 ; p = 4e-04)
Linear regression: y = 0.13 log10([DON]) + 1.17 (R2 = 0.03)
log10(Brevianamid.F in µg/kg)
log10(DON in µg/kg)
Spearman ρ = 0.14 (n = 434 ; p = 0.0041)
Linear regression: y = 0.1 log10([DON]) + 2.13 (R2 = 0.01)
log10(cyclo.L_Pro_L_Tyr. in µg/kg)
log10(DON in µg/kg)
Spearman ρ = 0.1 (n = 407 ; p = 0.037)
Linear regression: y = 0.07 log10([DON]) + 2.15 (R2 = 0.01)
log10(Tryptophol in µg/kg)
log10(DON in µg/kg)
Spearman ρ = 0.59 (n = 417 ; p = 2.5e-40)
Linear regression: y = 0.78 log10([DON]) + 0.59 (R2 = 0.41)
log10(Aurofusarin in µg/kg)
log10(DON in µg/kg)
Spearman ρ = -0.01 (n = 418 ; p = 0.91)
Linear regression: y = 0.03 log10([DON]) + 0.7 (R2 = 0)
log10(Emodin in µg/kg)
log10(DON in µg/kg)
Spearman ρ = 0.04 (n = 430 ; p = 0.39)
Linear regression: y = 0.08 log10([DON]) + 1.32 (R2 = 0)
log10(Enniatin.B1 in µg/kg)
log10(DON in µg/kg)



Supplementary Figure S3. Scatter plot showing the correlation between the concentrations of BRV-F, Cyclo, TRPT, AFN, EMO, ENN-B1, ENN-B, BEA, DON, ENN-A1 and API in pig feed samples.



Supplementary Figure S4. Dose effect curve of individual toxicity of 9 selected emerging mycotoxins (BRV-F, Cyclo, TRPT, AFN, EMO, ENN-B1, ENN-B, BEA, ENN-A1) and DON. Data are mean ± SEM of three biological replicates.

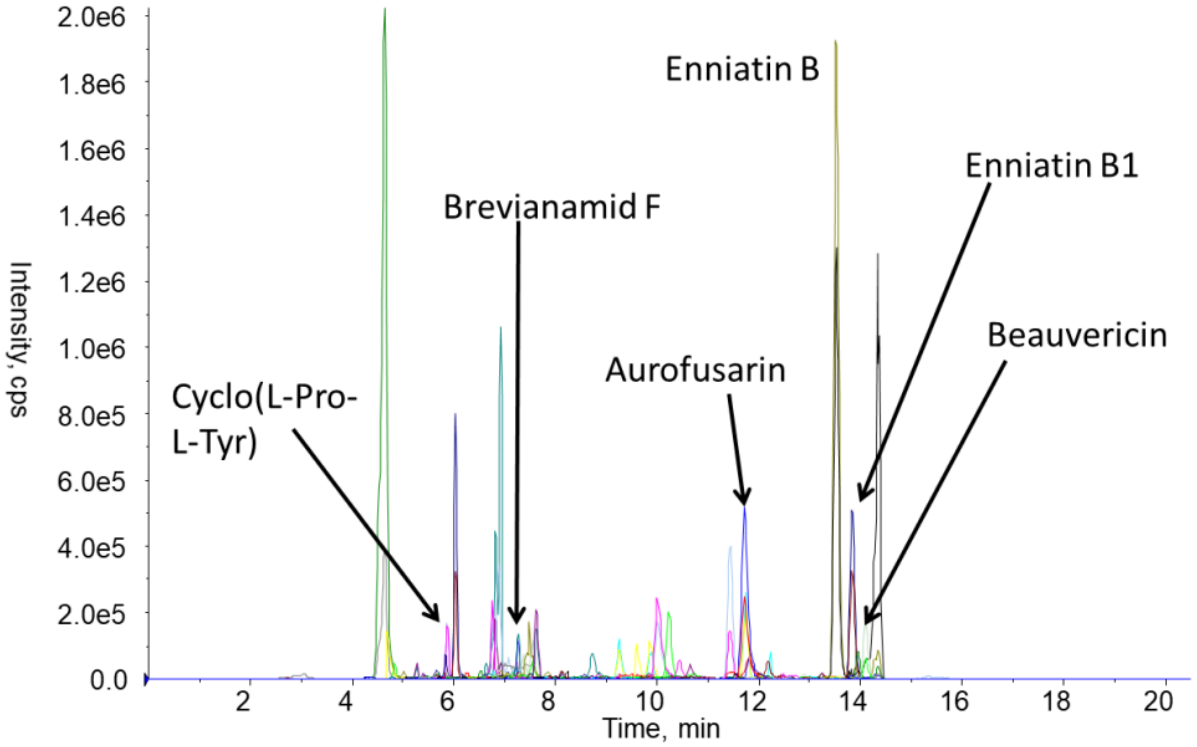


Figure S5a. LC-ESI(+)-MRM chromatogram (overlay of all acquired MRMs) of AT5-4153-009 (only compounds for toxicity tests are annotated).

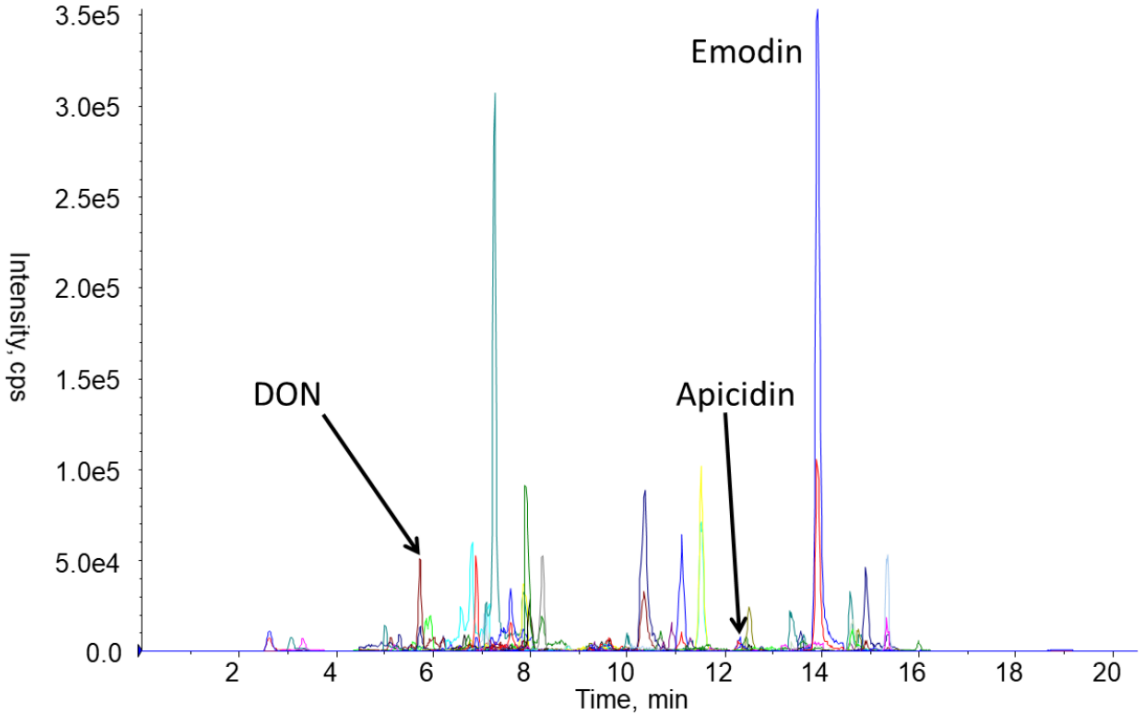
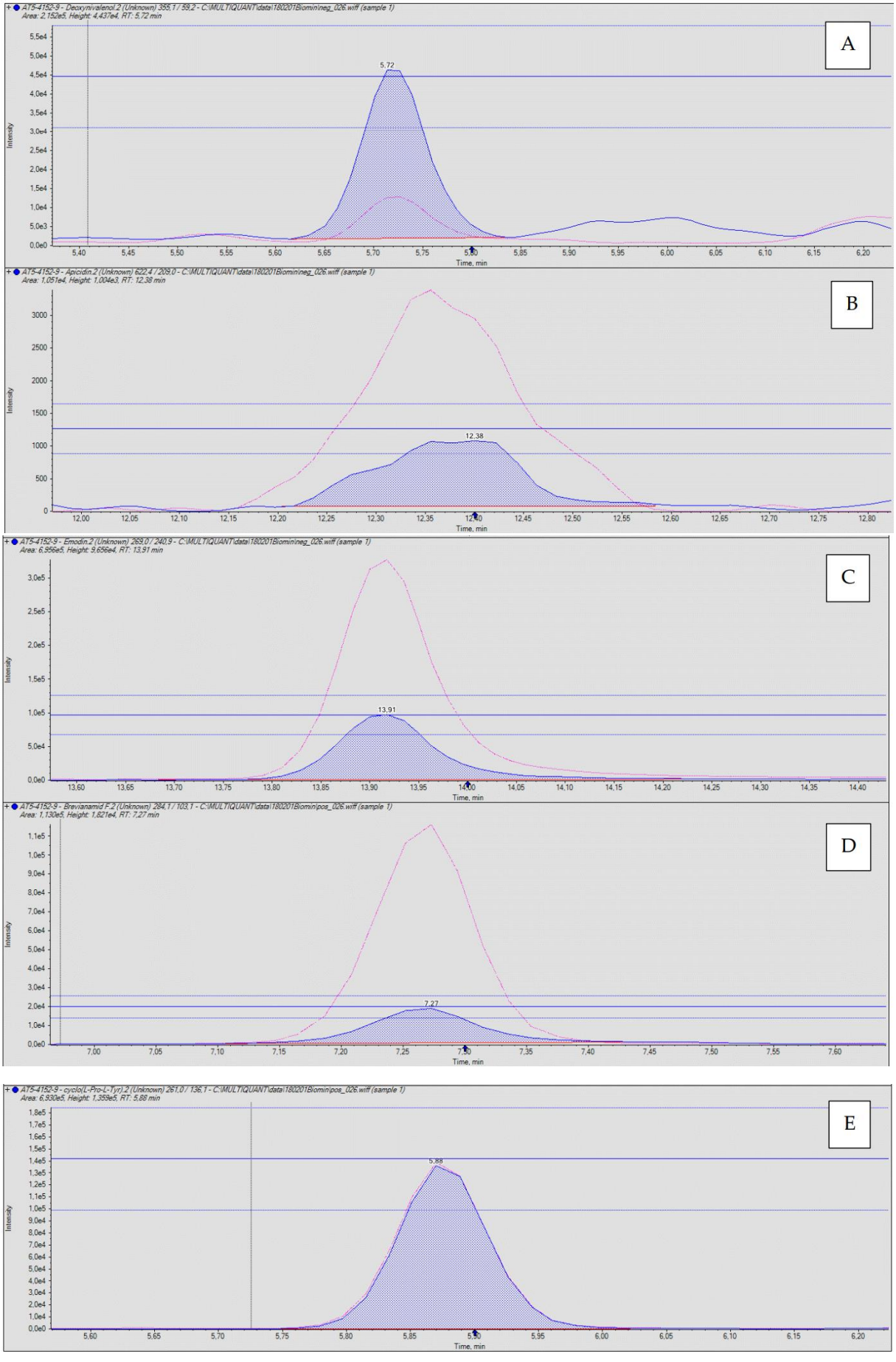
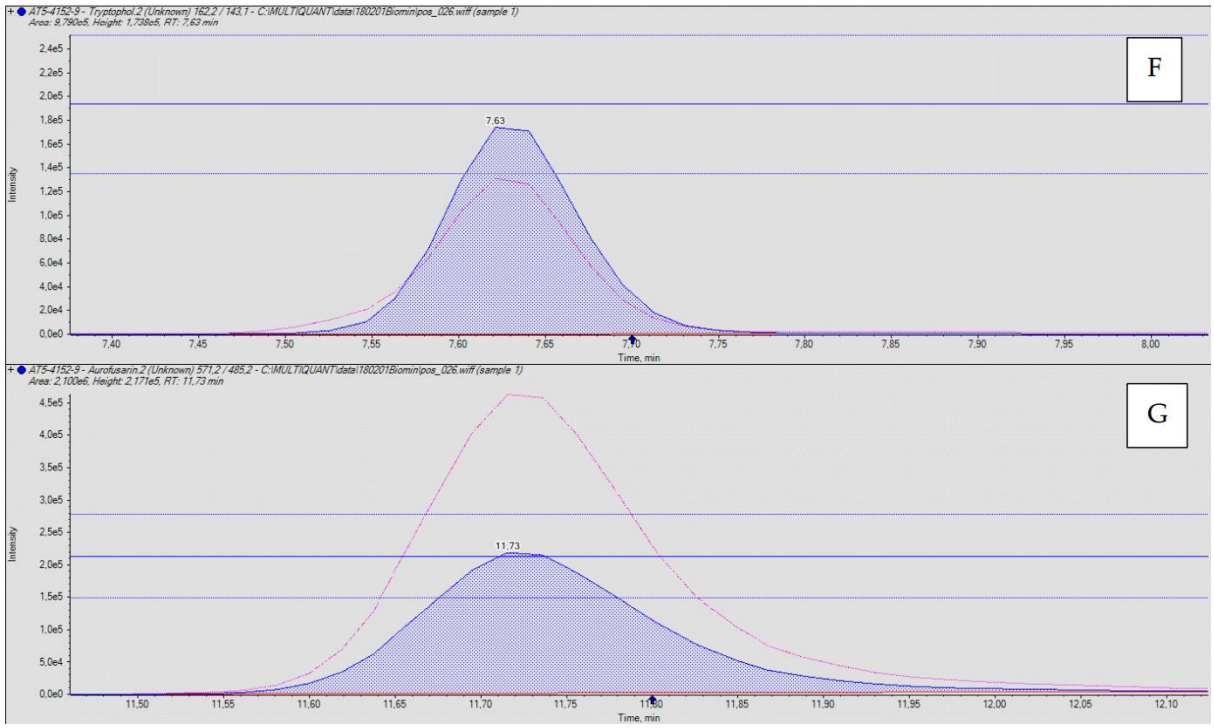
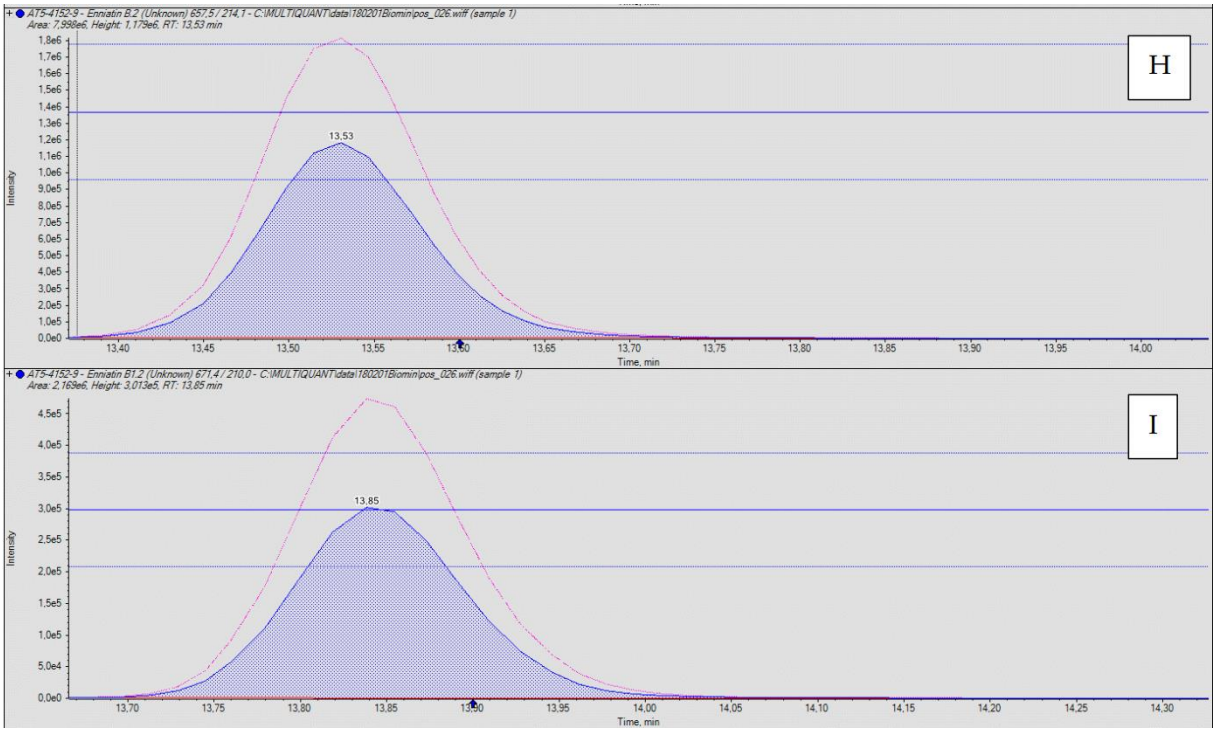
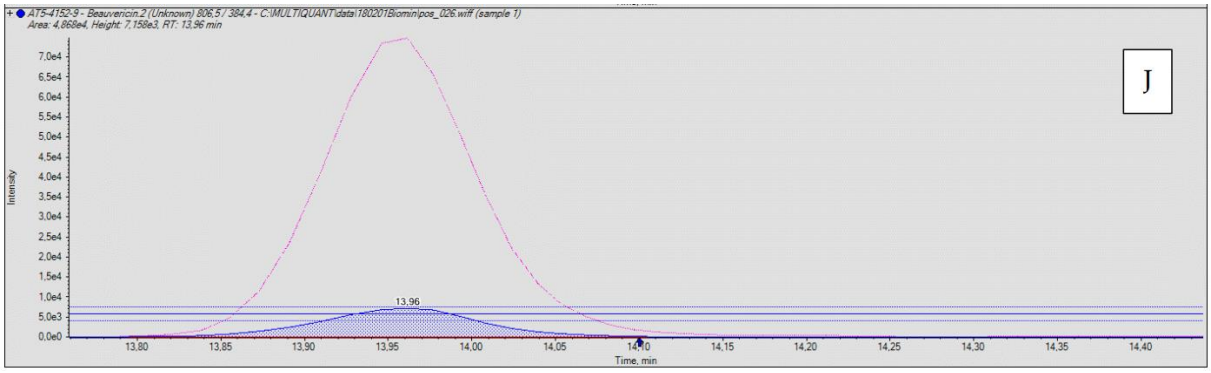


