THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Institut National Polytechnique de Toulouse (Toulouse INP)

Discipline ou spécialité :

Pathologie, Toxicologie, Génétique et Nutrition

Présentée et soutenue par :

Mme SU LUO

le lundi 23 septembre 2019

Titre :

Individual and combined toxicity of a mycotoxin, the deoxynivalenol and a trace metal, the cadmium on the intestine

---

Ecole doctorale :

Sciences Ecologiques, Vétérinaires, Agronomiques et Bioingénieries (SEVAB)

Unité de recherche :

Toxicologie Alimentaire (ToxAlim)

Directeur(s) de Thèse :

MME ISABELLE OSWALD

Rapporteurs :

M. GUNNAR SUNDSTOL ERIKSEN, NORWEGIAN VETERINARY INSTITUTE
Mme NOLWENN HYMERY, UNIVERSITE DE BRETAGNE OCCIDENTALE

Membre(s) du jury :

M. FRANCOIS BLACHIER, INRA PARIS, Président
Mme ISABELLE OSWALD, INRA TOULOUSE, Membre
M. PHILIPPE PINTON, INRA TOULOUSE, Membre

Résumé Français

Le déoxynivalénol (DON) est une mycotoxine de type trichothécène de type B, principalement produite par le genre Fusarium. C'est l'une des mycotoxines les plus répandues, elle est largement trouvée dans les céréales et les produits dérivés des céréales. Le cadmium est un composant de la croûte terrestre et un polluant environnemental courant. C'est un métal trace non essentiel et toxique pour la santé des humains et des animaux. Bien que la toxicité individuelle du DON et du Cd ait été bien étudiée, leur effet combiné est peu étudié. L'intestin étant le premier organe ciblé par les contaminants alimentaires, le but de cette étude est d'explorer l'effet combiné du DON et du Cd sur la fonction de barrière intestinale à l'aide de modèles in vitro, in vivo et ex vivo.

In vitro, des cellules épithéliales intestinales humaines Caco-2 ont été traitées avec une série de concentrations de DON et de Cd (0-30 µM) seules ou en combinaison. La fonction de barrière des cellules Caco-2 a été évaluée par la mesure de la résistance électrique transépithéliale (TEER), de la perméabilité paracellulaire et des protéines jonctionnelles. Le mélange DON, Cd et DON+Cd a diminué le TEER et augmenté la perméabilité paracellulaire de manière dépendante de la concentration. L'abondance des protéines jonctionnelles E-cadhérine et occludine a été considérablement réduite dans les cellules exposées au DON, au Cd et au DON+Cd, tandis que l'expression de ZO-1 et de claudine-3 et -4 est restée inchangée. Le mélange DON Cd a eu des effets légèrement supérieurs ou similaires à ceux du contaminant le plus toxique.

In vivo, les rats ont été exposés à des aliments contaminés par du DON (8,2 mg / kg), et à de l'eau de boisson contaminée par du Cd (5 mg / L) ou au mélange DON+Cd pendant 4 semaines. Les résultats n'ont montré aucun effet sur la prise de poids corporel au cours de l'expérience. Des dommages morphologiques légers caractérisés par un œdème au niveau de la lamina propria et un aplatissement et une fusion des villosités ont été découverts chez le rat exposé à chaque contaminant. Le score lésionnel du jéjunum était plus élevé chez tous les animaux traités que chez les animaux témoins. Une diminution significative de la profondeur de la crypte jéjunale a été observée chez les rats exposés au DON, au Cd et au DON+Cd, alors que la hauteur des villosités n'était pas affectée. Une immunomarquage plus faible de l'E-cadhérine dans le jéjunum de rats exposés à des contaminants seuls ou en association a également été observée, alors que l'occludine n'a diminué que chez les rats exposés au DON et au DON+Cd. Comme

indiqué in vitro, l'exposition in vivo au DON et au Cd a induit des effets similaires à ceux du contaminant le plus toxique.

Des explants jéjunaux de porcs ex vivo ont été exposés au DON (0-24 µM), au Cd (0-96 µM) et à la combinaison de DON+Cd. Le DON seul et le mélange DON Cd ont stimulé la réponse immunitaire chez le jéjunum en régulant positivement l'expression d'ARNm de IL-1β, IL-1α, IL-8 et TNF-α de manière dose-dépendante, tandis que le Cd seul n'a pas affecté ces gènes. L'expression génique des métallothionéines (MT), y compris MT1A et MT2A, était régulée positivement de manière dose-dépendante par le Cd seul et le mélange, mais n'était pas affectée par le DON seul. La régulation à la hausse des gènes de cytokines et de MT induite par le DON+Cd était similaire à celle obtenue par le DON ou le Cd seul.

En conclusion, le DON et le Cd modifient tous deux la fonction de barrière intestinale et l'effet combiné est similaire avec leur effet individuel. L'effet du mélange n'a démontré aucune synergie, ce qui suggère que la réglementation sur chaque contaminant protège suffisamment les consommateurs exposés aux mélanges de DON et de Cd.

Mots clés: déoxynivalénol, cadmium, effet combiné, fonction de barrière intestinale, protéines jonctionnelles, expression génique

Summary

Deoxynivalenol (DON) is a type B trichothecene mycotoxin mainly produced by *Fusarium* genus. It is one of the most prevalent mycotoxins widely found in cereals and cereal-derived products. Cadmium is a component of earth's crust and also a common environmental pollutant. It is a non-essential trace metal and toxic for humans and animals health. Although the individual toxicity of DON and Cd has been well investigated, their combined effect is poorly studied. As intestine is the first organ targeted by food contaminants, the aim of this study is to explore the combined effect of DON and Cd on the intestinal barrier function using *in vitro*, *in vivo* and *ex vivo* models.

In vitro, the human intestinal epithelail cells Caco-2 were treated with a series of concentrations of DON and Cd (0-30 µM) alone or in combination. The barrier function of Caco-2 cells was assessed through the measurement of transepithelial electrical resistance (TEER), paracellular permeability and junctional proteins. DON, Cd and DON+Cd mixture decreased the TEER and increased the paracellular permeability in a concentration-dependent manner. The abundance of junctional proteins E-cadherin and occludin was considerably reduced in cells exposed to DON, Cd and DON+Cd, while the expression of ZO-1, and claudin-3 and -4 remained unchanged. The mixture DON+Cd induced slightly higher or similar effects than the most toxic contaminant.

In vivo, rats were exposed to DON-contaminated feed (8.2 mg/kg feed), and Cd-contaminated drinking water (5 mg/L) or to the mixture DON+Cd for 4 weeks. The results showed no effect on body weight gain during the experiment. Mild morphological damage characterized by edema in lamina propria and villi flattening and fusion was found in rat exposed to each contaminant. The lesional score of jejunum was higher in all the treated animals than that in control animals. A significant decrease of jejunal crypt depth was observed in rats exposed to DON, Cd and DON+Cd, whereas villi height remained unaffected. A lower immunostaining of E-cadherin in the jejunum of rats exposed to contaminants alone or in combination was also observed, whereas occludin was only decreased in rats exposed to DON and DON+Cd. As shown *in vitro*, *in vivo* exposure to both DON and Cd induced similar effects than the most toxic contaminant.

Ex vivo, jejunal explants of pigs were exposed to DON (0-24 µM), Cd (0-96 µM) and in combination DON+Cd. DON alone and mixture DON+Cd stimulated immune response in jejunum by upregulating mRNA expression of IL-1β, IL-1α, IL- 8 and TNF-α in a dose-

dependent manner, while Cd alone did not affect these genes. Gene expression of metallothioneins (MTs) including MT1A and MT2A was dose-dependently upregulated by Cd alone and mixture, but not affected by DON alone. The upregulation of cytokine and MTs genes induced by DON+Cd was similar than by DON or Cd alone.

In conclusion, both DON and Cd alter intestinal barrier function and the combined effect is similar with their individual effect. The effect of the mixture did not demonstrate any synergy, suggesting that regulation on individual contaminant is protective enough for consumers exposed to DON and Cd mixtures.

Keywords: Deoxynivalenol, cadmium, combined effect, intestinal barrier function, junctional proteins, gene expression

Acknowledgements

Firstly, I would like to express my deep gratitude to my thesis supervisors Dr. Isabelle Oswald and Dr. Philippe Pinton, for their continuous support of my doctoral study, for their patient guidance, enthusiastic encouragement and valuable critiques of this research work and thesis writing. Their continued support promoted me keep moving forward. Without their guidance and help, this work could not be carried out.

Besides my supervisors, my sincere thanks also goes to the other members of my thesis committee, Dr. Laurent Ferrier and Dr. El Hassan AJANDOUZ, for their insightful comments and questions, helpful suggestions and kind encouragement on my study.

I would also like to thank Professor Ana-Paula Brcarense for her help in tissue section preparation and immunohistochemical analysis. My grateful thanks are also extended to Dr. Chloe Terciolo for her precious advice and assistance in immunofluorescence analysis, to Dr. Delphine Payros for her kind help in sampling in vivo experiments.

My particular thanks also goes to professor Hymery, professor Eriksen who are also my rapporteurs and professor Blachier, my jury members, for their constructive suggestions and comments on my thesis manuscript, which help a lot to improve this manuscript.

I thank my friends and colleagues, Manon Neves, Joelle Laffitte, Sylvie Puel for their collaboration and technical support for my research. Also I thank other members in our team, Laura Soler-Vasco, Le Thanh Huong, Sorphie Lorber, Christelle El Hajj Assaf, Amaranta Carvajal-Campos, Olivier Puel, Ophélie Rocher, Chrystian Zetina who helped me a lot in my lab life.

Finally, I would like to thank my husband, my parents and my brothers for their understanding and unconditional support for my study!