Figure S5b. LC-ESI(-)-MRM chromatogram (overlay of all acquired MRMs) of AT5-4153-009 (only compounds for toxicity tests are annotated).


A
5.72
Intensity
Time, min
B
12.38
Intensity
Time, min
C
13.91
Intensity
Time, min
D
7.27
Intensity
Time, min
E
5.88
Intensity
Time, min


AT5-4152-9 - Tryptophol,2 (Unknown) 162,2 / 143,1 - C:\MULTIQUANT\data\180201Biomin\pos_026.wiff (sample 1)
Area: 9,790e6, Height: 1,738e5, RT: 7,63 min
F
7,63
Intensity
Time, min
AT5-4152-9 - Aurofusarin,2 (Unknown) 571,2 / 485,2 - C:\MULTIQUANT\data\180201Biomin\pos_026.wiff (sample 1)
Area: 2,100e6, Height: 2,171e5, RT: 11,73 min
G
11,73
Intensity
Time, min


AT5-4152-9 - Enniatin B,2 (Unknown) 657,5 / 214,1 - C:\MULTIQUANT\data\180201Biomin\pos_026.wiff (sample 1)
Area: 7,998e6, Height: 1,179e6, RT: 13,53 min
H
13,53
Intensity
Time, min
AT5-4152-9 - Enniatin B1,2 (Unknown) 671,4 / 210,0 - C:\MULTIQUANT\data\180201Biomin\pos_026.wiff (sample 1)
Area: 2,169e6, Height: 3,013e5, RT: 13,85 min
I
13,85
Intensity
Time, min


AT5-4152-9 - Beauvericin,2 (Unknown) 806,5 / 384,4 - C:\MULTIQUANT\data\180201Biomin\pos_026.wiff (sample 1)
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J
13,96
Intensity
Time, min

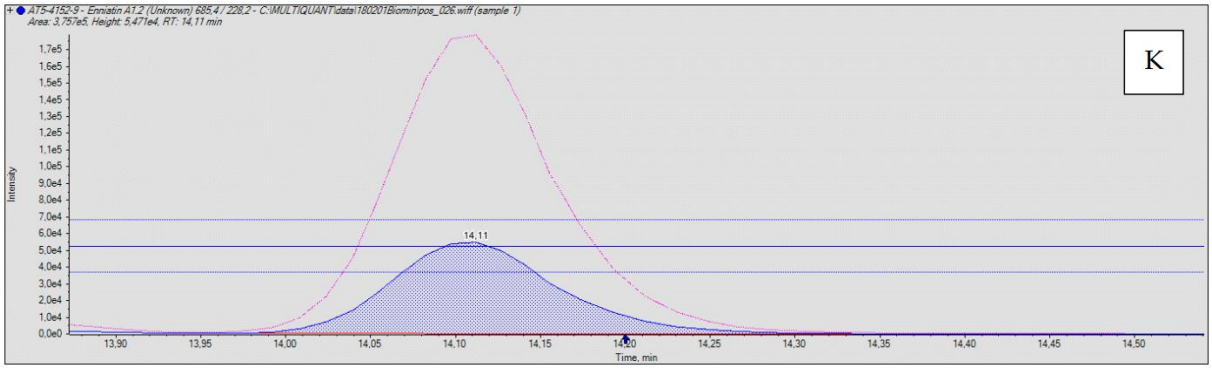

Figure S6. Overlay of the Extracted Ion Chromatograms of both quantifier (pink) as well as qualifier (blue; filled) of the 11 investigated compounds in sample AT5-4152-9; the horizontal lines denote the target height of the qualifier (± 30% tolerance) calculated from the height of the quantifier and the target ion ratio calculated from the standards.

Supplementary Table S1. Concentrations of DON and emerging mycotoxins converted in μM and the corresponding different ratios.

Metabolites	Concentrations at selected quartiles (μM)			Ratio DON/emerging mycotoxin		
	P25	P50	P75	Ratio 1	Ratio 2	Ratio 3
DON	0.38	0.70	1.31			
BRV-F	0.06	0.10	0.16	21.7	7.1	2.4
Cyclo	0.45	0.75	1.42	2.9	0.9	0.3
TRPT	0.74	1.22	1.98	1.8	0.6	0.2
AFN	0.15	0.37	0.96	8.7	1.9	0.4
EMO	0.01	0.02	0.04	122.7	36.5	9.9
ENN-B1	0.02	0.06	0.13	87.4	12.2	2.8
ENN-B	0.01	0.05	0.13	95.3	13.9	2.9
BEA	0.005	0.01	0.02	258.3	70.0	21.9
ENN-A1	0.01	0.02	0.05	184.0	29.4	7.7
API	0.006	0.01	0.02	236.2	63.4	20.5

The concentrations corresponding to the quartiles P25, median, P75 of emerging mycotoxins in μg/kg in pig feed were converted in μM assuming their dilution in 1 L of gastrointestinal fluid. Ratio 1 was calculated using the P25 (1st quartile) concentration of emerging mycotoxin and P75 (3rd quartile) concentration of DON. Ratio 2 was calculated using the median (2nd quartile) concentration of DON and each emerging mycotoxin. Ratio 3 was calculated using the P75 concentration of emerging mycotoxin and P25 concentration of DON.

5. Development of an *ex vivo* model for studying the effects of mycotoxins on liver: the Precision Cut Liver Slices

Liver is an important organ for the uptake, biliary excretion and metabolism of xenobiotics and food contaminants. The main target organ for toxicity and carcinogenicity is the liver (Peraica *et al.* 1999). In most of the cases, toxicity of liver go unnoticed due to the absence of good predictive models and specific toxicity biomarkers. *In vivo* methods imply the use of a large number of animals. Therefore, more attention has been given to design and validate alternative models for toxicity testing that lead to reduction and eventually replacement of animal use. Thus, there is interest in developing reliable liver models for early detection of toxic effects of toxins (Starokozhko *et al.* 2015). In order to explore liver toxicity of mycotoxins, we developed precision-cut liver slices (PCLS). PCLS are viable *ex vivo* explants of tissue with well-defined thickness. PCLS technique is easily transferable to mouse, mini-pig, monkey or pig liver, and human liver. Slices can be remained viable up to 96 h when incubated under carbogen (95% O_2/5% CO_2) and appropriate conditions (Graaf *et al.* 2010).

PCLS contain all cell types of the tissue in their natural environment and natural architecture, with intercellular and cell-matrix interactions remaining intact (Zimmermann *et al.* 2009). In addition, PCLS has already been proven to be functional and efficient in numerous metabolism and toxicity studies (Elferink *et al.* 2008). They are mainly used to study the metabolism and toxicity of xenobiotics, but they are suitable for many other purposes, such as endogenous metabolism, biotransformation and its induction and transport of drugs, as well as for toxicological studies, and for assessing the efficacy of drugs in diseased tissues.

The main objective of the development of this model was first to determine whether liver slices could be incubated and maintained viable during a reasonably long time of incubation and then, to expose pig liver slices to DON and assess its toxic effect through different markers.

We measured at different time points, the total protein and ATP contents in the liver slices. We have also collected the incubation medium in order to measure different liver damage markers. Ultimately, we assessed the effect of DON on the expression of selected genes.

5.1. Materials and Methods

Liver slices experiments were performed on pig and the first aimed to optimize the conditions of cutting and incubation of slices. Three piglets (5 weeks -old) were slaughtered in consecutive

days. Liver was sampled and a piece of the right lateral lobe (Figure 4) was perfused with cold sodium chloride (NaCl 0.9%) (Sigma St Quentin Fallavier, France) using a 50 mL syringe. Perfused liver was placed under the cylinder-shaped coring tool and liver cores were prepared using a drill and a tissue coring tool (Ø 10 mm) (Figure 5a). Then, cores were transferred to the cylindrical core holder of the Krumdieck slicer (Figure 5b–d). Slices were in thickness of 250-350 µm and average weight was 50-70 mg (Figure 5e) (Krumdieck *et al.* 1980). Coring and slicing was carried out into "Krebs-Henseleit" buffer supplemented with $NaHCO_3$ (Sigma). Krebs was bubbled in advance with carbogen. Good quality slices were round, equally thick at all sides and had smooth edges (Figure 5f). Slices were transferred to 12-well plates using a spatula to avoid damages (Figure 5g-h) (Graaf *et al.* 2010).

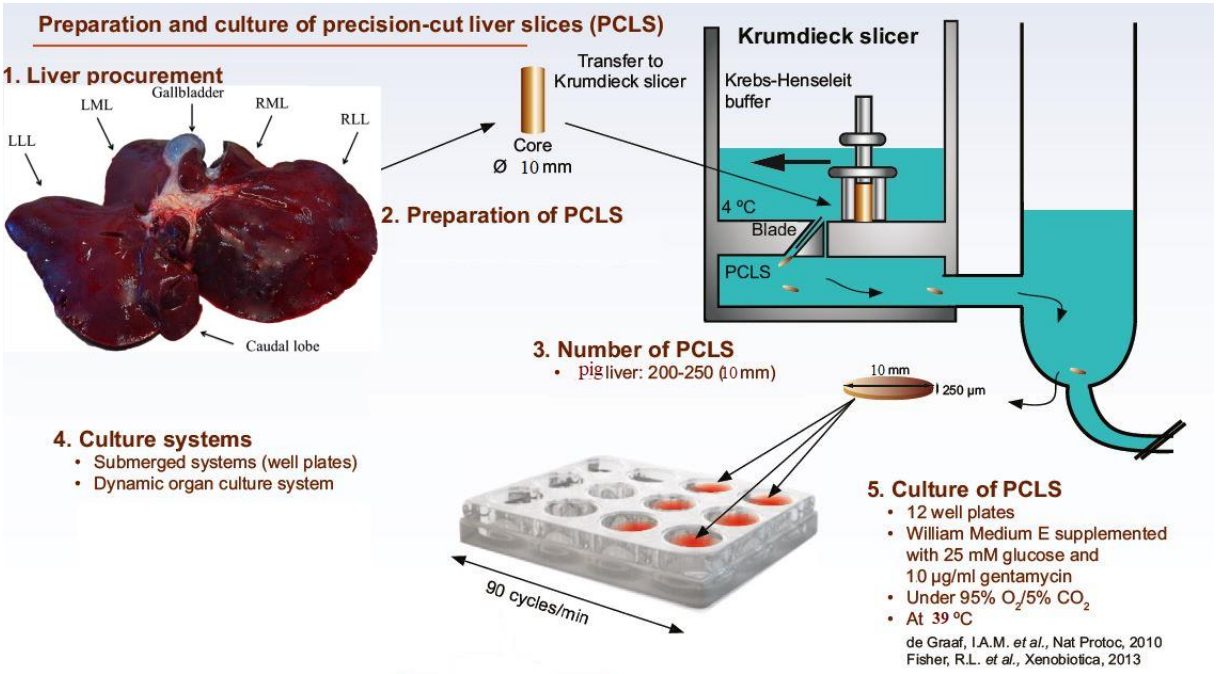

Figure 4: Four steps (an overview) of PCLS preparation (Olinga and Schuppan 2013).

In parallel, William medium E (WME) (Sigma) supplemented with 0.5% Gentamycin (10 mg/ml) and 1% Glutamine (200 mM) (Eurobio, Courtaboeuf, France) was also bubbled in carbogen, 1 h in advance.

Slices were maintained in Krebs buffer under carbogen bubbling for 1 h of regeneration phase, to maintain the quality and remove the debris. Surface and edges of the slices were smoother after regeneration. Then slices were immersed individually in 12 wells plate containing 2 mL WME containing or not DON (Sigma) dissolved in DMSO (1%) (Sigma). Control slice were incubated with same concentrations of vehicle in each incubation time. Plates were incubated

at 39°C for 2, 4, 6, 8 and 20 h in presence of DON or without DON (control) under orbital shaking. In the space between wells, few mL of medium were added and bubbled with carbogen in order to maintain high humidity and oxygen concentration. Incubation medium was refreshed after 8 h in order to maintain quality of medium. At each incubation time, incubation medium was collected to assess liver damage markers such as: alkaline phosphatase (ALP), aminotransferases such as aspartate transaminase (AST), and alanine transaminase (ALT), lactate dehydrogenase (LDH) and total protein over the incubation. PCLS were also collected in microtubes, snap frozen in liquid nitrogen and stored at – 80 °C for assessment of gene expression, total protein and ATP contents.

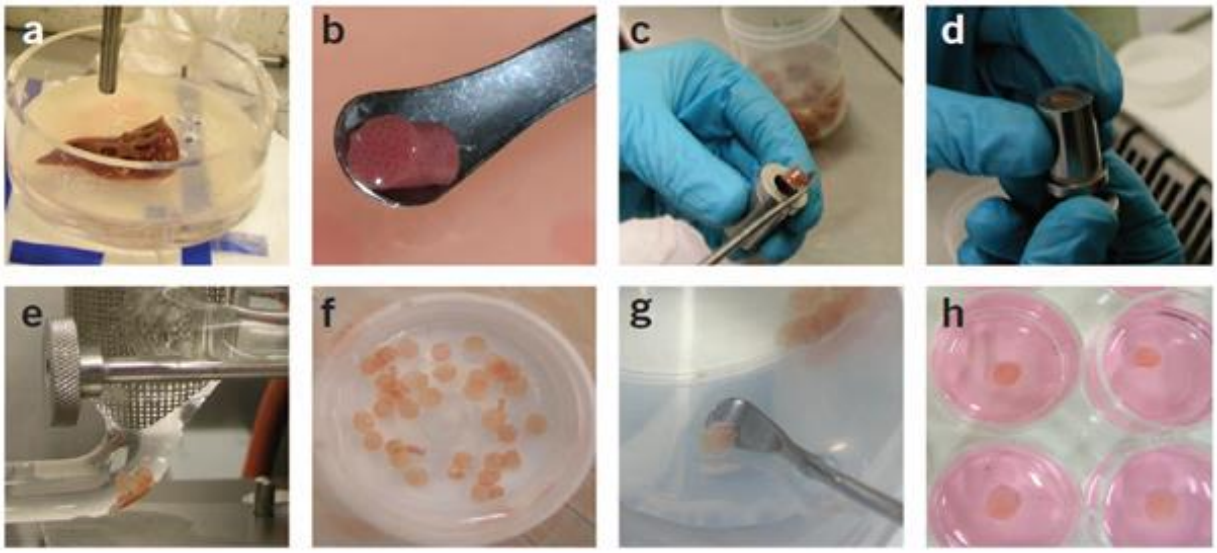

Figure 5: Preparation and incubation of liver slices. Liver cores are prepared using a drill and a tissue coring tool and transferred to the cylindrical core holder of the Krumdieck slicer (Graaf *et al.* 2010).

5.2. Statistical Analysis

One-Way Analysis of Variance (ANOVA) non-parametric test was used to find significant difference between the mean group across the incubation time or treatment and significant differences between the control and treated samples were analyzed via Bonferroni's multiple comparison tests, using statistical software GraphPad (La Jolla, CA, USA).

5.3. Analysis of Liver damage markers in culture medium

The main goal of this experiment was to assess quality of PCLS through several sensitive liver damage markers such as ALP, AST, ALT, LDH and total proteins, in order to know whether incubation (time) or treatment (DON 10 µM) influence the quality of PCLS.

5.3.1. Methods

Incubation medium was collected in different time (2, 4, 6, 8 and 20 h). Medium was renewed after 8 h of incubation. Samples were analyzed by GenoToul-Anexplo platform (Toulouse, France) using a Pentra 400 Clinical Chemistry benchtop analyzer (Horiba, Les Ulis, France). The reported values were expressed as means ± SEM of the three biological replicates.

5.3.2. Results and discussion

Overall, we observed that the release of enzymes was numerically but not statistically higher in the treated samples (DON 10 µM) than the control samples. Release of enzymes was gradually increased from 2 h up to 8 h. Medium was renewed at 8 h. Thus, similar result was obtained at 8 h and 20 h, whereas the release of AST and LDH was lower at 20 h compared to 8 h. Overall, no significant difference between the control and treated samples was observed whatever the duration of incubation (Table 21).

Table 21: PCLS damage markers (ALP, AST, ALT, total protein, LDH) level in the incubation medium

	Controls					**DON 10** µM				
Markers	**2 h**	**4 h**	**6 h**	**8 h**	**20 h**	**2 h**	**4 h**	**6 h**	**8 h**	**20 h**
ALP (U/L)	2.9±0.5	4.4±1.8	4.8±2.3	3.9±1.3	4.1±2.5	4.6±1	4.7±2.2	5.1±1.8	4.3±2.6	4.5±1.2
ALT (U/L)	4.7±0.6	5.0±1	6.3±1.5	6.3±0.6	5.3±2.3	5.3±0.6	6.3±0.6	6.0±0	5.3±0.6	6.7±0.6
AST (U/L)	77±40	103±27	112±57	147±34	64±20	98±23.6	117±15	115±38	152±40	84±38
LDH (U/L)	43±4.4	73±31	85±43	97±33	32±5	67±28	87±24	83±44	117±31	50±17
Total proteins (g/L)	5.2±0.2	5.2±0.3	5.4±0.4	5.1±0.1	5.3±0.5	5.2±0.2	5.2±0.2	5.3±0.4	5.0±0.2	5.7±0.3

Samples were treated to DON 10 µM over 20 h. Treated samples were compared to corresponding control at eac incubation time.

The level of ALP, AST, ALT, total protein, LDH was not significantly impacted by time or DON treatment. PCLS could be damaged during preparation or incubation. This potentially causes the release in the incubation medium of some enzymes ALP, ALT, AST, LDH as well total proteins (Pratt and Kaplan 2000; Limdi 2003). These markers are sensitive indicators of hepatocellular injury (Pratt and Kaplan 2000). ALP originates mainly from two sources, liver and bone. The physiological role of these enzymes is not entirely clear but their production increases in tissues undergoing metabolic stimulation (Limdi 2003). LDH is a cytosolic enzyme mainly present in periportal hepatocytes and released when the cells are lysed by hepatotoxins. The amount of released enzyme is proportional to the extent of damage caused to the cell (Naik *et al.* 2004). ALT, a cytosolic enzyme is found in its highest concentrations

in the liver and is a more specific indicator to liver injury. AST is found, in decreasing order of concentration, in liver, cardiac muscle, skeletal muscle, kidneys, brain, pancreas, lungs, leukocytes, and erythrocytes (Pratt and Kaplan 2000).

AST, ALT, ALP, LDH, and total protein are used to evaluate patients with known or suspected liver disease (Dufour *et al.* 2000; Starokozhko *et al.* 2015). These markers are also used to evaluate the liver slices. After 5 days of incubation, the protein content and the hepatic markers AST and ALT were modestly decreased (17–22%) relatively to their initial levels at day 0. In contrast, the levels of the ALP increased substantially (3-fold) and LDH also increased up to 25–28% (Behrsing *et al.* 2003). In another study, human liver slices were incubated and LDH release into the medium after 120 h of incubation was assessed in two different incubation medium, WME and RegeneMed®. LDH release was about 9–11% after 24 h. Thereafter it remained relatively stable (4–6% per day). The elevated LDH release during the first hours of incubation can be explained by the damage of the outer cell layers during the slicing and handling procedures. No difference was observed between slices incubated in WME and RegeneMed® (Starokozhko *et al.* 2015). When we assessed the LDH release at different incubation time (2 h – 20 h), we observed a maximum release at 8 h, whereas after 8 h release of LDH remained stable.

5.4. Analysis of total Proteins and ATP content in PCLS

The main goal of this experiment was to assess effect of DON on total proteins and ATP contents in PCLS during short (2 h) and longer (20 h) exposure period.

5.4.1. Methods

Tubes with ceramic beads (Bertin Technologies, St. Quentin en Yvelines, France) containing PCLS were taken out from the freezer -80 ºC. Then, 1 mL of ice-cold CelLytic™ MT buffer (Sigma) was added. All the tubes were transferred into FastPrep-24™ 5G Classic Homogenizer (MP Biomedicals). Tissues were immediately homogenized without thawing for 40 seconds (speed 6.0 m/s). Homogenates were placed on ice and centrifuged at 10000 g (gravitational acceleration) at 4 °C for 10 min to remove cellular debris. In total 0.85-0.9 ml of supernatant was transferred into a clean microtube. Proteins contents were measured directly by spectrophotometer using Nanodrop ND1000 (Labtech International) directly at 280 nm. The concentrations were determined by using a bovine serum albumin (BSA) standard curve (0.1 –

1.2 mg/mL), and the proteins content was expressed as mg/mL per slice. Data was obtained as mean ± SEM of three biological replicates.

ATP contents were also assessed in the same lysis extraction buffer from a single slice using 96-white well flat-bottom cell culture plates (Costar, Cambridge MA, USA). Serial ratios of lysed samples and CellTiter-Glo® (CTG) Luminescent (Promega, Charbonnières-les-Bains, France) were prepared for the measurement of ATP. Ratio 1 was 1/1 (100 µL CTG + 100 µL lysis sample). Ratio 2 was, 1.66/1 (125 µL CTG + 75 µL lysed solution), ratio 3 was 3/1 (150 µL CTG + 50 µL lysed solution) and ratio 4 was 7/1(175 µL CTG + 25 µL lysed solution) respectively. The aim of testing serial dilutions was to verify whether the amount of CTG in the mixture was limiting confirm that test work correctly and respect to their dilution factor, the final value of diluted or not diluted samples was almost similar. ATP was measured by luminescence using spectrophotometer (Tecan Infinite M200 ProTM equipped with the Tecan iControl TM software). Blank was subtracted from all the wells. Luminescence of treated slices was compared with untreated one.

5.4.2. Results and discussion

We first investigated the effect of DON and incubation time on total proteins. As shown in Figure 6, incubation time did not affect the total protein content. By contrast, 20 hours of exposure to DON 10 µM decreased by 50% the total proteins content, whereas 2 hours exposure had no effect.