Contents

Introduction **9**

1. Intestine 9
2. DON 11
   - 2.1. Occurrence, exposure and regulation of DON in food and feed 12
   - 2.2. Toxicokinetics of DON 13
   - 2.3. Mode of action of DON 14
   - 2.4. Toxicity of DON 15
     - 2.4.1. Immunotoxicity 16
     - 2.4.2. Genotoxicity 16
     - 2.4.3. Reproductive and developmental toxicity 17
     - 2.4.4. Neurotoxicity 17
     - 2.4.5. Intestinal toxicity 17
       - 2.4.5.1. DON-induced morphological lesions in intestine 17
       - 2.4.5.2. DON-induced modification of intestinal immunity 18
       - 2.4.5.3. DON-induced oxidative stress on intestine 19
       - 2.4.5.4. Effects of DON on the intestinal barrier function 20
       - 2.4.5.5. Effect of DON on the intestinal microbiota 25
3. Cadmium (Cd) 26
   - 3.1. Occurrence, exposure and regulation of Cd in food and feed 26
   - 3.2. Toxicokinetics of Cd 28
   - 3.3. Mode of action of Cd 29
   - 3.4. Toxicity of Cd 31
     - 3.4.1. Immunotoxicity 31
     - 3.4.2. Genotoxicity 32
     - 3.4.3. Reproductive and developmental toxicity 32
     - 3.4.4. Neurotoxicity 32
     - 3.4.5. Intestinal toxicity 33
       - 3.4.5.1. Cd-induced morphological lesions in intestine 33
       - 3.4.5.2. Cd-induced modification of intestinal immune response 33
       - 3.4.5.3. Cd-induced oxidative stress on intestine 34
       - 3.4.5.4. Effects of Cd on the intestinal barrier function 35
       - 3.4.5.5. Effect of Cd on the intestinal microbiota 36

Experimental work **38**

1. *In vitro* and *in vivo* effects of a mycotoxin, deoxynivalenol, and a trace metal, cadmium, alone or in mixture on the intestinal barrier 41

2. Specific intestinal toxicity of deoxynivalenol and cadmium: analysis on pig jejunal explants 68

General Discussion and conclusion 81

Perspectives 93

References 98

Annex 135

Abbreviations list

DON: Deoxynivalenol

FUM: fumonisins

Afla: Aflatoxins

OTA: ochratoxin A

PAT: Patulin

TCT: trichotehcenes

ZEN: Zearalenone

MAPKs: Mitogen-activated protein kinases

ERK: Extracellular signal-regulated protein kinase

JNK: Jun N-terminal kinase

PKR: protein kinase R

Hck: Hematopoietic cell kinase

Ig: Immunoglobulin

EFSA: European Food Safety Authority

ASTDR: Agency for Toxic Substances and Disease Registry

TDI: Tolerable daily intake

TWI: Tolerable weekly intake

DNA: Deoxyribonucleic acid

RNA: Ribonucleic acid

qPCR: Quantitative polymerase chain reaction

IEC: Intestinal epithelial cells

Caco-2: Human colon carcinoma cells

MDCK: Madin-Darby Canine Kidney cells

ROS: Reactive oxygen species

RNS: Reactive nitric species

iNOS: Inducible nitric oxide synthase

NO: Nitric oxide

GSH: Glutathione

SOD: Superoxide dismutase

CAT: Catalase

GPx: Glutathione peroxidase

XOR: Xanthine oxidoreductase

HIF1α: Hypoxia-inducible factor 1

HMOX: Heme-oxigenase

IFN-γ: Interferon gamma

TNF-α: Tumor necrosis factor alpha

IL: Interleukin

NF-κB: Nuclear factor-kappa B

IPEC-1/IPEC-J2: intestinal porcine epithelial cells

TEER: transepithelial electrical resistance

TJs: tight junction proteins

AJs: adherens junction proteins

ZO: Zonula occludens

kD: kilo Dalton

µM: Micromole

µg/kg b.w.: Microgram per kilogram of body weight

Ω: Ohms

FITC: Fluorescein isothiocyanate

MLC: Myosin light chain

Cd: Cadmium

MTs: Metallothioneins

Introduction

1. Intestine

The gastrointestinal tract (GT) is the interface for the interaction of external and internal environment. Its primary functions include food digestion, nutrient absorption, hormone secretion and immune response. These functions are supported by the unique structure of layered GT, which is divided into four layers from the inside to the outside, including the mucosa, the submucosa, the muscularis propria and the serosa (Rao and Wang 2010) (Fig.1). Mucosa is the deepest layer being consisted of three layers. The first layer is composed of a single layer of epithelial cells covering the second layer, the lamina propria, which consists of connective tissue and lymph nodes, and the third layer is muscularis mucosae. Below the mucosa is the submucosa, which contains inflammatory cells, lymphatics, autonomic nerve fibers, and ganglion cells, as well as arteries and small venous channels. Beneath the submucosa is the muscularis propria, which rests on the outermost layer called serosa. These different layers are not isolate from each other but combined by connective tissue, nerves and vessels and function together (Rao and Wang 2010).

Among these layers, the first layer of mucosa named as epithelial cells or epithelium is structurally and functionally the pivotal part. This single-cell layer lining the gut lumen, efficiently provides a selective filter, preventing the permeation of harmful luminal molecules and affording the appropriate absorption of nutrients and water (Groschwitz and Hogan 2009; Suzuki 2013). The intestinal epithelium is renewed rapidly and frequently. The mature and absorptive enterocytes on the top of villi die and exfoliate in 3-5 days, which is beneficial to remove the damaged cells. The stem cells on the base of intestinal crypt continually divide and provide proliferating cells, which differentiate into mature enterocytes during migrating upward to replace the dead cells in villi (Wells et al. 2017). The balance between cell apoptosis and proliferation plays an important role in maintenance of intestinal homeostasis and barrier function.

The barrier function of intestinal epithelium is achieved by tight and adherens junction proteins (Fig.2) , which connect to the cellular cytoskeleton linking the adjacent cells to tightly seal the intercellular space (Groschwitz and Hogan 2009; Suzuki 2013). The tight junction proteins (TJs) located in apical side consist of transmembrane proteins including claudins, junctional adhesion molecules (JAMs) and occludin and intracellular scaffold proteins such as zonula occludens (ZO-1, -2 and -3). ZOs and occludin play an important role in regulation and

maintenance of TJ assembly (Suzuki 2013), while claudins act as both tightening (Claudin-3, -4, -7 ect.) and pore-forming (Claudin-2 and -15) protein in TJ, which positively and negatively impact the paracellular permeability (Schumann et al. 2012; Lu et al. 2013). The adherens junction proteins (AJs) beneath the TJs like E-cadherin initiate and maintain the cell-cell adherion and support TJ formation (Garcia et al. 2018). In addition, junctional proteins interact with the cytoskeleton network not only to regulate paracellular solute and water flux but also to integrate such diverse processes as gene transcription, tumor suppression, cell proliferation, and cell polarity (Schneeberger and Lynch 2004). Disruption of these proteins, followed by increased paracellular permeability, induces activation of intestinal immune system and tissue injury leading to disturbance of homeostasis and systemic diseases (Suzuki 2013). Therefore, understanding of the alteration of junctional proteins is important on revealing the dysfunction of intestinal barrier caused by food contaminants.

The gut is the first barrier line to prevent organisms from the external harmful substances, which also makes it the first target of food contaminants, such as pathogens, toxins and other chemicals (Pinton and Oswald 2014; Payros et al. 2017). These contaminants can change intestinal morphology, modulate intestinal immunity, and disturb intestinal microbial homeostasis, consequently leading to dysfunction of intestine. Among these toxic food contaminants, mycotoxins and heavy metals are very common and the most studied ones. In the present study, we are going to investigate the toxicity of a mycotoxin, Deoxynivalenol, and a trace metal, cadmium or their combination on intestinal function.

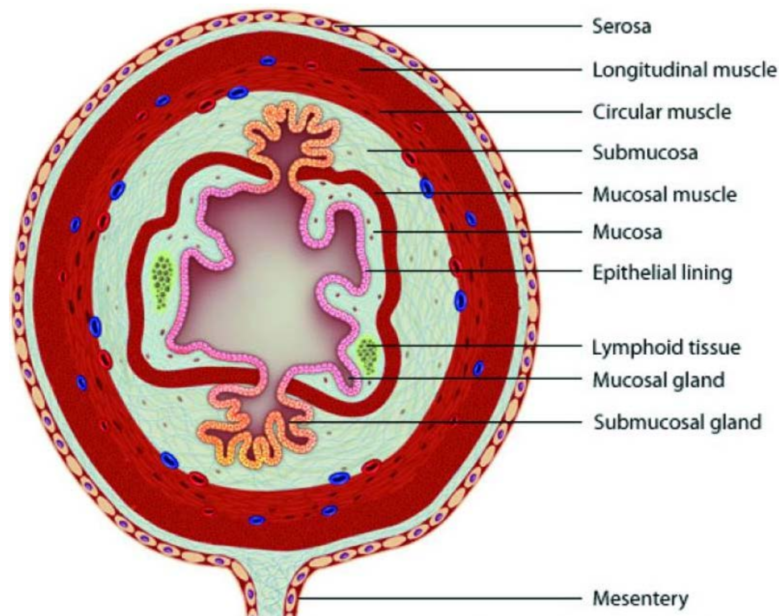


Figure 1 The structure of gastrointestinal tract (Rao and Wang 2010)