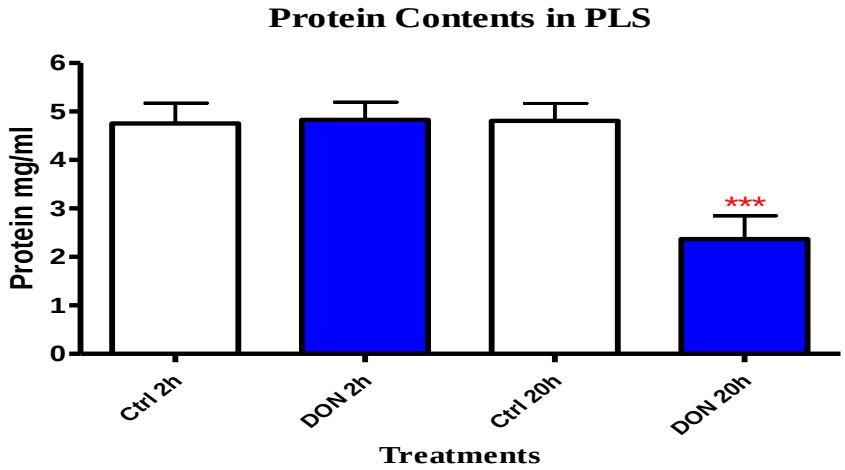

Figure 6: Total protein in PCLS. Samples were treated by DON 10 µM during 2 h and 20 h of incubation treatments. Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference between control and treated groups * P<0.05

This result demonstrated that incubation had no effect on the total protein contents of PCLS, whereas DON 10 µM in longer incubation (20 h) significantly decreased total protein contents. Mainly, at the cellular and subcellular level, DON binds to the ribosome, inhibits protein and nucleic acid synthesis and triggers ribotoxic stress, leading to the activation of mitogen-activated protein kinases (MAPKs) and their downstream signaling pathways (Pierron *et al.* 2016b). Thus, decrease of proteins in PCLS confirms that DON inhibits synthesis of proteins, and inhibition was clearly observed at longer incubation.

We then analyzed the effect of DON and incubation on ATP content. Results demonstrated that a short DON exposure (2h) did not have any effect on ATP content. By contrast a long incubation time (20h) with or without DON decreased ATP contents by more than 70% (Figure 7).

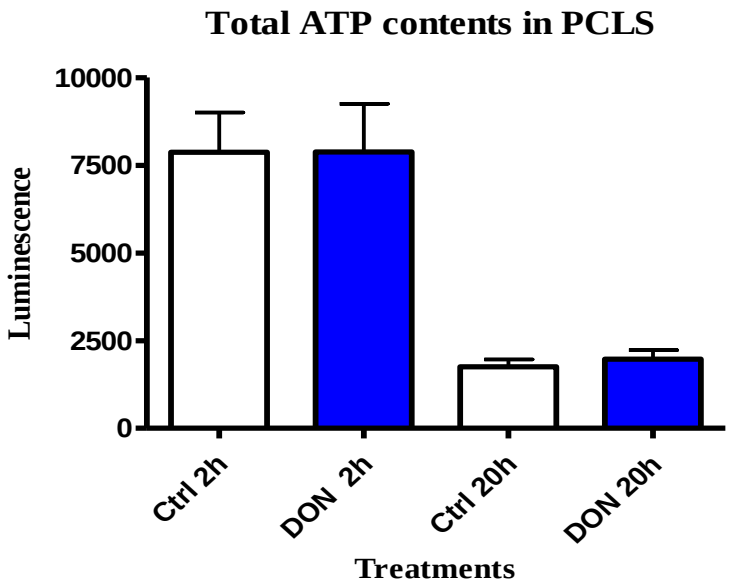

Figure 7: Total ATP content in a PCLS. Samples were treated by DON 10 µM during 2 h and 20 h of incubation treatments. Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference between control and treated groups * P < 0

Overall, total protein contents of PCLS was reduced just due to the treatment, whereas unlikely to protein, ATP was highly reduced due to the incubation (20 h).

The viability of PCLS is usually assessed via ATP and proteins content. Some studies have demonstrated that in longer incubation, a decrease of weight, thickness, total proteins and ATP was observed. In rat PCLS incubated for 24 h the wet weight significantly decreased from 17.6 mg per slice to 9.0 mg (~51%), thickness was reduced from 218 µm (fresh slices) to 184 µm

(~84%) and total proteins content was reduced significantly from 1.58 mg per slice to 1.03 mg(~66%). However, ATP was slightly decreased (17%) compared to the one of control (Granitzny *et al.* 2017). In addition to the treatments, incubation medium also play a role in the degradation of ATP. When human liver slices were incubated in two different mediums, WME and RegeneMed® after 120 h, the amount of ATP was dissimilar. Slices incubated in RegeneMed® had a consistently lower ATP level over time compared to WME. However, the incubation time had no effect on ATP levels. These lower ATP levels in slices incubated in RegeneMed® medium may be explained by the formation of the large necrotic regions in the inner part of the slices (Starokozhko *et al.* 2015). Furthermore, ATP level was measured in rat and mouse PCLS incubated in WME and DMEM medium for 72 h. Both mouse and rat slices lost more than 60% ATP level within 72 h in WME medium, however in DMEM with growth factors and hormones ATP level instead increased within 72 h (Koch *et al.* 2014).

Our results on the total proteins and ATP contents are not in accordance with already published results. We incubated slices up 20 h, but we did not see any effect of incubation on total protein contents, whereas we observed the significant effect of incubation on the ATP. Previous stated results are from human, rats and mouse, whereas we assessed the behavior of pig liver slice. These variations might be dependent to the animal species.

5.5. Assessment of the time and dose dependent effects of DON on the expression of selected genes

The main objectives were (i) to assess the effect of DON 10 µM on the expression of genes of PCLS at different time of exposure (2 h, 4 h, 6 h, 8 h and 20 h) and (ii) to assess the doses effect of dose (DON 3 µM and 10 µM) at 20 h. Treated samples were compared to corresponding controls at each incubation time.

5.5.1. Methods

5.5.1.1. Extraction

A single PCLS was lysed in 1 mL Extract-all reagent (Eurobio) in a microtube with ceramic beads (Bertin Technologies, St. Quentin en Yvelines, France) using FastPrep-24™ 5G Classic Homogenizer (MP Biomedicals). Rapidly, the lysate was transferred into a new microtube. Then, 200 µL of chloroform (Sigma) were added into lysate, shacked vigorously several times and incubated for 5 minutes at room temperature. Afterward, microtubes were centrifuged for 15 min in 13,000 g. The solution was separated into aqueous and organic phases. RNA

remaining in the aqueous phase was collected and recovered by precipitation with isopropyl alcohol and washed with ethanol 75% (Sigma) before dilution in RNase-free water. RNA concentration and purity with A260/A280 and A260/A230 ratios >1.95 were determined using spectrophotometer Nanodrop ND1000. In addition, quality of PCLS RNA was assessed via RNA integrity number (RIN) using Agilent 2100 Bioanalyzer (Agilent Santa Clara, CA USA). An aliquot of total RNA (1 µL) was analyzed using RNA Nano LabChips (Agilent). RIN value is determined by an algorithm for assigning integrity values to RNA measurements using the 28S-to-18S-Ribosomic RNA ratio.

5.5.1.2. Reverse transcription and PCR

Reverse transcription (RT) was performed with the Kit High Capacity cDNA-RT (Fisher Scientific, Illkirch, France). Each reaction contained 10 µL RNA (2 µg RNA) and 10 µL of the mixture (2 µL of 10X RT buffer; 0.8 µL of 25X dNTP MIX 100 µM; 2 µL of 10X RT random primer; 1 µL of RNase inhibitor, 1 µL of multiSribe reverse Transcriptase and 3.2 µL Nuclease-free H_2O) (Thermo Fisher, Life Sciences). The conditions of RT reaction were 25°C for 10 min, 37°C for 2 h, 85°C for 5 min, maintain at 4°C. Ultimately, 5 µL of cDNA were diluted in 95 µL of sterile water. Diluted cDNA (5 ng/µL) was stored at -20 ºC for the next steps of qPCR (quantitative polymerize chain reaction).

Quantitative real time polymerize chain reaction (qRT-PCR) was performed to assess the relative mRNA expression levels. Each sample was tested in duplicate and the potential genomic DNA contamination was verified through a non-template control (NTC). For one reaction, 2.5 µL of SyberGreen Power® SYBR Green qPCR master MIX (Sigma-Aldrich) and 1.5 µL of Primers (reverse and forward) and 1 µL of cDNA (5 ng/ µL) was prepared. The specificity of qPCR products was assessed at the end of the reactions by analyzing the dissociation curves using QuantStudio^{-TM} (Realtime PCR Software v.1.1, 2014). Data were exported into an Excel file. LinRegPCR v2014.3 program was used for qPCR data analysis to calculate N0 (the initial RNA concentration) and amplification efficiency (Ruijter *et al.* 2014) and then the data were exported back as an Excel file. For quantification and statistical analysis, target genes mRNA expression was normalized with the housekeeping genes (HKG) using N0 ($=10^{intercept}$) with LinRegPCR. Three HKG, ribosomal protein L32 (RPL32), Beta-2-Microglobulin (B2-microglobulin) and TATA-Box Binding Protein (TBP) were used for data normalization. The best housekeeping genes were determined using Normfinder program which calculates a stability value based on the combined estimate of both intra- and intergroup values

(Andersen *et al.* 2004). In brief, NormFinder and LinRegPCR are the best approaches for reference gene selection and efficiency determination, respectively.

5.5.2. Results and discussion

5.5.2.1. Quality of the RNA

The assessment of RNA integrity is a critical first step before the following steps of RT and qPCR. RNA-Integrity (RIN) was obtained from each control slice. Analysis of the Bioanalyzer profile of total RNA indicated that the 28S and 18S rRNA bands were visible for each individual in each incubation time except for one sample at 20 h (Figure 8). The RIN for an RNA sample ranges from 1 to 10. A RIN of 10 is fully intact and a RIN of 1 is completely degraded (Fleige and Pfaffl 2006). Average RIN of 3 liver slices (biological replicates) were calculated. Initial RIN (sample without incubation) was 7.5 whereas; the final RIN (20 h incubation) was obtained 6.2. This moderate decrease demonstrates that the quality of PLCS were maintained stable after different time of incubation (Figure 9). Thus, the RIN we obtained allowed us to go further and perform the qPCR and perform assessment of gene expression.

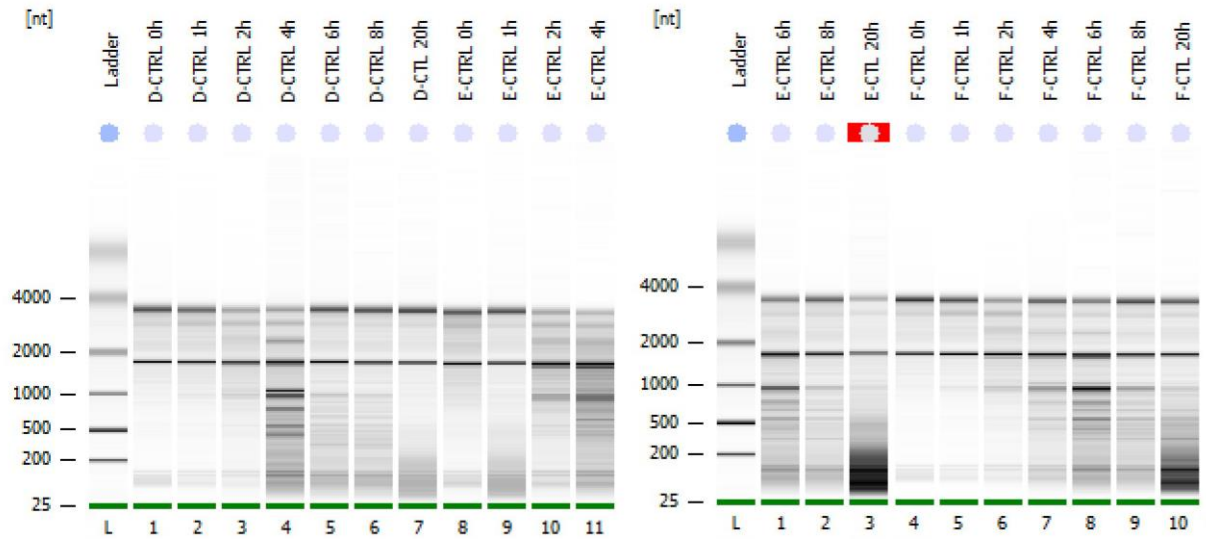

Figure 8: RIN obtained from an individual control PCLS in different time of incubation: Each letter (D, E, F) represent a biological replicate (animal).

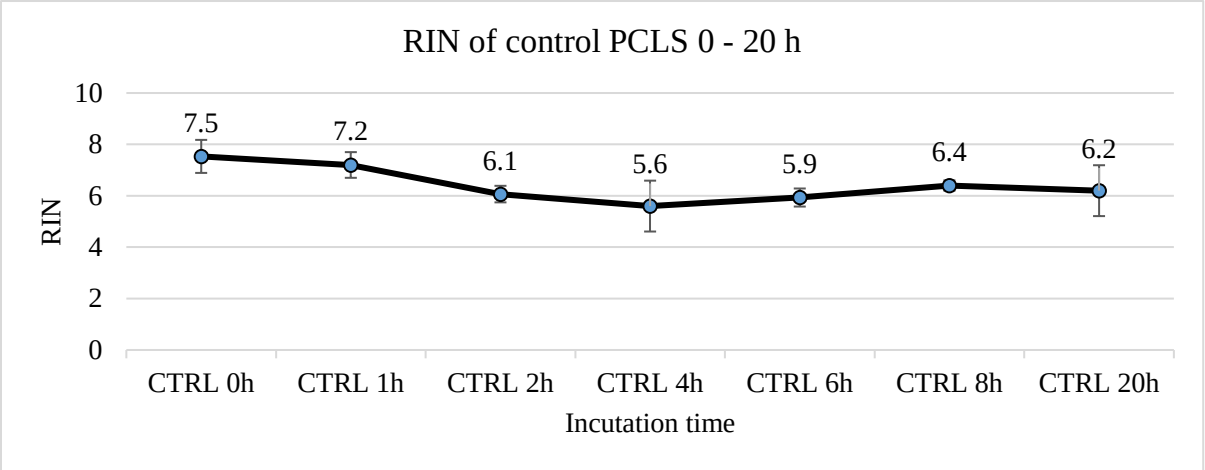

Figure 9: RIN curve of three controls animals over 20 h of incubation. Data are mean ± SEM of three biological replicates.