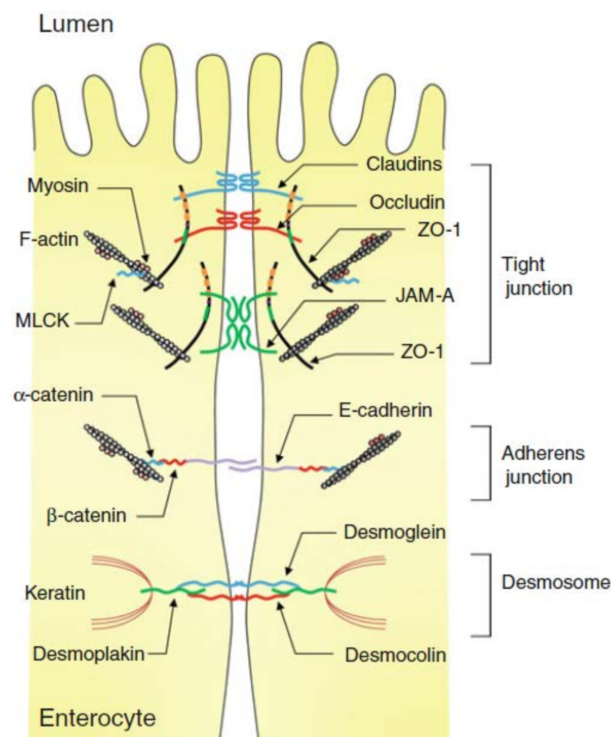


Figure 2 Molecule structure of the intercellular junction of intestinal epithelial cells (Suzuki 2013)

2. DON

Fungi like *Aspergillus*, *Fusarium* and *Penicillium* are common pathogens infecting crops in the fields or during storage. They can produce various toxic secondary metabolites known as

mycotoxins, including zearalenone (ZEN), fumonisins (FUM), aflatoxins (Afla) , patulin (PAT), ochratoxin A (OTA) and trichothecenes (TCT) (Pinton and Oswald 2014; Payros et al. 2016). Among them, TCT mainly produced by *Fusarium* are a big family of over 150 toxins classified into A, B, C and D groups according to their molecular structure (McCormick et al. 2011; Proctor et al. 2018). Deoxynivalenol (DON) belongs to type B TCT and has a worldwide occurrence in cereals and cereals-derived products (Maresca 2013; Pierron et al. 2016a). Due to its property of emesis induction following acute exposure, DON is also called vomitoxin (Maresca 2013). DON possesses heat-resistance making it persistent throughout the food chain and it raises a worldwide concern (Pinton and Oswald 2014).

2.1. Occurrence, exposure and regulation of DON in food and feed

Data collected from 21 European countries have shown that 44.6%, 43.5% and 75.2% of unprocessed grains of undefined end-use, food and feed were contaminated by DON, with the highest concentration in maize, wheat and oat grains and derived products (EFSA 2013). Among these grains, wheat is more sensitive to DON accumulation. In a survey on the worldwide occurrence of mycotoxins, up to 55% wheat samples from northern and central Europe were positive for DON (Rodrigues and Naehrer 2012). The latest Biomin global mycotoxin survey showed that in 2895 tested finished feed and raw cereal samples, 64% of them was positive with DON in Europe. In each part of Europe the contamination of DON appears similar. DON is also the most prevalent mycotoxins in North and South America, as well as in Asia and Africa (BIOMIN 2019). The main contributors of DON exposure for human are grain-based products as bread, rolls and pasta. The average chronic exposure to DON ranges from 0.2 to 2.0 µg / kg body weight (b.w.) / day (EFSA 2017). The tolerable daily intake (TDI) of DON was set at 1µg / kg b.w./day (EFSA 2017). However, the exposure level for young children is close to or even higher than the TDI. For example, in Germany the high consumers in the group of 4-6 years old children are chronically exposed to DON at a level of 2.7 fold higher than the TDI (EFSA 2013). Chronic and acute exposure to DON for animals range from 3.9 to 43.3µg / kg b.w./day and from 11.6 to 317.9µg / kg b.w. (EFSA 2013).

To protect human and animal health, many countries established the maximum concentration of DON in food and feed. European Commission regulated the level of DON in food for human direct consumption ranges from 0.2 to 0.75 mg / kg depending on the food category and age groups of exposed population, in complementary and complete feedstuffs from 0.9 to 5 mg / kg depending on the groups of exposed animal population and species (Table 1) (European Union 2006a, b). The US Food and Drug Administration has determined an

advisory level of 1 ppm of DON on finished wheat products intended for human consumption, 10 ppm on grains and grain by-products used for cattle and chicken, 5 ppm in grain-based feed for pig and other animals (Food and Drug Administration 2010).

Table 1. Recommendation for DON in foodstuffs/feedstuffs in Europe (European Union 2006a, b)

Foodstuffs	Maximum levels (mg/kg)
Unprocessed cereals other than durum wheat, oats and maize	1.25
Unprocessed durum wheat and oats	1.75
Unprocessed maize	1.75
Cereals intended for direct human consumption, cereal flour (including maize flour, maize meal and maize grits) bran as end product marketed for direct human consumption and germ, with the exception of foodstuffs	0.75
Pasta dry	0.75
Bread (including small bakery wares), pastries, biscuits, cereals snacks and breakfast cereals	0.5
Processed cereal-based foods and baby foods for infants and young children	0.2
Feedstuffs	Maximum content in mg/kg relative to a feed with a moisture content of 12 %
Feed materials	
- Cereals and cereal products with the exception of maize by-products	8
- Maize by-products	12
Complementary and complete feedstuffs with the exception of:	5
- complementary and complete feedstuffs for pigs	0.9
- complementary and complete feedstuffs for calves (< 4 months), lambs and kids	2

2.2. Toxicokinetics of DON

How DON is transported into the intestinal epithelial cells is still unclear. It is likely to be transported by passive diffusion as the cell entry of DON was not saturated when Caco-2 cells were exposed to 5 to 30 µM of DON (Videmann et al. 2007; Maresca 2013). Pig is the most sensitive farm animal to the toxicity of DON and its absorption in pig is rapid. *In vitro* intestinal absorption of DON analyzed by a dynamic *in vitro* GI-model is 51%, and most absorption take place in the jejunal compartment (Avantaggiato et al. 2004). In another *in vivo*

study, chronic exposure to DON (5.7 mg/kg diet, 5-8 weeks) showed a higher oral bioavailability with 89% than acute exposure (5.7 mg/kg diet, single bolus) with 54% in pig. DON was detected in serum samples as early as 15 min after chronic and actute exposure (Goyarts and Dänicke 2006). After feeding pigs with 4.2 mg DON/kg feed, the peak of DON concentration in serum was observed at 4.1 h. (Dänicke et al. 2004; EFSA 2017). DON can be transformed into less toxic metabolite DOM-1 by the microflora in colon of human and other monogastric animals, then excreted with feces. However, human microflora has a lower transformation efficiency than rodents, such as mouse and rat. Large amounts of ingested DON is conjugated to glucuronides or sulfonates in the liver and is excreted with urine or feces (Fig.3) (Payros et al. 2016; EFSA 2017). DON is rapidly excreted within 24 h and the metabolite DON-15-glucuronide is the main urinary biomarker in human (Vidal et al. 2018).

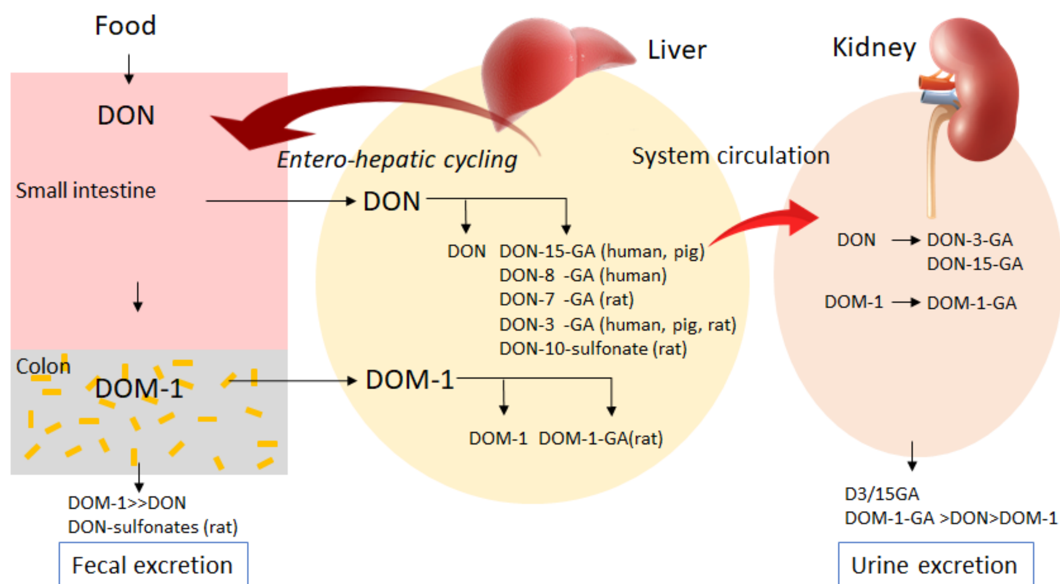


Figure 3 Absorption, metabolism and excretion of DON in human and monogastric animals. DOM-1, de-epoxide metabolite of DON; DON-GA, glucuronide derivetives of DON; DOM-1-GA, glucuronide derivetives of DOM-1 (Payros et al. 2016).

2.3. Mode of action of DON

The most frequent reported modes of action on DON are ribotoxic stress and oxidative stress. It is known that via its epoxy group DON binds to the 60s ribosomal subunit and target on the peptidyl transferase. This interaction inhibits the initiation and elongation of peptide chains of protein synthesis resulting in an inhibition of RNA, DNA and protein synthesis, which called 'ribotoxic stress' (Waśkiewicz et al. 2014; EFSA 2017). Upon DON binding to ribosomes, the ribosome-associated double-stranded RNA-dependent protein kinase (PKR) and

the hematopoietic cell kinase (Hck) are activated within 5 minutes. Subsequently the downstream sensor of PKR eukaryotic initiation factor 2α (eIF2α) is phosphorylated, thereby inhibiting translation (Zhou et al. 2003b, 2014). On the other hand, DON is able to trigger ER stress by decreasing the glucose regulated protein 78 (GRP78) (Shi et al. 2009), followed by the activation of the PKR-like ER kinase (PERK), one of the sensors under ER stress, which induces phosphorylation of eIF2α leading to a global translation inhibition (Wek et al. 2006; Tsuru et al. 2016).

Following the ribotoxic stress, various downstream events occur. The DON-induced PKR and Hck activation triggers activation of the mitogen-activated protein kinases (MAPKs), including p38; extracellular signal regulated protein kinase 1 and 2 (ERK 1/2) and c-Jun N-terminal kinase 1 and 2 (JNK 1/2) (Pestka 2008). MAPKs activation induces cell apoptosis at high dose and mRNA expression of pro-inflammatory cytokine genes at low dose (Zhou et al. 2003a, 2005). In addition, recent reports demonstrated that DON induces rRNA cleavage by PKR/Hck-driven p-38 activation in the RAW264.7 cells, which could impair ribosome function and leading to cell apoptosis (Li and Pestka 2008; He et al. 2012).

Since the past several years, oxidative stress has also been considered as a potential toxicity mechanism of DON toxicity. Oxidative stress is triggered by over production of reactive oxygen species (ROS) including hydroxyl radical (HO^-), superoxide anion (O^{-2}) and perhydroxyl radical (HOO^-) or reactive nitrogen species (RNS), such as nitric oxide (NO) (Mishra et al. 2014). Several in vitro studies illustrated that DON exposure markedly induced DNA damage by inducing ROS production, inhibited activity of the antioxidant system including glutathione (GSH), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx), and increased lipid peroxidation leading to cell apoptosis (Zhang et al. 2009b; Dinu et al. 2011; Lautert et al. 2014). *In vivo* experiments using broiler chickens showed that DON (7.54±2.20 mg/kg feed) dietary exposure for 10 days induced mRNA expression of Xanthine oxidoreductase (XOR, associated with cellular defense enzyme systems) up-regulation, whereas that of hypoxia-inducible factor 1 (HIF1α) and Heme-oxigenase (HMOX) downregulation in the liver, both of which are sensitive markers of oxidative injury. In the jejunum the mRNA of HMOX and XOR were up-regulated, while in the ileum, only XOR was up-regulated (Osselaere et al. 2013). These data suggest that oxidative stress is an important mechanism of action of DON toxicity.

2.4. Toxicity of DON

Toxicity of DON depends on its capacity to break through such biological barriers as intestinal or blood brain barriers and to impact the functions and viability of the cells forming such organic systems (Maresca 2013). DON is able to impair the intestinal, immune and nervous systems thus exerts its immunotoxicity, genotoxicity, reproductive and developmental toxicity and neurotoxicity (EFSA 2017). The symptoms of DON poisoning, in the case of acute exposure, include diarrhea, emesis, abdominal stress, headache and dizziness; while in the case of chronic dietary exposure, include anorexia, decrease of body weight gain and low nutrition efficiency (Pestka 2008).

2.4.1. Immunotoxicity

DON has either an immunostimulatory or immunosuppressive effect depending on the duration and doses of exposure (Pestka et al. 2004; Liao et al. 2018). For instance, an increased IgA concentration in the serum and lower mRNA expression of IFN-γ and TGF-β in mesenteric lymph node of pigs exposed to DON (2.2-2.5 mg / kg feed) was observed. Meanwhile, DON exposure altered vaccinal immune response in animals (Pinton et al. 2008). DON exposure (0.5-2mg/kg b.w.) for 14 days increased the population of CD8+ cells in the spleen and CD4+ T cells in mesenteric lymph node (MLN) of BALB/c mice. The expression of TLR4 in spleen and TLR2-4 in MLNs was decreased. In addition, the level of IgA and IgE in serum was reduced and increased respectively, while the mucosal IgA was significantly increased in the duodenum. The level of inflammatory cytokines as IFN-γ, IL-2, IL-4 and IL-6 was increased in the serum in DON-exposed animals compared to control animals (Islam et al. 2013). A higher concentration of DON at 11.4 mg / kg feed exposure for 30 days, induced significant lymphocyte apoptosis and immunosuppression in the lymphoid organs of rats (Bracarense et al. 2016). These results indicate that the immune system in different species is extremely sensitive to the toxicity of DON.

2.4.2. Genotoxicity

DON exerts its genotoxicity by inducing oxidative damage to DNA. Human peripheral blood lymphocytes were exposed to DON (6.25-500 ng/mL, which equal to 0.02-1.7 μM) for 6, 12 and 24h, lipid peroxidation, DNA damage and protein expression of DNA repair genes were observed (Yang et al. 2014). DON (12.5-50 μM) exposure for 8h was also reported to induce genotoxicity in intestinal cells IEC-6 indicated by phosphorylated histone H2AX, a marker of DNA double-strand breaks (Payros et al. 2017). In human liver cell line HepG2, DON ranging from 3.75 to 30 μM induced DNA strand breaks after 1h exposure via ROS production (Zhang et al. 2009b). These *in vitro* studies demonstrated that DON possesses

genotoxicity properties. Nevertheless, no correlation was found between DON and tumor or cancer thus it was classified in group 3 by the International Agency for Research on Cancer (IARC 1993).

2.4.3. Reproductive and developmental toxicity

Recent studies in different animal models have highlighted the link between DON exposure and the deficiency of maternal reproduction and fetus development (Yu et al. 2017). An in vitro study performed on pig oocytes and zygotes demonstrated that 1.88 µM of DON markedly inhibited the meiotic progression and maturation of pig oocytes and delayed the development of pig zygotes (Alm et al. 2002). DON (4.2 mg/ kg) exposure of sows at 63 and 70 days of gestation, is able to pass placental barrier entering into placenta, which may change the population of leukocytes and lymphocytes of fetus (Goyarts et al. 2010). Oral exposure of female rats to DON (2.5 and 5 mg/ kg b.w/d) by daily gavage at 6-19d of gestation, significantly decreased the body weight gain, crown-rump length, and vertebral ossification of fetus (Collins et al. 2006). These *in vivo* data indicate that DON pass through the placental barrier, subsequently induces restriction of fetus development.

2.4.4. Neurotoxicity

There is a potential link between DON exposure and neuronal diseases. It was reported that DON (1-100 µM) exposure for 48h altered the functions and decreased the viability of microglia and enterocytes, which are responsible for brain homeostasis (Razafimanjato et al. 2011). In addition, DON is capable of reducing the viability of brain endothelial cells and destroying the blood-brain barrier. DON at 1 and 10 µM significantly decreased the TEER and increased the paracellualer permeability of primary porcine brain capillary endothelial cells, with a decreased viability at 10 µM after 48h exposure (Behrens et al. 2015). This process is contribute to entrance of DON into the central nervous system and alteration of brain homeostasis, thereby inducing neurotoxicity. Low dose of DON (0, 0.125, 0.25, 0.5, 1 and 2 µg/mL) is also able to inhibite proliferation and induce apoptosis of PC12 cells by mitochondrial apoptosis pathway after 24h of exposure (Wang *et al.*, 2016).

2.4.5. Intestinal toxicity

Due to its special function, intestine is the first target organ of many food contaminants after ingestion. Here, we are going to stress the general toxicity of DON on the intestine and its specific effect on intestinal barrier function.

2.4.5.1. DON-induced morphological lesions in intestine

The gut is the first barrier line to prevent organisms from the external harmful substances, such as antigens, toxins and other chemicals (Pinton and Oswald 2014; Payros et al. 2016). Thus, it is also the first target that can be exposed to a higher concentration of DON than other organs. On the other hand, DON is a protein synthesis inhibitor making it more toxic to such tissues with a rapid renewal as intestinal epithelium (Wells et al. 2017). The main morphological lesions in the intestine are multifocal atrophy, villi fusion and apical necrosis, which resulted in shorter and thicker villi (Bracarense et al. 2012; Pinton et al. 2012; Gerez et al. 2015). This suggests that DON induces an imbalance between proliferation and apoptosis of enterocytes. The impairment caused by DON on the intestinal morphology contributes to the decrease of nutrient absorption, consequently resulting in growth deficiency in animals (Alizadeh et al. 2015).

2.4.5.2. DON-induced modification of intestinal immunity

As described above, DON-induced inflammatory cytokine expression is mediated by MAPKs activation, which is associated with the phosphorylation of PKR and Hck. MAPKs plays a key role in signal transduction in the immune response. It has been proved that DON-induced p-38 and ERK activation modulate the gene expression involving in pro-inflammatory or inflammatory cytokines in neutrophils or macrophages (Chung et al. 2003; Islam et al. 2006; Gauthier et al. 2013). The intestine has its own immune network, which protects it from infections or other harmful injuries, thus inflammatory or immune response will occur in the intestine to counteract DON-induced damage.

Different intestinal cell lines and animal species have been used to investigate DON-induced inflammatory or immune response. In the human epithelial intestine 407 cells, DON (25, 500 and 1000ng/mL) exposure for 12h promoted IL-8 expression via ERK1/2 activation (Moon et al. 2007). The expression of IL-8 also observed in the DON-treated Caco-2 cells with absent changes in the expression levels of IL-1β and TNF-α (Kadota et al. 2013). However, in the DON-exposed porcine epithelial cells IPEC-1, the mRNA expression of IL-8, IL-1α, IL-1β and TNF-α significantly increased (Cano et al. 2013). Several ex vivo studies constructed by jejunum explants demonstrated that the DON-mediated up-regulation of inflammatory genes was associated with MAPKs regulation (Cano et al. 2013; Alassane-Kpembi et al. 2017a; García et al. 2018). In vivo studies carried out in piglets further supported the obtained conclusions of in vitro and ex vivo investigations. Swine exposed to DON exhibited a potent inflammatory response in the intestine. The regulated dose of 0.9 mg DON/kg feed, induced IL-10 and IL-1β mRNA increase in the duodenum and COX-2 increase in the cecum of growing

pigs (Alizadeh et al. 2015). Chronic ingestion of DON caused an up-regulation of IL-1β, IL-6, MIP-1β and IL-2 mRNA in jejunum of piglets. In addition, the mRNA of IL-1β, TNF-α and IL-6 was up-regulated in the ileum (Bracarense et al. 2012).