Quality of RNA were evaluated in different species (human, rat and mouse). Initial RIN for human, rat and mouse PCLS were 8.9, 8.3 and 8.2, whereas after 3 days of incubation the final RIN were 7.8, 6.8 and 7.3 respectively (Niehof *et al.* 2017). RIN is usually higher in cell lines rather than PCLS. For example, RIN were always ≥ 8.1 for tissue and ≥ 9.5 for cells during 4 h incubation (Delgado-Ortega *et al.* 2014).

It should be kept into account, the RIN number is not a tell-all with regard to downstream applications. For example, depending upon primer design, RT-PCR can be performed successfully when starting with partially degraded RNA when the amplicon is short. Similarly, RIN numbers are not particularly useful for nucleic acids that have been purified from archived pathology specimens. RNA recovered from formalin-fixed paraffin-embedded tissues and tissue sections also exhibit some degree of degradation (Farrel. Jr 2017).

5.5.2.2. Assessment of gene expression

Next, we selected several genes involved in different cellular function and processes (Table 22) including 3 HKG. Selection of the genes was based on the literature (Nielsen *et al.* 2009; Sahu *et al.* 2010; Kouadio *et al.* 2013; Osselaere *et al.* 2013; Wu *et al.* 2014b; Liu *et al.* 2016; Luo *et al.* 2017). The goal of the first experiment was to determine the quality of the PCLS over the incubation time. Therefore, we selected genes already investigated in previous studies to confirm those results and hence to evaluate the relevance of the model of PCLS.

Table 22[2]: List of selected genes assessed in PCLS in response of DON 10 µM

Inflammatory cytokines	Lipids Metabolism	Oxidative Stress	Junctional Proteins	Transcriptional Factor	Protease Inhibitors	Housekeeping Genes
CCL20	SCD1	SOD1	E-Cadherin	JUN	Serpin	RPL32
IL-6	SCARB-2	CAT	OCLN	FOS		B2-microglobulin
IL-1b	CYP7A1	CCS	CLDN-3			TBP
IL-10	LDLR	DUOX				
IL-22	LXR	NFKB				
IL-8	ABCG-8					
TNF-alpha	NR1H3					

First, we analyzed the effects of DON 10 µM on the gene expression at different incubation time (2, 4, 6, 8 and 20 h) (Figures 10-13). Genes were modulated after 4 h when PCLS were treated with DON 10 µM at different exposure time. Hereby, inflammatory cytokines were upregulated time dependently until 8 h. Most of the genes were upregulated time dependently compared to the controls one. CCL-20, IL-1b, IL-8 and IL-10 were significantly upregulated until 8 h of exposure, but at 20 h, their expression was decreased. TNF-alpha was upregulated only at 6 h of exposure. None of the tested inflammatory cytokines was expressed at 2 h (Figure 10). Obtained results were consistent to the previous results. DON at 2.5 mg/kg BW markedly upregulated TNF-a, IL-1b, IL-6, CXCL-2, CCL-2 and CCL-7 after 2 and 6 h in spleen (Wu *et al.* 2014b). DON at 24 µM also induced inflammatory cytokines IL-1b, IL-1a, IL-8, and TNF-a when jejunal explants were treated for 4 h (Luo *et al.* 2017). Altogether, DON induces inflammation in liver as in other organs.

Among the tested genes involved in cellular function of lipid metabolism (ABCG8 and SREBP2) were significantly upregulated and LXR was significantly downregulated time dependently (Figure 11). Our obtained results were in accordance with previous results. DON significantly affected lipids metabolism in HepG2 cells when treated with DON at 10 µM for 4 h -12 h periods. (Liu *et al.* 2016). Likewise, DON revealed disorders in lipid metabolism, when Swiss mice were orally administered with low doses of DON (45 µg/kg bw/day) during 7 days (Kouadio *et al.* 2013). In liver, disorders of lipid metabolism, may result in deposit of lipids in the walls of blood vessels, which can lead to atherosclerosis (a disease characterized by abnormal thickening and hardening of the walls of the arteries). In the consequence of gene

[2] Definition of abbreviations:
CCl-20 (C-C Motif Chemokine Ligand), IL (Interlukin), TNF-a (Tumor Necrossis Factor Alpha), SCD1 (Stearoyl-CoA Desaturase), SCARB-2 (Scavenger Receptor Class B), CYP7A1 (Cytochrome P450 Family), LDLR (Low Density Lipoprotein Receptor), LXR (Liver X Receptor), ABCG-8 (ATP Binding Cassette), NR1H3 (Nuclear Receptor Subfamily 1), SOD1 (Superoxide Dismutase), CAT (Catalase), CCS (Copper Chaperone), DUOX (Dual Oxidase), NFKB (Nuclear Factor kappa-light-chain-enhancer of activated B cells), OCLN (Occludin), CLDN-3 (Claudin-3) JUN (Jun Proto-Oncogene), FOS (Fos Proto-Oncogene), RPL32 (Ribosomal Protein L32), B2 (Beta-2)-Microglobulin, TBP (TATA-Box Binding Protein)

modulation that are involved in cellular function of lipid metabolism may cause an autosomal dominant disease. Mainly, this disease in liver is associated due to the deficiency of the low density lipoprotein.

Genes involved in the cellular function of oxidative stress were also modulated time dependently except SOD1. All three genes NFKB, CAT and CCS were upregulated significantly compared to the corresponding control (Figure 12). These results confirm that DON induces oxidative stress in liver. Obtained results were in accordance with previous results. DON at 10 µM for 4 h-12 h induced oxidative stress in HepG2 cells (Liu *et al.* 2016). Likewise, similar cells were exposed to DON at 25 µM for 6h-24 h, and was associated with oxidative stress (Sahu *et al.* 2010). Oxidative stress is traditionally defined as an imbalance between pro-oxidant compounds and antioxidant defenses (Kunsch Charles and Medford Russell M. 1999). Oxidative stress is thought to play a role in the genesis of hepatic encephalopathy found in patients who have liver failure (Webb and Twedt 2008).

DON had moderately effect on gene modulation involved junctional protein of PCLS. Only E-Cadherin was significantly up regulated in longer incubation time (20 h) (Figure 13). Obtained results were consistent and confirmed previous results. DON at 7.5 mg/kg feed, fed during 3 weeks to broiler chickens, up-regulated CLDN-5 in the jejunum, while no effect was seen on genes involved in cells junction (Osselaere *et al.* 2013). It is well known that in eukaryotic cells, at the cellular and subcellular level, DON binds to the ribosome, and inhibits protein synthesis (Pierron *et al.* 2016b). Tight junction (TJ) proteins exert their functional role as integral proteins in forming barriers in the liver. A causative role of TJ proteins has been established in the pathogenesis of colorectal cancer and gastric cancer. Among the best characterized roles of TJ proteins in liver disease biology is their function as cell entry receptors for HCV (hepatitis C virus)—one of the most common causes of hepatocellular carcinoma (Zeisel *et al.* 2019).

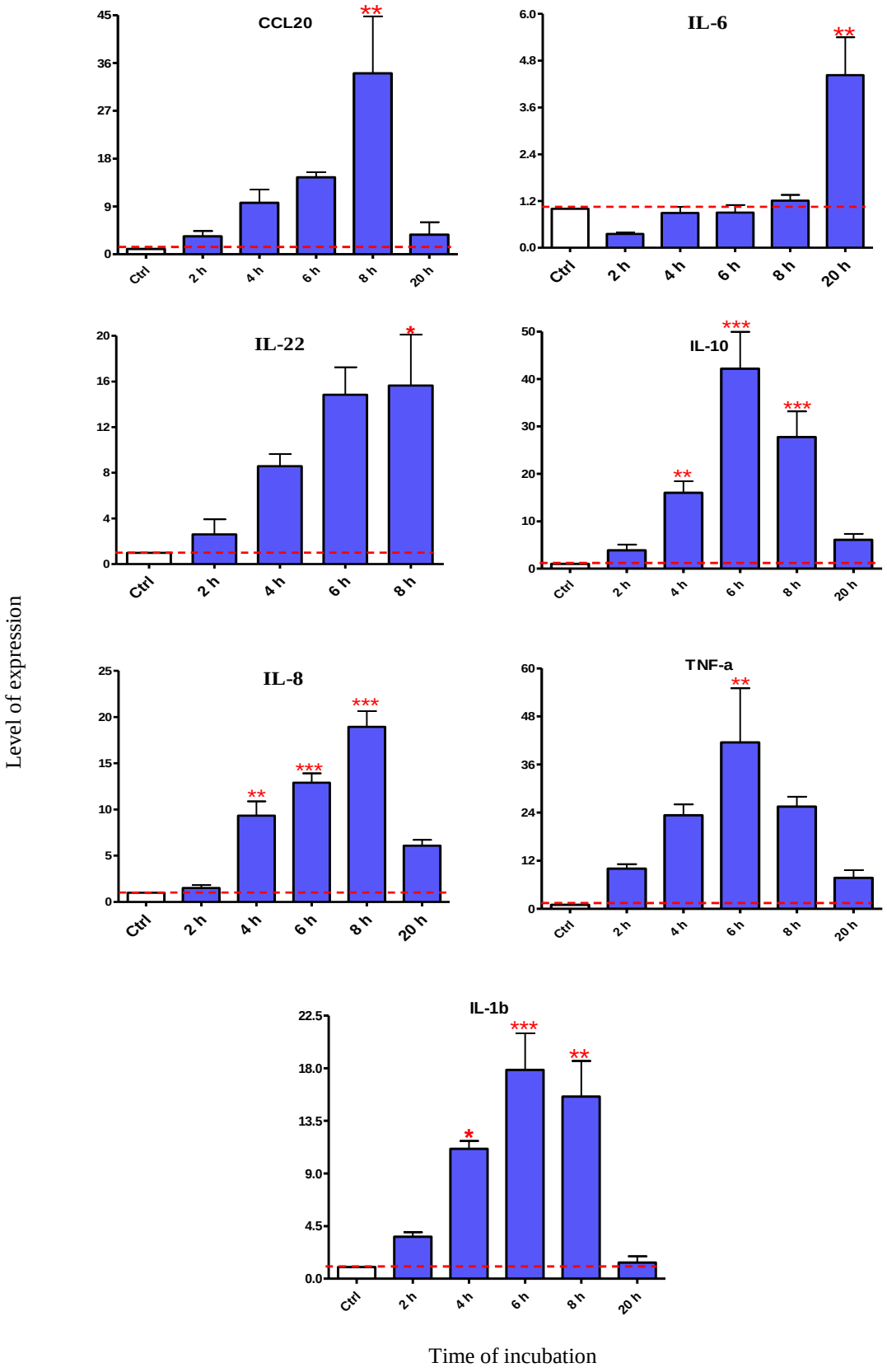

Figure 10: PCLS treated with DON 10 µM. Inflammatory cytokines, gene modulation: Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference between treated and corresponding control at each time point * P < 0.05, ** P < 0.01, *** P < 0.001