2.4.5.3. DON-induced oxidative stress on intestine

Oxidative stress is a non-specific response to cell injury caused by toxins or inflammation. The production of ROS can degrade the membrane phospholipids of cells or organelles, which is responsible for damage of intestinal integrity and increase of intestinal barrier permeability (Wu et al. 2014).

Several intestinal cell lines were used to test the capacity of DON to induce oxidative stress. Human HT-29 cells exposed to DON (250 and 500ng/mL) for 24h, exhibited a higher level of ROS and nitrite production compared to the non-treated cells. The higher production of ROS and nitrite was co-occurrence with NF-κB activation (Krishnaswamy et al. 2010). DON (1, 2.5 and 5μM) exposure of the non-tumorigenic rat intestinal epithelial cell line IEC-6, induced an evident increase of ROS generation, which could be exacerbated by nivalenol (Del Regno et al. 2015). The application of NF-κB inhibitor showed that NF-κB activation was involved in DON-mediated ROS release (Del Regno et al. 2015), which could explain the NF-κB activation in the previous study (Krishnaswamy et al. 2010). The two studies demonstrated that NF-κB plays an important role in DON-mediated oxidative stress. In the latter study, NF-κB activation mediated the increase of iNOS (inducible nitric oxide synthase) protein expression leading to the release of NO, which reacted with superoxide anion resulting in peroxynitrite formation. Peroxynitrite can nitrate tyrosine residues in proteins, an alternative to phosphorylation, thereby changing the structure and function of proteins, consequently altering the cytoskeletal organization and impairing cell signal transduction (Del Regno et al. 2015). All these reactions contribute to intestinal barrier dysfunction.

On the other hand, DON induced Nif2 activation, an oxidative stress sensor, increasing the expression of NQO1 and HO-1, which are the main products of Nif2 activation for protecting cells from oxidative stress injuries (Del Regno et al. 2015). However, another study performed on the human Caco-2 cell model lead to different result in terms of iNOS expression. DON (0.1 and 1μM) exposure for 3, 6 and 12h induced a time- and dose-dependent increase in iNOS mRNA expression, whereas the iNOS protein and the NO production were undetectable. These were caused by proteasome-dependent degradation of iNOS protein, in which the PKR, MAPKs (p-38 and ERK1/2) and NF-κB signal pathways were involved (Graziani et al. 2015). These contradictory conclusions suggest that DON could use different mechanisms to exert its

toxicity in different conditions depending on cell types or the doses and duration of exposure. The oxidative injury was also observed in DON-treated (0.9mg DON/kg feed treated for 10 days) piglets, with upregulated and downregulated mRNA expression of HMOX1, one of the most sensitive indicator of oxidative stress, in the colon and jejunum (Alizadeh et al. 2015). These data revealed that low dose of DON efficiently induces oxidative stress in the intestine and intestinal cells.

2.4.5.4. Effects of DON on the intestinal barrier function

The intestinal barrier is an epithelium monolayer with the tight junction proteins (TJs), adherens junction proteins (AJs) and desmosomes connecting the adjacent cells, which are responsible for sealing the intercellular space. The intestinal epithelium functions as a selective permeable barrier allowing the dietary nutrients, electrolytes and water absorption and against bacteria, virus and other toxic molecules entrance, thereby maintaining the intestinal homeostasis (Groschwitz and Hogan 2009). Due to their important role in maintaining the barrier function, TJs and AJs can be targets of the food contaminants, such as DON. DON is able to damage the intestinal barrier by disturbance and redistribution of TJs, AJs and the cytoskeleton structure (Pinton et al. 2012; Akbari et al. 2014).

A study carried out on Caco-2 cells demonstrated that the transepithelial electrical resistance (TEER) of the monolayer and the expression of TJs(claudin-1, -3 and -4) were dose-dependently decreased in cells exposed to DON (1.39, 4.17 and 12.5μM), whereas the expression level of occludin and ZO-1 remained unchanged (Akbari et al. 2014). Treated piglets with 3mg DON/kg feed for 35d, induced a significant decrease of the expression of AJ protein E-cadherin and TJ protein occludin in the ileum (Bracarense et al. 2012) Nevertheless, a later study showed that DON elevated occludin expression in the duodenum, jejunum and colon of DON-fed (0.9mg/kg feed) pigs (Alizadeh et al. 2015). This distinction may be caused by different dose and duration of exposure.

The underlying mechanisms of adverse effects on intestinal barrier function caused by DON have been investigated using various cell lines. The porcine intestinal epithelial cell line (IPEC-1) is highly sensitive to DON. DON (30 μM) significantly declined the expression of claudin-4 and the TEER of the monolayer, and raised the permeability to 4kD-dextrant after 48h exposure. When treated for 1h, an obvious augmentation of ERK phosphorylation was observed. Interestingly, when the cells were pretreated with ERK inhibitor U0126 for 2h before DON addition, the alteration of claudin-4 expression, TEER and permeability was restored. They therefore demonstrated that ERK activation mediated decrease of claudin-4 expression

(Pinton et al. 2010). This conclusion was supported by Lei et al. (Lei et al. 2014), who pointed out that ERK activation reduced the expression of TJ proteins in the LPS-exposed Caco-2 cells. However, this is contrast with another study executed on the Caco-2 cells, which showed that claudin-4 decrease was independent of ERK activation by DON and U0126 simultaneous exposure (De Walle et al. 2010). The different results may due to the routes of ERK inhibitor U0126 application. A recent study has reported that DON (20 μM) induced intestinal barrier dysfunction in IPEC-J2 cells by reduction of claudin-1 and claudin-3 expression. The decrease of claudin-3 not claudin-1 expression partially caused by ERK activation (Springler et al. 2016). In contrast, Zhang et al. demonstrated that 20 μM DON exposure of IPEC-J2 caused a reduction of TEER and expression of claudin-4, and promoted p-38 activation and inhibited ERK activation (Zhang et al. 2016). This clearly suggests that different concentrations of DON selectively affects specific TJs in different cell line, and the activation of MAPKs is not the only mechanism of intestinal barrier damage.

On the other hand, ERK activation was reported to increase the expression of ZO-1 and occludin and decrease the permeability to FITC-dextran in the intestinal epithelial cells IEC-6 (Yang et al. 2005). Basuroy demonstrated that ERK activation inhibited H2O2-induced redistribution of ZO-1 and occludin in Caco-2 cells thereby maintain the normal barrier function (Basuroy et al. 2006). An interesting study conducted by Aggarwal et al. revealed that ERK activation in under-differentiated Caco-2 cells promoted tight-junction disruption, whereas in the differentiated cells enhanced tight junction integrity (Aggarwal et al. 2011). The results indicate that the effects of ERK on intestinal tight junction integrity depends on differentiation state of cells.

It is notable that DON has different sensitive target TJ and AJ proteins in different cell lines. Thus, DON may impede intestinal barrier function via alternative mechanisms depending on cell types. Although these studies obtained different or even discrepant conclusions, it still can be seen that MAPKs/ERK plays an important role in DON-induced intestinal barrier dysfunction. Due to the role of ERK in regulation of intestinal barrier function is still controversial, further studies are needed to illustrate its mechanisms of toxic actions.

Table 3 Effect of DON on intestinal barrier function

Model	Dose and time exposure	Effect on barrier function	References
In vitro			
Caco-2 cells	2 μg/ml, 48h 0.125-6 μg/ml, 24h	Decrease in TEER values	(Sergent et al. 2006)

Caco-2 cells	1.39–12.5 µM 24 h	Decrease in TEER values Decrease in horizontal impedance Increase in permeability of LY and 4 kDa FITC-dextran Increase in transcript level of claudin3, claudin4, occludin and ZO-1 Decrease in protein expression of claudin1, claudin3 and claudin4 Redistribution of claudin1, claudin3, claudin4, occludin and ZO-1.	(Akbari et al. 2014)
Caco-2 cells	0.16–16 µM 24 h	Decrease in TEER values Increase in permeability of mannitol Increase in transcript level of claudin4 and occludin Decrease in protein expression of claudin4	(De Walle et al. 2010)
Caco-2 cells	5–100 µM 48 h	Decrease in TEER values Increase in permeability of 4 kDa FITC-dextran Increase in translocation of pathogenic Escherichia coli (strain 28C) Decrease in protein expression of claudin4	(Pinton et al. 2009)
Caco-2 cells	1–100 µM 12 h	Decrease in TEER values Increase in permeability of HRP and 4 kDa FITC-dextran Increase in translocation of commensal Escherichia coli (strain k12)	(Maresca et al. 2008)
Caco-2 cells	0.37–1.5 µM 6–120 h	Decrease in horizontal impedance value of undifferentiated cells	(Manda et al. 2015)
Caco-2 cells T84 cells	0.16–0.67 µM 14 days	Decrease in TEER values Increase in permeability of LY	Kasuga et al. (1998)
HT-29-D4 cells	0.001–100 µM 48 h	Decrease in TEER values	(Maresca et al. 2002)
IPEC-1 cells	30 µM 48 h	Decrease in TEER values Increase in permeability of 4 kDa FITC-dextran Decrease in protein expression of claudin3, claudin4 Redistribution of claudin4	(Pinton et al. 2010, 2012)
IPEC-1 cells	5–50 µM 48 h	Decrease in TEER values Increase in permeability of 4 kDa FITC-dextran Decrease in protein expression of claudin3 and claudin4	(Pinton et al. 2009)
IPEC-1 cells IPEC-J2 cells	0.67–6.7 µM 48 h	Decrease in protein expression of ZO-1 Redistribution of ZO-1	(Diesing, *et al.*, 2011 b)
IPEC-J2 cells	6.74 µM 48 h	Decrease in TEER values Decrease in protein expression of claudin3, occludin and ZO-1 Redistribution of ZO-1	(Gu et al. 2014)
IPEC-J2 cells	0.67–13.4 µM 24–72 h	Decrease in TEER values Decrease in protein expression of claudin3 and ZO-1 Redistribution of claudin3	(Diesing, *et al.*, 2011 a)
IPEC-J2 cells	1.68–33.7 µM 72 h	Decrease in TEER values Increase in permeability of doxycycline and paromomycin	(Goossens et al. 2012)

IPEC-J2 cells	0.33–3.3 µM 24 h	Increase in translocation of pathogenic Salmonella typhimurium (strain 112910a)	(Vandenbroucke et al. 2011)
IPEC-J2 cells	4 µM 12 h	Decrease in TEER values Increase in permeability of 4 kDa FITC-dextran Increase in translocation of commensal Escherichia coli (strain ATCC® 25922™) Increase in transcript level of claudin1, claudin4, occludin and ZO-1 Decrease in protein expression of claudin3 and claudin4	(Ling et al. 2016)
IPEC-J2 cells	20 µM 72h	Decrease in protein expression of claudin1 and claudin4	(Springler et al. 2016)
IPEC-J2 cells	20 µM 1h	Decrease in TEER values Decrease in protein expression of claudin4	(Zhang et al. 2016)
IPEC-J2 cells	0.2 – 2 µg / mL 6, 12, 24h	Decrease in mRNA expression of ZO-1, occludin and claudin1	(Liao et al. 2017)
Ex vivo			
Pig jejunal explants	5–50 µM 2 h	Increase in permeability of 4 kDa FITC-dextran	(Pinton et al. 2009)
Pig jejunal explants	10 µM 4h	Decrease in E-cadherin expression	(Basso et al. 2013)
Pig jejunal explants	10 µM 3h	Increase in permeability of fluorescein sodium salt	(García et al. 2018)
Pig jejunal explants	10 µM 4h	Decrease in E-cadherin expression	(Da Silva et al. 2019)
In vivo			
Piglet	3 mg/kg feed 5 weeks	Decrease in protein expression of E-cadherin and occludin in ileum	(Bracarense et al. 2012)
pig	2.85 mg/kg feed 5 weeks	Decrease in protein expression of claudin4 in jejunum	(Pinton et al. 2009)
pig	3.5 mg/kg feed 6 weeks	Decrease in transcript level of claudin3, claudin 4 and occludin in ileum	(Lessard et al. 2015)
pig	0.9 mg/kg feed 10 days	Increase in transcript level of CLDNs (cecum), OCLD (duodenum, ileum, cecum and colon) and ZOs (duodenum and colon) Decrease in transcript level of claudin 4, occludin, ZO-1 and ZO-2 in jejunum Increase in protein expression of occludin in duodenum, jejunum and colon	(Alizadeh et al. 2015)
Mouse	25 mg/kg bw 6 h	Increase in permeability of 4 kDa FITC-dextran Increase in transcript level of CLDN2, claudin 3 and claudin 4 in distal small intestine Redistribution of claudin 1-3 in distal small intestine	(Akbari et al. 2014)
Mouse	5 mg/kg bw 24 h	Increase in transcript level of CLDN2 and claudin 3 in duodenum Decrease in protein expression of claudin 3 in duodenum	(Bol-Schoenmakers et al. 2016, p.)
Broiler chicken	7.5 mg/kg feed 3 weeks	Increase in transcript level of CLDN5 in jejunum and claudin 1, CLDN5, ZO-1 and ZO-2 in ileum	(Osselaere et al. 2013)
Broiler chicken	10 mg/kg feed 3 weeks	Decrease in mRNA level of jejunal occludin and claudin1	(Wu et al. 2018)
Atlantic Salmon	5.5 mg/kg feed 8 weeks	Decrease in mRNA level of claudin25b, occludin and tricellulin in middle and distal intestine	(Moldal et al. 2018)

From the description above, it is not difficult to imagine that DON-induced inflammatory injury, oxidative stress or other potential mechanisms of toxicity may also involve in DON-mediated damage on intestinal barrier function.

In the intestine of patients suffering inflammation bowel disease (IBD), Crohn's disease or ulcerative colitis, the intestinal barrier is always defective. This aroused the interest to investigate the link between inflammation and intestinal barrier dysfunction. The interferon gamma (IFN-γ) was the first inflammatory cytokine that was observed to induce TJ disruption in human derived intestinal cells (Madara and Stafford 1989). Since then, researchers continue to explore the effects of IFN-γ on the intestinal barrier and the underlying mechanisms. They found that IFN-γ induced occludin endocytosis from the TJ complexes leading to TJ barrier disruption. This process requires myosin light chain (MLC) phosphorylation and Rho kinase activation. Both NF-κB and PI-3 kinase inhibition restored the adverse effects caused by IFN-γ indicating that the two pathways are involved in IFN-γ induced increase of intestinal permeability and decrease of occludin expression (Al-Sadi et al. 2009).

In addition to IFN-γ, other cytokines' effects on intestinal barrier were also investigated. The permeability of Caco-2 monolayer was evidently increased by a physiological concentration of IL-1β via NF-κB pathway. This was further proved by NF-κB p65 depletion, which completely abolished IL-1β induced increase of TJ permeability (Al-Sadi and Ma 2007). Further, TNF-α was reported to promote opening of Caco-2 TJ barrier via MLCK expression mediated by NF-κB activation (Ye et al. 2006). Later, TNF-α was proved to active MAPK/ERK signaling pathway to promote transaction factor Elk-1binding to MLCK, leading to MLCK activation and expression. These reactions resulted in a decreasd TEER on Caco-2 monolayer and an increased intestinal permeability in mouse (Al-Sadi et al. 2013). Since NF-κB activation is the downstream event of MAPKs activation, it is reasonable to hypothesize a main pathway for TNF-α induced intestinal barrier disruption: TNF-α - MAPKs/ERK - NF-κB – MLCK - TJ disruption. These data indicate that DON induced inflammation in intestine is contribute to barrier dysfunction.

Oxidative stress is also able to trigger intestinal injury and barrier disruption. In the intestinal cells, oxidative stress caused an increase of permeability of Caco-2 monolayer by reorganization of occludin-ZO-1 and E-cadherin-β-catenin complexes from the intercellular junctions and dissociation occludin from ZO-1 (Rao et al. 2002; Basuroy et al. 2006).

In fact, inflammation and oxidative stress can affect one another (Fig.4). Oxidative stress-mediated tissue damage could induce overexpression of inflammatory cytokines in human intestine who suffered intestinal inflammation disease (Alzoghaibi 2013; Moret et al. 2014; Oyinloye et al. 2015; Vitali et al. 2015). It is reported that hydrogen peroxide H_2O_2 upregulated the mRNA expression of IL-8 and TNF-α and increased protein expression of IL-8 in IPEC-J2 cells (Paszti-Gere et al. 2012). NO, one of the reactive nitrogen species, could induce inflammatory cytokines production by activation of NF-κB or AP-1(Korhonen et al. 2005). In turn, inflammatory cytokines also mediate ROS or NOS production in the inflammatory or epithelial cells (Federico et al. 2007).

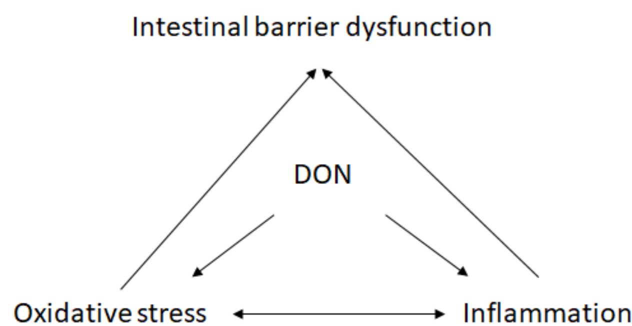


Figure 4 The relationship between DON-induced inflammation, oxidative stress and intestinal barrier dysfunction

2.4.5.5. Effect of DON on the intestinal microbiota

Gut microbiota have a marked influence on the the physiology functions of hosts and dysbiosis induces malnutrition, systematic diseases and chronic inflammatory diseases (Guinane and Cotter 2013; Thursby and Juge 2017). The effects of DON on the intestinal microbiota have been investigated in various animal models, such as rodents, pigs and chickens. Germ-free rats inoculated with human fecal flora were daily exposed to 100μg DON/kg b.w. for four weeks. It was found that the abundance of *Bacteroides/Prevotella* was significantly increased, while the concentration of *Escherichia coli* was decreased (Saint-Cyr et al. 2013). DON (10 μg/kg b.w./day) exposure of mice for 9 months caused an elevation of *Proteobacteria* and a reduction of *Bacteroidetes* (Vignal et al. 2018). DON dietary challenge altered the cecal microbiota of broiler chickens with a decrease of *Proteobacteria* and an increase of *Firmicutes* (Lucke et al. 2018). In pigs, it was reported that DON exposure increased the number of aerobic mesophilic bacteria and did not affect the abundance of anaerobic sulfite-reducing bacteria at 28d post exposure (Waché et al. 2009). It is worth noting that the decrease of the number of

Bacteroidetes and the increase of *Proteobacteria* was frequently observed in the IBD and Crohn's diseases, where are involved in intestinal inflammation diseases. The study of Wang et al. demonstrated that intestinal dysbacteriosis induced losses of barrier function and increased bacteria translocation, which can induce systematic inflammation (Wang *et al.*, 2014). It suggests that DON-induced alteration of microbiota causes breaking of intestinal barrier that favor bacteria translocation leading to inflammation in intestine, which in turn, having a negative effect on the intestine defection of barrier function.