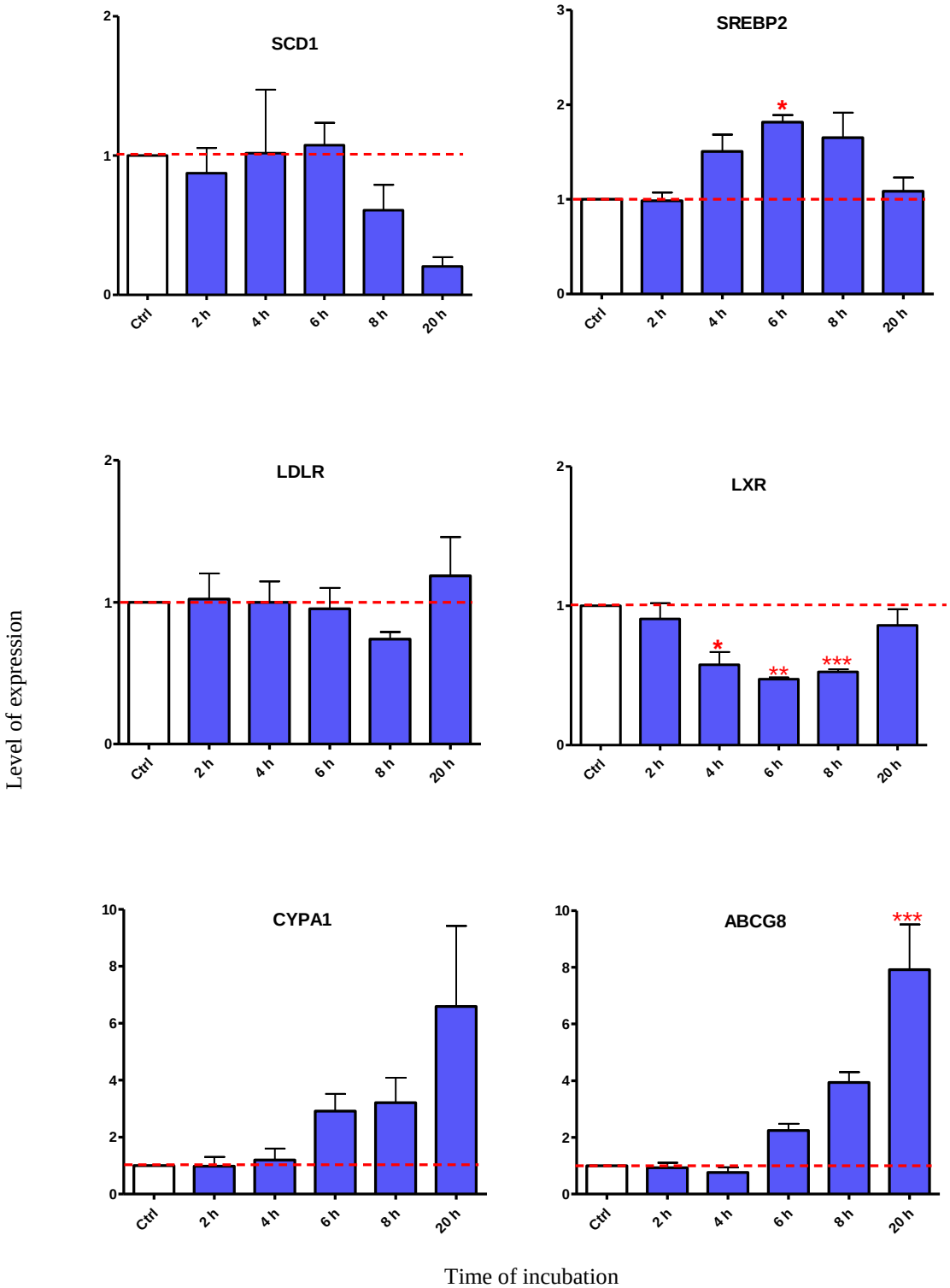

Figure 11: PCLS treated with DON 10 µM. Lipids metabolisms, gene modulation. Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference between treated and corresponding control at each time point * P < 0.05, ** P < 0.01, *** P < 0.001

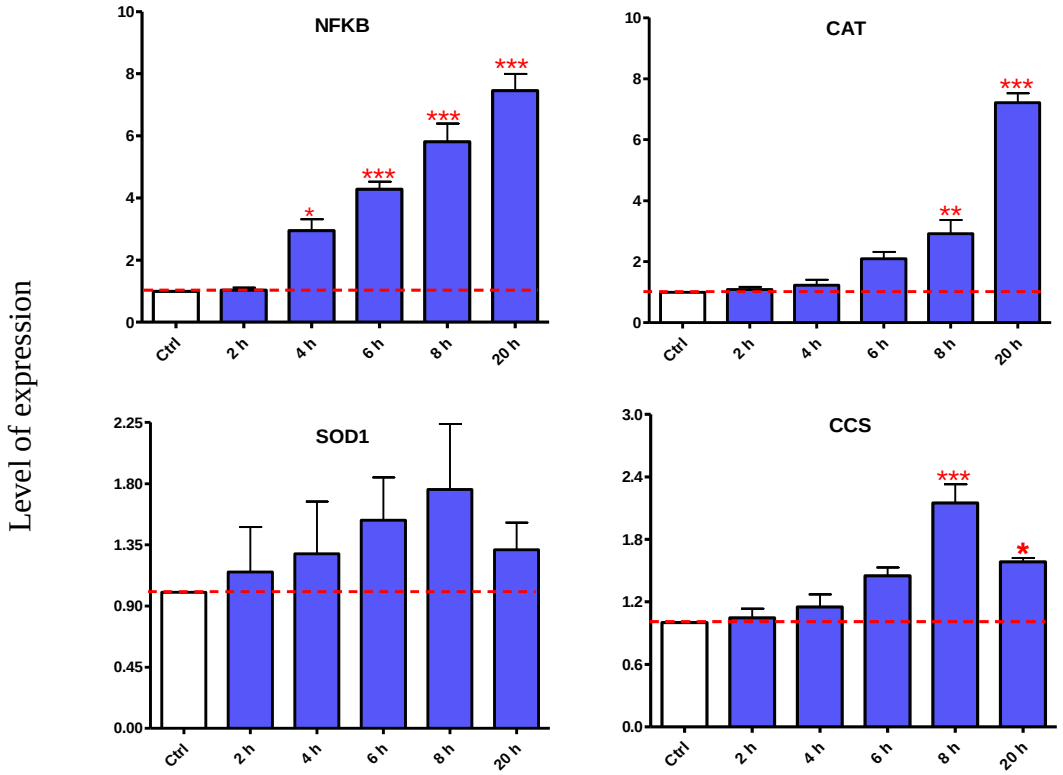

Figure 12: PCLS treated with DON 10 µM. Oxidative Stress, gene modulation

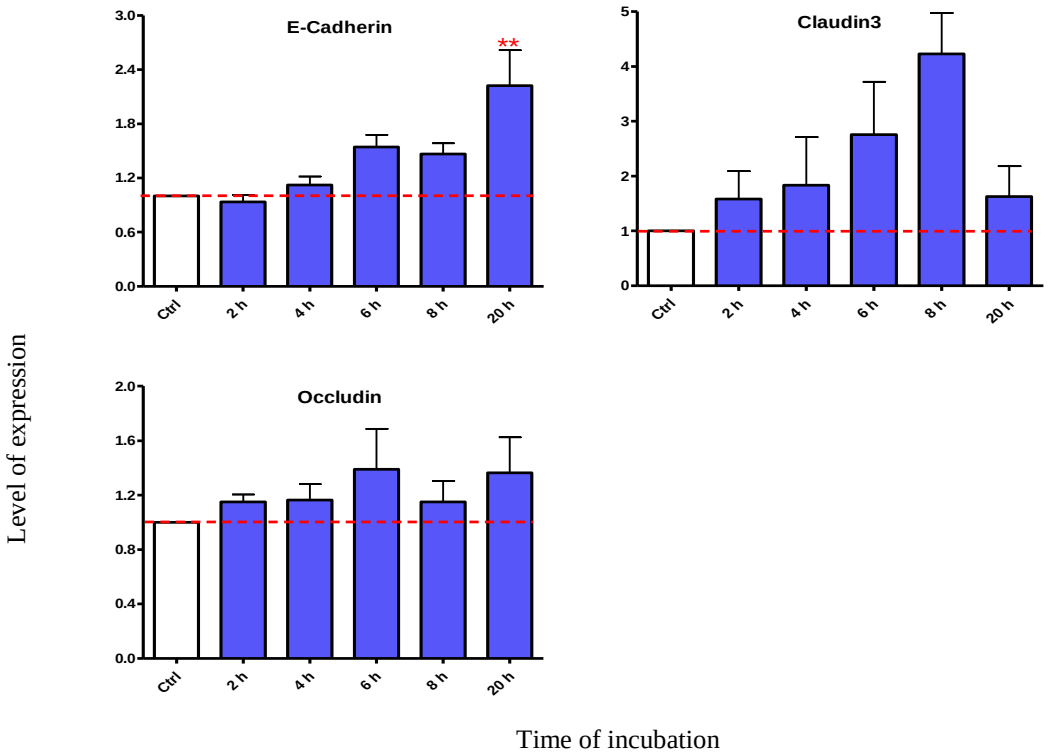

Figure 13: PCLS treated with DON 10 µM. Protein junction, gene modulation. Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference between treated and corresponding control at each time point * P < 0.05, ** P < 0.01, *** P < 0.001

Then the effect of the doses of DON 3 µM and 10 µM was assessed on gene regulation at one incubation time 20 h (Figure 14). Genes were selected involved in different cellular function or process such as (inflammatory cytokines, lipids metabolism, oxidative stress, junctional proteins, transcriptional factor and protease inhibitor) (Table 22). All the genes were regulated dose dependently except CCS and SOD1 genes involved in cellular function of oxidative stress and Serpin which is the largest and most broadly distributed superfamily of protease inhibitors. Expression of some studied genes was modulated when PCLS were treated with DON 10 µM. However, expression of some genes seems to be modulated with DON 3 µM but not significantly. Only one gene of inflammatory cytokine (CCL20) was significantly regulated when PCLS were treated with DON 3 µM (Figure 14).

Overall, genes from different pathways were up regulated at 20 h of exposure in response to DON 10 µM. Most of the genes were up-regulated in longer exposure time, whereas some of the genes were down-regulated. Likewise, most of the genes were regulated dose dependently.

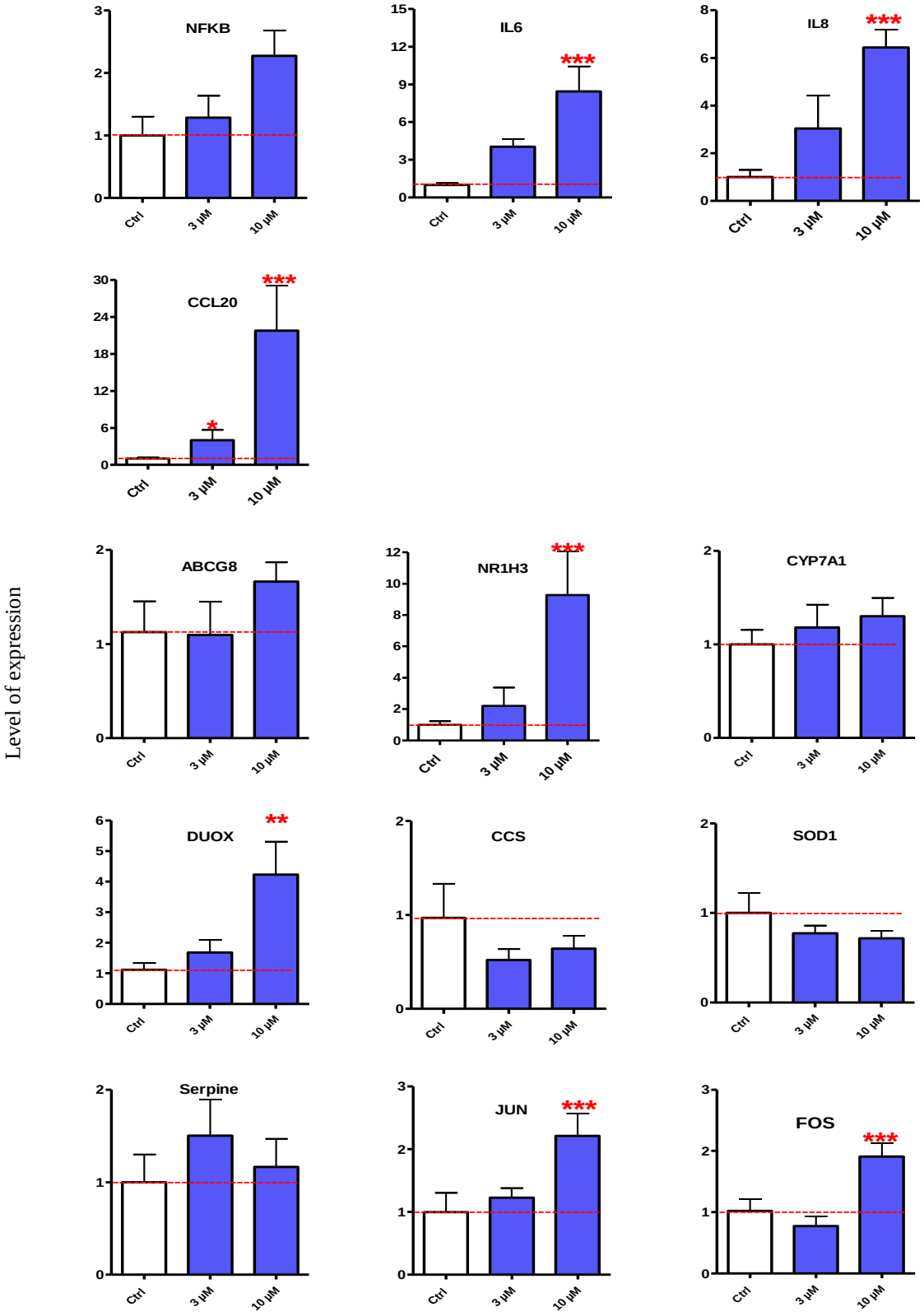

Figure 14: PCLS treated with DON 3 µM and 10 µM at 20 h incubation. Data are mean ± SEM of three biological replicates. Bonferroni multiple comparison test. Significant difference treated and corresponding control at each time point * P < 0.05, ** P < 0.01, *** P < 0.001

However, it is reported that inflammatory cytokines are regulated due to DON in the intestinal cells (Stadnyk 2002; Wan *et al.* 2013), but we found that DON can induce other genes in hepatic cells in addition to inflammatory cytokines. Other studies have also shown that in addition to inflammatory cytokines, DON can induce genes from other pathways. DON modulated significantly inflammatory cytokines IL-1β, IL-1α, IL-8, and TNF-α as well genes associated with oxidative stress MT1A, MT2A, when intestinal jejunal explants were treated with DON 0.75-24 µM for 4 h (Luo *et al.* 2017). Furthermore, modulation of inflammatory cytokines (IL-1β, IL-1α, IL-8, TNF-α) were observed following DON exposure *in vitro* and *in vivo* (Chung *et al.* 2003; Islam *et al.* 2006).