3. Cadmium (Cd)

Cadmium (Cd) is a toxic and carcinogenetic heavy metal widely distributed in the environment. Both natural (volcanic eruption, weathering and erosion) and anthropogenic activities contribute to Cd contamination and Cd-related health problems (EFSA 2009; ATSDR 2012).

3.1. Occurrence, exposure and regulation of Cd in food and feed

Cd naturally exists in the earth's crust as a consequence of volcano eruption, minerals and rock exfoliation. Industrial emission is the main source of Cd pollution. Cd from nature and industries can directly enter into air, water and soil and absorbed by the roots of cereals or vegetables, consequently arriving and accumulating in the edible part (EFSA 2009; ATSDR 2012). A survey performed between 2003 and 2007 in 20 European countries on 137, 200 samples of raw cereals, vegetables and meat indicated that 66% were contaminated with Cd. While 5% of them even exceed the maximum level of 0.2 mg/kg for bran, germ, wheat and rice and of 0.1 mg/kg for other raw cereals specified by European Commission (European Union 2006a; EFSA 2009). In the 73rd report of JECFA, all the 1503 wheat samples tested from 19 European countries and 11 other countries worldwide were contaminated with Cd (JECFA 2011). The smokers and the occupational workers are exposed to Cd via smoking and inhalation during work process (ATSDR 2012). Cd-contaminated food and water are the primary sources for the general population (EFSA 2009). Grain and cereal products, as well as fish and offal were the major contributor for human Cd dietary exposure (Fig.5) (Filipic et al. 2006; ATSDR 2012). A tolerable weekly intake (TWI) for Cd of 2.5µg/ kg b.w. was established (EFSA 2009, 2011). The mean dietary exposure to Cd for adults across European countries was close to or slightly exceeding this TWI, and subgroups, such as vegetarians, children, smokers exceed the TWI by about 2-fold (EFSA 2009).

In Europe, the guidance concentration in cereal grains used for food ranges from 0.1 to 0.2 mg / kg depending on the grain species, no more than 0.5 mg / kg in the complete feed, and 0.005 mg / L in water (Table 2) (European Union 2013, 2014).

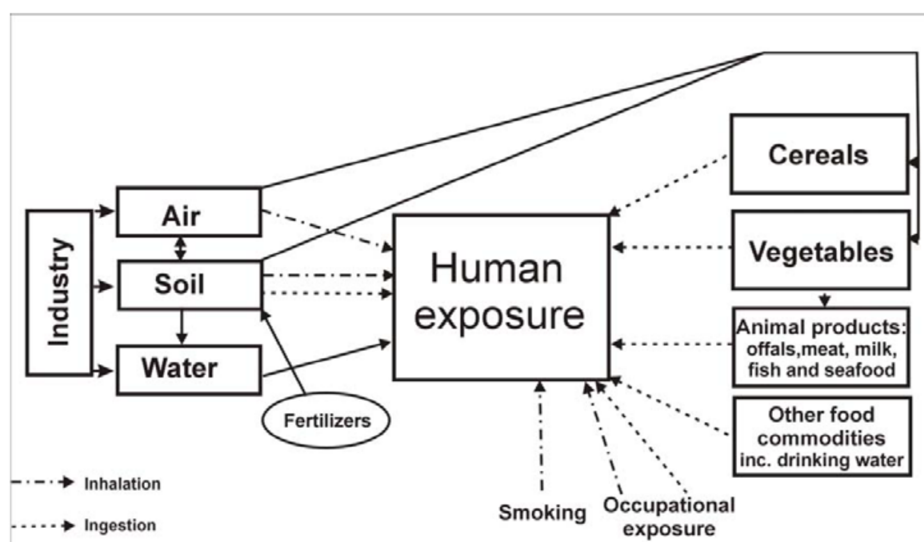


Figure 5 Sources of human exposure to cadmium (EFSA 2009)

Table 2. Recommendation for Cd in foodstuffs/feedstuffs in Europe (European Union 2013, 2014)

Foodstuffs	Maximum levels (mg/kg wet weight)
Cereal grains excluding wheat and rice	0.1
Wheat grains, rice grains; Wheat bran and wheat germ for direct consumption; Soybeans	0.2
Vegetables and fruit, excluding root and tuber vegetables, leaf vegetables, fresh herbs, leafy brassica, stem vegetables, fungi and seaweed	0.05
Root and tuber vegetables (excluding celeriac, parsnips, salsify and horseradish), stem vegetables (excluding celery)	0.1
Leaf vegetables, fresh herbs, leafy brassica, celery, celeriac, parsnips, salsify, horseradish and the following fungi: *Agaricus bisporus, Pleurotus ostreatus, Lentinula edodes*	0.2
Meat (excluding offal) of bovine animals, sheep, pig and poultry	0.05
Horsemeat, excluding offal	0.2
Liver of bovine animals, sheep, pig, poultry and horse	0.5
Kidney of bovine animals, sheep, pig, poultry and horse	1.0
Processed cereal-based foods and baby foods for infants and young children	0.04
Feedstuffs	Maximum content in mg/kg relative to a feed

	with a moisture content of 12 %
Feed materials of vegetable origin	1
Feed materials of animal origin	2
Complete feed with the exception of: - complete feed for cattle (except calves), sheep (except lambs), goats (except kids) and fish; - complete feed for pet animals	0.5 1 2

3.2. Toxicokinetics of Cd

Since Cd is not an essential element for human or animals, a specific transporter for Cd does not exist. Some transport proteins for essential metals, such as iron, zinc and calcium are reported to be responsible for Cd absorption. Divalent metal transpoter 1 (DMT 1) was highly induced by Fe deficient diet in rat duodenum, which was associated with the higher tissue Cd concentration (Park et al. 2002; Ryu et al. 2004). DMT1 knockdown Caco-2 cells displayed a 50% reduction of Cd uptake (Bannon et al. 2003). Knockdown of Zinc transporters Zrt/Irt-related protein 8 (ZIP8) and ZIP14 significantly decreased Cd uptake in mouse kidney proximal tubule cells (Fujishiro et al. 2012). Calbindin D9k, the major calbindin isoform in the enterocyte is also considersred as a candidate transporter for Cd. These transporters are mainly expressed in duodenum and jejunum where most Cd is absorbed (Vesey 2010). The absorption rate for Cd through gastrointestinal tract was estimated between 3% and 7% in adult human (Vesey 2010). The ingested Cd entries into the blood and binds to proteins such as albumin and metallothioneins (MTs). Then, Cd firstly arrives in the liver where it induces synthesis of liver MTs. The Cd-MT complexes are released to blood again after hepatocytes necrosis or apoptosis. Meanwhile, some parts of these complexes may be excreted with bile and reenter the small intestine through the entro-hepatic cycle. The Cd-MT complexes released by hepatocytes then reach the kidney via blood circulation. These complexes are filtrated in the glomerulus and reabsorbed in the proximal tubules. It then remains in tubule cells for a long time (Godt et al. 2006). Cd was mainly excreted with urine and feces even though the excretion of Cd is very scarce (Fig.6).

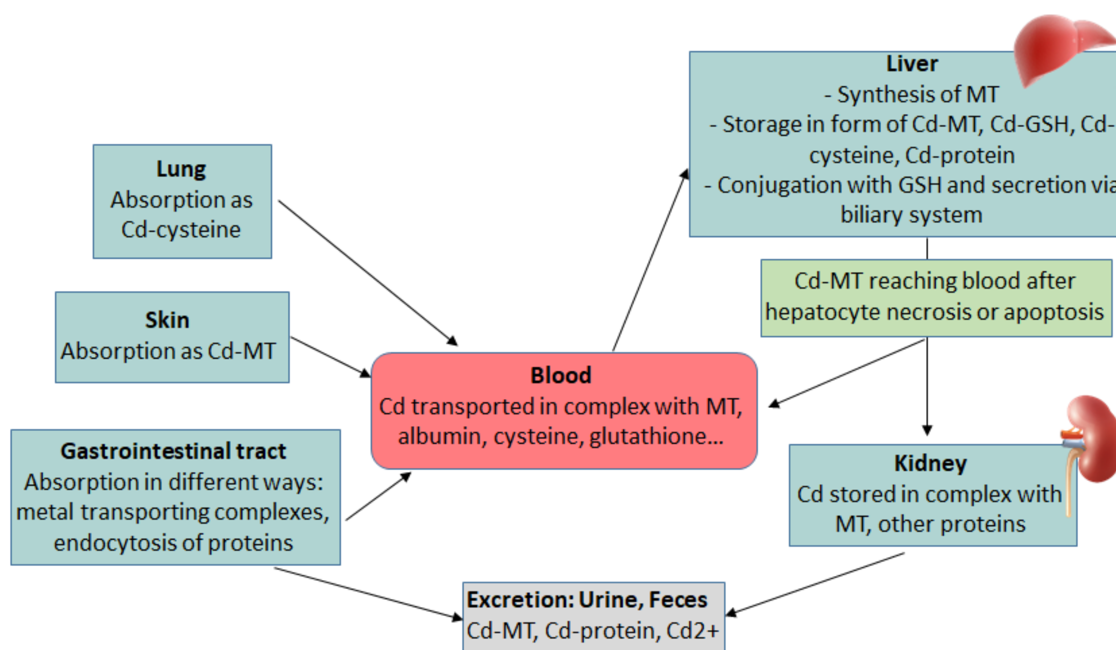


Figure 6 Metabolism, storage and excretion pf Cd in human body (Godt et al. 2006)