5.6. Discussion on PCLS

The model of tissue slices was invented in 1923 to measure cell metabolism and oxygen consumption in tumor tissue and then explored to study amino acid metabolism in various organs and species including human (Li *et al.* 2016). PCLS have been used by many researchers because they represent an organ mini-model that closely resembles the organ from which it is prepared, with all cell types present in their original tissue-matrix configuration (Graaf *et al.* 2007). Before, PCLS were used for a dynamic organ culture system as incubation system (Dogterom 1993). Whereas now, PCLS have been shown to be a valuable tool to predict metabolism of novel drugs, metabolism and toxicity of xenobiotics and as well for many other purposes (Graaf 2006; Graaf *et al.* 2007). This model has been developed long time ago.

We developed the PCLS model in order to assess toxicity of mycotoxins through several biomarkers, such as ATP and total proteins contents, enzyme release such as ALP, AST, ALT and LDH, and as well gene expression. Although we used piglet liver, this model it is easily transferable to mouse, guinea pig, monkey, dog, and as well to human (Graaf *et al.* 2010). We found that liver slices is a good model for long incubation time under constant presence of carbogen. On the opposite, certain organs such as intestine cannot be incubated for longer incubation (Graaf *et al.* 2010).

Our results of liver damage markers, ALP, AST, ALT, LDH and total protein contents in the incubation medium were not affected either by incubation or treatments. We chose a time of exposure of 20 h in order to maintain quality of RNA in PCLS and avoid its degradation. The RNA molecule is inherently fragile in the natural environment and constantly degrade into smaller molecules through hydrolysis, preventing the faithful reproduction (Spurway and Wackerhage 2006; Fiore 2019).

Then, we assessed effect of DON 3 µM and 10 µM on gene expression of PCLS. Genes were selected based on literature involved in different cellular functions or processes. We confirmed that the effect of DON on the gene expression was dose and time dependent. In longer incubation and in high concentration of DON, regulation was significant compared to their corresponding controls. Most of the genes were modulated time dependently compared to the controls. Modulation was mainly pronounced after 2 h of incubation. Most of inflammatory cytokines, i.e. CCL-20, IL-1b, IL-8 and IL-10, were significantly upregulated until 8 h of exposure. Among the tested genes involved in cellular function of lipid metabolism, ABCG8 and SREBP2 were significantly upregulated and LXR was significantly downregulated time dependently. Genes involved in the cellular function of oxidative stress (NFKB, CAT and CCS) were also modulated time dependently except SOD1. DON had a moderate effect on gene modulation involved in junctional protein of PCLS. Only E-Cadherin was significantly up regulated at longer incubation times.

To our knowledge, this is the first study that assess the effect of DON on the PCLS. However, effect of DON have been addressed on hepatic cells *in vivo* and *in vitro* (Sahu *et al.* 2008; Grenier *et al.* 2011; Osselaere *et al.* 2013; Bracarense *et al.* 2017). Effect of low dose (commonly found in crops) of DON was examined on five weeks old piglets. DON contaminated diets induced significant liver lesions when compared to animals fed control diet (Grenier *et al.* 2011). Effects of 1.75 mg/kg or 11.4 mg/kg of DON in contaminated diet was assessed in rats exposed for 7, 14 or 30 days, using histological and immunohistochemical analyses on liver. Ingestion of contaminated feed induced a significant increase in the lesional score in the liver. The main histological findings observed were cytoplasmic vacuolization and hepatocellular megalocytosis. A significant increase in hepatocyte proliferation was observed in rats that received 1.75 mg/kg of DON. The results of the morphological and immunohistochemical analyses suggest that the ingestion of DON can induce functional hepatic impairment and immunosuppression in a dose- and time-dependent manner (Bracarense *et al.* 2017).

In conclusion, the liver slices appear as a promising model to study the effects of food contaminants on liver tissue. It allows the assessment of different markers into the tissue or released in the culture medium. This tool may also prove useful for identification of genes in liver-resident cells that support cancer or invasion in liver. Moreover, this tool can partially supplant *in vivo* pig experiments for prescreening liver diseases.

5.7. Discussion on Experimental models

A model is "a representation of a real or actual subject". In the field of toxicology, experimental scientific models are used to understand toxicological mechanisms. Toxicology is an experimental laboratory science that has traditionally utilized various animal models to study the effects of chemicals. Historically, toxicology studies relied heavily on various histopathological and biochemical endpoints in whole animals to draw conclusions on the effects of a chemical on living systems (Choudhuri *et al.* 2018). However, the general adoption of the principles of 3R (Replace, Reduce, Refine) provided the impetus for the development of *in silico, ex vivo* and *in vitro* models in toxicity testing (Figure 15). Their relative contributions to the scientific and animal welfare implications as well the opportunities they offer in the research are different. As each of them facilitated research in one way, on the other hand they have disadvantages and limitations.

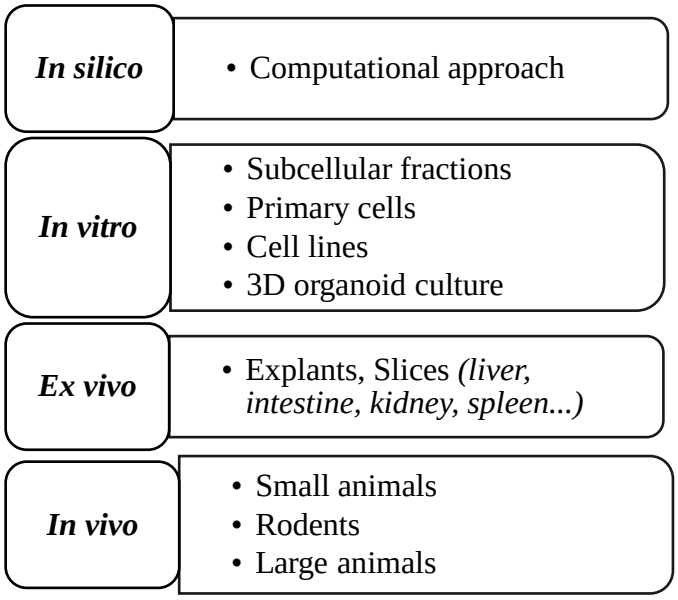

Figure 15: Experimental models

5.7.1. *In silico* models

In silico toxicology methods are computational approaches that analyze, simulate, visualize or predict the toxicity of chemical products. These methods are based on the chemical structure of the compounds, and are usually used together with other toxicity tests. The purpose of building a model for use in *in silico* prediction of toxicity is to derive a relationship between the properties of the compounds and their biological effect (Cronin and Madden 2010). *In silico* toxicity is assessed using a wide variety of tools (i.e., methods, algorithms, software, data, etc) to analyze beneficial or adverse effects of a compound (Yap *et al.* 2019). *In silico* models are

used to solve many problems in predictive toxicology and furthering knowledge and reducing the need to resort to animal testing. Predictions are done based on the chemical structure and selected properties of a compound. So, modelling and simulation via *in silico* contribute to reduce, refine and replace animal experimentation. Mainly modelling and simulation are based on experimental data, quantitative structure activity relationship and scientific data (Cronin and Madden 2010). Following are briefly listed pros and cons of this model:

In Silico model	
Pros	**Cons**
• Predictions for a large set of compounds in a high-throughput mode • Simulation based on the structure of a compound • Performing without animals • Less cost	• Difficult to extrapolate result to *in vivo/ex vivo* • Need special software and expert people

The building of *in silico* models is an exciting field as it draws on so many areas of science and is dynamic and continually evolving. Looking to the future, there are many opportunities for further development of this field. This will include the use of more successful computational techniques to gain insight into fundamental molecular and chemical processes (Cronin and Madden 2010).

5.7.2. *In vitro* models

The term *in vitro* presents "the technique of performing a given procedure in an artificial environment outside the living organism". The major aims of *in vitro* systems are to develop, simulate and predict biological reactions to materials when placed into or on tissue in the body. The *in vitro* methods not only assist in hazard identification and risk assessment but also in understanding dose response relationship and evaluating a new product before its market launch or supplying to researchers or pharmacological companies (Dhawan and Kwon 2017). Although, the *in vitro* studies can be criticized for being very different from the natural environment, they present several advantages.

In vitro models	
Pros	**Cons**
• Reduced number of animals • Lower costs • Optimal dose test • Rapid and easy to perform • Allow to analyze the mode of action • Adapted to assess interaction	• Effect on a single cells does not represent effect on the whole body • Difficult to assess pharmacokinetics/ absorption/excretion • Difficult to extrapolate results to *in vivo*

5.7.3. *Ex vivo models*

Ex vivo models represent a tissue or slices culture of an organ, where the natural original three-dimensional architecture and original cell composition and intercellular connections are retained. An *ex vivo* model is ethically advantageous, requires no postsurgical animal care, enables more reproducibility, and provides a tightly controlled artificial environment for toxicological studies (Dhawan and Kwon 2017). Although, *in vitro* and *ex vivo* appear somehow similar, as both testing methods involve experiments on biological matter, conducted outside of a living organism in an artificial environment. For instance, *ex vivo* models are much more complex than *in vitro* in terms of cell diversity, and therefore closer to *in vivo* conditions. Despite of many advantages of *ex vivo* models, they also has some limitations as well.

Ex vivo models	
Pros	**Cons**
• Reduce animal number • Closest to *in vivo* • Cytoarchitecture retained • Numerous explants from a single tissue • Cell remain fully differentiated • Physiological function maintained	• Limited incubation time • Require fresh explant for each experiment • Difficult to extrapolate results from animal to human

5.7.4. *In vivo models*

In vivo models are defined as “living organisms with an inherited naturally-acquired or induced pathological process that closely resembles the same phenomenon in human” (Chow *et al.*

2008). Their application in biomedical research, and pathophysiological and toxicological studies, provide the opportunity to determine pharmacokinetics of toxic compounds and to observe other clinical symptoms such as feed and water intake. In order to interpret and validate the results of other experimental models (*in vitro, in silico, ex vivo),* it is essential to perform *in vivo* assays. *In vivo* models are complex and integrated models that includes ingestion, absorption, metabolization and excretion processes. Following are briefly pointed out the main pros and cons of this model:

In vivo model	
Pros	**Cons**
• Presence of all metabolic process of host mechanism • Study animal behaviour against toxins • Assessment of zootechnical parameters • Pharmacokinetics assessment • Sampling of different organs	• Authorization of protocol from Ethical Committee • Expertise required • Large number of animals for statistical analysis • Costly

The two forms of *in vivo* experiments are animal studies and clinical trials in toxicological and pharmacological studies. Animal models are classical toxicological models used as a mandatory element in preclinical (toxicological and pharmacological) studies of new compounds (Parvova *et al.* 2011). Animal models are used as preclinical tools for potential drug screening processes and translation into clinical trials. Preclinical testing includes testing of all materials and prototypical devices before testing or using in humans. This testing is conducted in order to provide an assurance of device safety prior to use in humans in a clinical trial (Belma *et al.* 2019).

The overall effect of the experiment on a living organism can be observed in *in vivo* models. Thus, *in vivo* experiments are more realistic than *in vitro* experiments with respect to the phenomenon and toxicity which comes out in the consequence of a toxicological experiment. Their main advantages are that they allow studying the toxicity of mycotoxins in presence of host defense mechanisms and integrated (ingestion, digestion, and excretion) system. In addition, *in vivo* assays provide the opportunity to assess the toxicity of mycotoxins through several endpoints simultaneously. For instance, suitable biomarkers for DON and ZEN exposure in porcine plasma, urine and feces and for DON, AFB1 and OTA in plasma and

excreta of broiler chickens were identified (Lauwers *et al.* 2019). The effects of DON and FUM alone or in combination during 5 weeks diet on hematological and biochemical blood parameters as well histopathological effects on the liver, lungs and kidneys were investigated (Grenier *et al.* 2011). A sub-chronic toxicity study of NIV was conducted in male and female rodents for 90 days to assess histopathology of the hematopoietic and immune organs, body weight gain, white blood cell and red blood cell counts as well the hemoglobin concentration (Takahashi *et al.* 2008).

So far, numerous experimental models have be introduced. Each model has its justification at some points when applying to investigate toxicity (Sande 1999). Thus, a number of animal and non-animal models are used in research over the last years. It is established worldwide that *in vitro* testing models are of extreme importance for screening as well as to elaborate the mechanism and set of causes of various diseases (Jain *et al.* 2018).

Among all the model previously presented, first we chose a cell culture model in order to investigate individual and combined toxicity of DON and emerging mycotoxins in porcine intestinal cells *in vitro*. Then, we developed precision-cut liver Slices (PCLS) model *"ex vivo"* in order to assess hepatotoxicity of DON in liver slice, as briefly discussed their implication, pros and cons as following ahead.

5.7.5. *Use of intestinal cells to assess the toxicity of emerging mycotoxins*

In our study, first we used an *in vitro* model of intestinal cells, the first target of ingested contaminants, to characterize the potential toxicity of emerging mycotoxins. We used the porcine intestinal epithelial cells (IPEC-1) and this *in vitro* model allowed us to assess serial doses-toxic effects of mycotoxins either alone or in mixture. In the following part, we focused on the pro and cons of this cell culture model.

Intestinal Epithelial Cell culture model

Pros	Cons
• Standard culture condition • Low cost • Unlimited lifespan (immortalized cell lines) • Obtained from different target organs	• Altered genomic contents • Represent the only smallest part of an organ • Changing characteristics after several passages

• Physio-chemical environments precisely controlled	• Need validation via *in vivo*

In vitro presents a rapid, easy and economic model that is widely used to investigate toxicity of mycotoxins. For instance Novak *et al.* assessed the individual toxicity of emerging and regulated mycotoxins in porcine intestinal cells (IPEC-J2) using an *in vitro* model (Novak *et al.* 2019). On the base of iso-dose effect, the combined toxicity of several ENNs (A, A1, B and B1) were assessed individually or in combination using Caco-2 cells (Prosperini *et al.* 2014a). Binary ENN+DON (1:1) and quaternary mixture of ENNS (A, A1, B, B1) were tested in ratios 1:1:1:1 in Caco-2 cells (Prosperini *et al.* 2014a). Likewise, binary mixture of ENN+BEA, DON+BEA and DON+ZEA were tested in Caco-2 cells using *in vitro* (Ficheux *et al.* 2012; Fernández-Blanco *et al.* 2016).

5.7.6. Development of PCLS model to assess toxicity of mycotoxins

Precision-cut liver slices (PCLS) are viable *ex vivo* explants of tissue with a reproducible, well defined thickness. They represent a mini-model of the organ under study and contain all cells of the tissue in their natural environment, leaving intercellular and cell-matrix interactions intact, and are therefore highly appropriate for studying multicellular processes (de Graaf *et al.* 2010). We used a liver slice model to assess the toxicity of DON. We aimed to develop the PCLS model in order to make screening and assess toxicity through several markers such as ATP, total protein contents and gene modulation. With an increasing public demand to limit the number of animals used in research and to reduce the distress to the experimental animals (3R concept), PCLS represent a promising method with numerous advantages for toxicological studies.

PCLS model	
Pros	**Cons**
• Lobular structure preserved (all enzyme equipment preserved) • Selective intralobular effects detectable • Studies on human liver possible • Studies on several compounds at different concentrations	• Viability: 6 h to 2 days • No collection of bile possible • Not all the cells preserved similarly (inter-assay variability)

PCLS represent the closest *in vitro* model of the *in vivo* situation. The major advantages are that the three-dimensional architecture is preserved. PCLS have been applied by many researchers (Graaf *et al.* 2007). One of the great advantages of the PCLS model is that numerous slices can be prepared from a single liver. On the other hands, PCLS can be employed for studies of xenobiotic metabolism, xenobiotic-induced cytotoxicity and genotoxicity, and to evaluate the effects of xenobiotics on hepatic enzyme activities (Price *et al.* 1998).

Moreover, slices can be prepared from a range of tissues (including the heart, kidney, lung, intestine and spleen) from both experimental animals and human organs depending on the purpose of the study. For example, the effects of 4 h exposure of ENN-B1 alone and mixed with the most toxic trichothecene the T-2 toxin were assessed on the histopathology of piglets intestinal explants (Kolf-Clauw *et al.* 2013). Intestinal explants mounted in Ussing chambers were used to investigate the effect of mycotoxins on paracellular passage of FITC-dextran (Pinton *et al.* 2009). Mouse precision-cut liver slices (mPCLS) were incubated for 24 h to assess drug-induced liver injury (Hadi *et al.* 2012). Furthermore, splenic explants were used to characterize the clinical, immunological, and parasitological features of the hamster spleen infected with the parasite *Leishmania donovani* (Osorio *et al.* 2011).

We investigated the toxicity of DON in the PCLS model. To the best of our knowledge this is the first study, investigating toxicity of mycotoxins using PCLS. Our aim in developing PCLS model was to introduce an appropriate alternative to the *in vivo* model contributing to the reduction of animal number and adapted to the simultaneous study of several toxins.

In conclusion, all above mentioned models are widely used whereas none of them can be considered as ideal model. Each model has its own advantages and limitations, but all those models give us a wide and global perspectives to use and implement them in the research. The purpose of this discussion was to explore the historical evolution of different culture models and the reasons why the continues development of different models exists.

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6. General Conclusion

Maintaining a safe global food and feed supply is a critical issue facing society. Cereal grains and their processed food products are frequently contaminated with mycotoxins. The diverse nature of mycotoxigenic fungi contribute to the worldwide occurrence of mycotoxins. Recent finding and surveys have shown that 60 – 80% of food, feed and cereal grains are contaminated by well-known as well as by emerging mycotoxins (Gruber-Dorninger *et al.* 2019; Eskola *et al.* 2019; Abdallah *et al.* 2017; Streit *et al.* 2013).

We collected more than 500 finished pig feed samples from the worldwide market during five years and analyzed them via LC-MS/MS method. We found that more than 95% of the samples were contaminated by at least one mycotoxin and 88% of them were naturally co-contaminated by DON and other regulated and emerging mycotoxins. The co-occurrence of mycotoxins represents the reality of fungal contamination of cereals and cereal-derived food products (Lee and Ryu 2017).

We analyzed the prevalence and concentration of mycotoxins in compound feed which exact composition was unknown. The composition of feed might have an influence on the prevalence and as well on the concentration of mycotoxins. Although, more than one mycotoxin, produced by a single or several fungal species, may occur in various combinations in a given sample or food. Thus, compound feed that are made of several and various commodities, could be more contaminated rather than the raw materials (Streit *et al.* 2012). However, some authors have claimed that the concentrations of mycotoxins tend to be lower in processed food products; the incidences varied depending on the individual mycotoxins, possibly due to the varying stability during processing and distribution of mycotoxins (Lee and Ryu 2017).

DON is one of the most wide distributed regulated mycotoxins across the world (Knutsen *et al.* 2017; Gruber-Dorninger *et al.* 2019; Novak *et al.* 2019). Among farm animals, pig is one of the most sensitive species to mycotoxins (Pinton *et al.* 2009; Pierron *et al.* 2016b). When exposed to DON, pig performances and immune response are altered. This lead the CONTAM Panel to determine the LOAEL of 1.3 mg DON/kg feed for pig using the reduction of feed intake as the most sensitive endpoint (Knutsen *et al.* 2017).

In addition to regulated mycotoxins, emerging mycotoxins also occur frequently and in high concentrations in cereals and cereal-derived products (Springler *et al.* 2016b; Malachová *et al.*

2014; Vaclavikova *et al.* 2013). Emerging mycotoxins are usually co-produced with regulated mycotoxins (Hussein and Brasel 2001) and the hazard of exposure to mycotoxin mixtures is gaining interest. The individual toxicity of mycotoxins is reported in different cell types (Ivanova *et al.* 2006; Alshannaq and Yu 2017; Olleik *et al.* 2019; Novak *et al.* 2019), whereas at the present very little is known about the health risk of mycotoxins mixture.

As we confirmed the co-contamination of finished pig feed by emerging mycotoxins and DON, we investigated the combined toxicity of DON and the 10 most prevalent and co-occurring with DON emerging mycotoxins. We focused on the effects on the intestine as a first barrier against food contaminants. When feed contaminated with mycotoxins is ingested, the gastro-intestinal tract is the first possible site of interaction (Fraeyman *et al.* 2018) and intestinal epithelial cells can be exposed to high concentrations of toxins. We tested the individual and combined effects of emerging based on their realistic concentrations on IPEC-1 cells. Indeed, the potential risk of chronic exposure to multiple mycotoxins bringing the same mechanism of action and/or targeting the same organs may lead to synergistic or at least additive effects (Speijers and Speijers 2004). Their individual effects lead to identify 3 classes of toxicity: Class #1 (non-toxic) included BRV-F, Cyclo and TRPT, Class #2 (moderately toxic) included AFN, EMO and ENN-B1 and Class #3 (highly toxic) included ENN-A1, B, BEA, and API. Despite of the high percentage of co-occurrence with DON, there was very low correlation between the concentration of emerging mycotoxins and the one of DON. Thus, we tested the toxicity of the mixtures based on 3 scenarios representing plausible to situations of exposure for animals. First scenario was low concentration of emerging mycotoxins + high concentration of DON (P25/P75), second scenario was median concentration of emerging mycotoxins + median of DON, whereas the 3rd scenario was the reverse case of scenario #1 (P75/P25). In most of the cases, the combined effect was similar to the individual effect of DON, or lesser than DON.

In addition to intestine, liver is the main site of detoxification for xenobiotics, including mycotoxins and represents a target organ for food contaminants. So, we developed an *ex vivo* model to study the effects of mycotoxins on liver, the Precision Cut Liver Slices (PCLs). The first steps of developing this tool aimed to verify the maintenance of the quality of the tissue during time. We confirmed through analyzing liver damage makers (AST, ALP, AST, LDH) and as well via RIN that quality of tissue maintained stable over time of incubation. This tool was then used to assess the toxicity of DON (3 and 10 µM) at different incubation times (0 to 20 h), by studying gene expression, ATP and total proteins content. Exposure to DON for 4 h or more led to induce in the expression of certain genes. The total proteins contents were

significantly decreased upon exposure to DON 10 µM in longer incubation time in accordance with its well-characterized inhibition of protein synthesis. At the cellular and subcellular level, DON binds to the ribosome, inhibits protein and nucleic acid synthesis and triggers ribotoxic stress, leading to the activation of mitogen-activated protein kinases (MAPKs) and their downstream signaling pathways (Pierron *et al.* 2016b). PCLS are considered as closely reflect the *in vivo* situation and are potentially useful for studying the toxicity of mycotoxins on liver-resident cells. Application of the PCLS technique in the field of mycotoxicosis analysis however, has not yet been well developed. We confirm that PCLS is a promising model to assess hepatotoxicity of mycotoxins.

7. Perspectives

Toxic effect of emerging mycotoxins on PCLS

This study showed that most of the tested emerging mycotoxins were toxic, some of them being more toxic than DON. We analyzed the hepatic toxic effects of DON on several endpoints such as gene expression, ATP degradation and total proteins contents using PCLS. However, the toxic effect of emerging mycotoxins on above-mentioned markers through PCLS is still to be performed to obtain a picture of their potential toxicity on the liver.

Some of emerging mycotoxins such as ENNs and BEA have ionophoric properties and increase ions permeability (Na^+ K^+ and Ca^{2+}) in biological membrane. They increase oxidative stress by altering membrane ion permeability and uncoupling oxidative phosphorylation (EFSA 2014). BEA can induce genes from different pathways. A toxicogenomic study of the effects of BEA on gene expression changes in Jurkat cells and human lymphoblastic T cells showed changes in the expression of genes involved in to apoptosis and programmed cell death (Escrivá *et al.* 2018). Furthermore, ENN altered the gene expression profile of primary rat hepatocytes (Jonsson *et al.* 2016). This demonstrates the need of analyzing the overall effect of emerging mycotoxins on gene expression using whole transcriptome approach.

Combined toxicity of DON and emerging mycotoxins on PCLS

Recently, due to the natural co-occurrence of mycotoxins, the hazard of exposure to mycotoxin mixtures is increasing day by day. The potential risk of chronic exposure to multiple mycotoxins, when the mechanism of action is similar, or if they target the same organs may lead to a synergistic or at least an additive effect when combined together (Speijers and Speijers 2004). However, in our study performed on intestinal cells, we reported that emerging mycotoxins did not exacerbated the toxicity of DON in mixture. In order to improve the knowledge on the combined toxicity of emerging mycotoxins and DON using the *ex vivo* model of PCLS is the continuation of the present work.

Slices from other organs

The Krumdieck tissue slicer, which underwent regular improvements, is currently one of the most widely used instruments and can produce hundreds of slices in a semi-automatic procedure within 1 hour. Precision-cut tissue slices (PCTS) are widely used by many researchers as an *ex vivo* model of different animals including rat, mouse, hamster, guinea pig, cow, pig, deer, dog,

monkey, trout and humans. Slices can be harvested from different tissues (liver, intestine, lung, kidney, brain, spleen, heart, thyroid gland, prostate, lymph node and several types of tumours) (Groothuis *et al.* 2014). If the effects of mycotoxins have already been investigated *ex vivo* using intestinal explants (Pinton *et al.* 2009; Alassane-Kpembi *et al.* 2017a) their toxicity on explants from other organs is poorly investigated. Thus, PCTS of other organs, such as kidney, spleen or reproductive tissue could provide original and useful results.

Investigation of toxic effect of emerging mycotoxins in vivo

It is important, ultimately to confirm the results obtained with *in vitro* or *ex vivo* approaches by performing *in vivo* trials. Contaminants that show certain effects *in vitro* may therefore have different effects *in vivo*. In contrast to *in vitro* studies, *in vivo* studies are needed to evaluate how the body as a whole, will respond to a particular substance (Goodwin 2007). The toxic effects of DON have been already addressed via several endpoints *in vivo*, whereas the toxicity of emerging mycotoxins *in vivo* are fragmentary. We reported the occurrence and co-contamination of emerging and regulated mycotoxins in pig feed. Perform *in vivo* experiments with pigs exposed to feed contaminated at realistic level and, of course, upon regulations and ethics in animal studies, is of high interest for obtaining useful data for risk assessment.

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9. Publications/Communications

9.1. Publications

toxins

MDPI

Article

Co-occurrence of DON and Emerging Mycotoxins in Worldwide Finished Pig Feed and Their Combined Toxicity in Intestinal Cells

Abdullah Khan Khoshal [1], Barbara Novak [2], Pascal G. P. Martin [1], Timothy Jenkins [2], Manon Neves [1], Gerd Schatzmayr [2], Isabelle P. Oswald [1,*] and Philippe Pinton [1,*]

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9.2. Oral Presentations

a. Combined Toxicity of Emerging and Regulated Mycotoxins on intestinal epithelial cells (IPEC-J2) and intestinal explants

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Graduate School of Science and Technology, AU – Folum , Aarhus University, Denmark (August 17, 2017 Summer School Programm)

b. Toxicity of Emerging and Regulated Mycotoxins on intestinal epithelial cells (IPEC-J2) and intestinal explants

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BIOMIN Holding GmbH, Tulln , Austria (November 17th 2017, Scientific Visit)

c. Co-occurrence of DON and Emerging Mycotoxins in Worldwide Finished Pig Feed and Their Combined Toxicity in Intestinal Cells

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