3.3.Mode of action of Cd

Oxidative stress is considered as the effective molecular basis of Cd toxic actions. Cd exposure induces a significant increase of ROS production in vivo (rat) and in vitro (primary rat hepatocytes), which contributes to hepatocyte apoptosis (Liu et al. 2011; Wang et al. 2014b). In fact, Cd can neither participate directly in redox reaction nor generate ROS, as it is a redox-stable metal. However, Cd is able to substitute with copper, zinc and iron in various cytoplasmatic and membrane proteins, thereby increasing the components of free redox-active metal that can induce oxidative stress (Dorta et al. 2003; Liu et al. 2009). These metals induce ROS generation through Fenton-type chemical reaction (Desurmont 1983; Lee et al. 2012). Although Cd is redox-stable, the production of hydroxyl radical has an extremely strong oxidizing ability, which is highly toxic to human health.

$$\text{metal}^{\,n+} + H_2O_2 \longrightarrow \text{metal}^{\,n+1} + \text{-OH} + OH^-$$

On the other hand, Cd interferes with antioxidant enzymes activity, such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPx) through binding their thiol groups (Filipic et al. 2006; El-Boshy et al. 2015). Oral exposure to Cd (40mg/L) for 30 days decreased the content of GSH, SOD, CAT and GPx in serum (El-Boshy et al. 2015), meanwhile intraperitoneal injection of Cd (6.5mg Cd/kg bw / d) for 5 days markedly reduced the activity of these antioxidant enzymes in hepatic and renal tissues (Dkhil

et al. 2014). These studies suggest that Cd exposure through different routes induces oxidative stress by inducing ROS production in an indirect way and compromises the antioxidant system by directly binding to their thiol groups.

Cd-induced ROS generation is responsible for the downstream events as DNA damage, apoptosis, inflammation and genomic instability.

Heavy metals interact directly with DNA or nuclear protein, causing DNA damage or conformational changes that may lead to carcinogenesis (Wang and Shi 2001; Beyersmann and Hartwig 2008; Qin et al. 2016). However, Cd did not induce DNA breaks on the pure DNA extracted from liver cells of mice, suggesting Cd may not be able to directly attack on DNA (Valverde et al. 2001), while in the intact cells exposed to Cd, the free radicals increased significantly (Valverde et al. 2001). In addition, ROS production especially the highly reactive hydroxyl radical (OH^-) can directly attack on DNA bases inducing DNA damage (Dizdaroglu and Jaruga 2012; Cadet and Wagner 2013), as well as hydrogen peroxide H2O2 (Gafter-Gvili et al. 2013). These data suggest that ROS plays an important role in Cd-induced DNA damage.

Cd not only induces DNA damage but also inhibits the DNA repair systems, including excision repair (base excision repair (BER) and nucleotide excision repair (NER)), mismatch repair (MMR) and recombination repair (homologous recombination (HR) and non-homologous end-joining (NHEJ)), the last system is involved in DNA double-strand breaks (DSBs) (Giaginis et al. 2006; Lei et al. 2015; Li et al. 2015). Together with inhibition of DNA repair, DNA damage is associated with genotoxicity, genomic instability and carcinogenesis of Cd contamination. Since the oxidative stress and the inflammation caused by Cd were repeatedly observed in literature (Liu et al. 2015; Ghosh and N 2018), it promotes to speculate that there may be a link between the two processes. In the human umbilical vein endothelial cells (HUVECs), Cd increased the expression and secretion of inflammatory cytokine TNF-α via p38/ MAPK activation (Dong et al. 2014). These results indicate that Cd-induced inflammation isregulated by oxidative stress-mediated MAPKs activation (Cd-ROS-MAPKs/p38-inflammation).

Many reports demonstrated that acute Cd-induced apoptosis is p53- and MAPKs-dependent (Yu et al. 2011). Cadmium exposure upregulated *p53* gene expression and increased p53 protein level *in vitro*, leading to apoptosis(Achanzar et al. 2000; Al-Assaf et al. 2013; Lee et al. 2016). In addition siRNA-induced *p53* silencing effectively inhibited cell apoptosis (Aimola et al. 2012), indicating that p53 plays a critical role in Cd-induced cell apoptosis. Other hypothesis proposed that Cd induces apoptosis via MAPK/JNK-mediated mitochondrial pathway, as the JNK inhibitor or JNK-specific siRNA interference protected cells from Cd-

induced apoptosis (Chang et al. 2013; Jiang et al. 2014; Yuan et al. 2015). This reveals that MAPK/JNK pathway is involved in Cd-mediated cell apoptosis. Nevertheless, JNK inhibition did not decrease the ROS generation, while the antioxidant N-acetylcysteine (NAC) restored Cd-induced events, including JNK activation (Chang et al. 2013). This clearly implies that Cd-induced events associated to apoptosis are mediated by Cd-generated ROS. These data guide to speculate that p53-and MAPKs-dependent ways of apoptosis may be in the same signaling pathway (Cd-ROS-MAPKs/JNK-p53-Bax-Mitochodria-caspases-apoptosis).

Apoptosis is an important process in organisms to eliminate transformed and mutated cells from the body. Thus, cancer cells have to develop various efficient mechanisms against apoptosis to keep survival. Chronic low-dose exposure of Cd to rat liver cells and human prostate cells can induce cell transformation with an apoptotic resistance property, which is due to the inhibition of JNK activation associated with MT or Bcl-2 overexpression (Qu et al. 2006, 2007). The abnormal proliferation of these DNA-damaged cells could elicit malignant transformation that finally develop to cancer.

3.4. Toxicity of Cd

Cd has various target organs, such as kidney, liver, lung, reproductive and nervous system. In these organs Cd is able to induce genotoxicity, cancer, immunotoxicity, neurotoxicity, reproductive and developmental toxicity (ATSDR 2012; Rani et al. 2014).

3.4.1. Immunotoxicity

There is still controversy on pro- or anti-inflammatory properties of Cd. Some studies demonstrated that Cd stimulate immune system and promote inflammatory reactions; while other studies suggested that Cd has non-inflammatory or anti-inflammatory properties (Olszowski et al. 2012). In vivo, Cd (40 mg/L drinking water) treated for 30 days evidently increased the secretion of inflammatory cytokines IL-1β, TNF α, IL-6 and IL-10 and decreased IFN-γ and the content of lymphocytes in serum of rats (El-Boshy et al. 2015). Similarly, the gene expression of TNF-α, IL-6 and IL-1β in larval zebrafish was up-regulated by Cd (1, 3, 10 μM) after 96h exposure (Jin *et al.*, 2015). While, in adult zebrafish, the release of TNF-α was increased in the liver, brain and ovary, the mRNA level of NF-κB was upregulated in the liver and ovary at 24h exposure of Cd (1 mg / L). By contrast, in Cd (3 μM) treated rat primary lung cells and rat alveolar macrophages, the mRNA expression of TNF-α and IL-1β was reduced after 20h exposure, while their protein levels were not affected. The expression and release of IL-6 in alveolar macrophages was downregulated and unaltered (Låg et al. 2010). These data

revealed both positive and negative effects of cadmium on immune modulation. To improve understanding of the immunotoxicity of Cd, more studies in mammals should be done.

3.4.2. Genotoxicity

Cd induces genotoxicity characterized by DNA damage through induction of ROS production, resulting in cell apoptosis or malignant transformation that may develop to cancer (Achanzar et al. 2001; Chang et al. 2013). In human bronchial epithelial cells, 5 μM of Cd exposed for several weeks triggered genomic instability, DNA breaks and down-regulation of DNA repair genes (*hMSH2, ERCC1, XRCC1*, and *hOGG1*). Meanwhile, the mutation of exons on these genes was also observed in tumorgenetic cells of nude mice (Zhou et al. 2013). Occupational exposure to Cd (blood concentration 5.4-30.8 µg/L) significantly increased the frequency of sister chromatid exchanges (SCEs) as well as the leukocytes with DNA fragmentation in lymphocytes compared with controls (Palus et al. 2003). This study is consistent with a previous study performed on mice, which demonstrated that single intravenous exposure of Cd 5.7 and 7.6 mg /kg body weight, evidently increased the percentage of polychromatic erythrocytes with micronuclei, the frequency of SCEs and the portion of chromosomal aberrations in mouse bone marrow (Fahmy and Aly 2000). These gene toxic effects caused by Cd are necessary prerequisites for cadmium-induced cancer.

3.4.3. Reproductive and developmental toxicity

Reproductive system is a key site of Cd toxicity both in male and female. Cd is able to inhibit oocyte development in ovary and decrease sperm motility. In addition, it induces implantation delay, consequently inhibiting embryo development (Thompson and Bannigan 2008; Zhao et al. 2017). A study performed with bovine oocytes proved that Cd (2 and 20 μM) exposure for 24h, efficiently decreased the oocytes maturation, as well as the post-fertilization cleavage rate in zygotes and blastocyst development (Akar et al. 2018). Another research illustrated that the motility of human and mouse sperm were significantly reduced by Cd (2.5 – 10 μg/ml) exposure for 6-24h in a dose- and time-dependent manner. Exposure to these doses of Cd for 30 min did not alter sperm motility but significantly decreased the fertilization rate of sperm in vitro. Besides, exposure to low dose of Cd at 0.625 and 1.25 μg/ml for 12-84h dramatically declined the blastocyst formation rate of embryo (Zhao et al. 2017). Cd is also reported to pass through placental barrier and accumulate in embryo, resulting in degeneration and decompaction in blastocysts or early pregnancy loss (Thompson and Bannigan 2008; Gundacker and Hengstschläger 2012).

3.4.4. Neurotoxicity

It is known that Cd induces opening of blood-brain barrier and enters into central nervous system leading to endocrine disruption and even neuronal cells death through ROS production (Kim et al. 2013a; Tobwala et al. 2014; Chouchene et al. 2016). For instance, Cd can interfere with estrogen signaling in vitro and in vivo. *In vitro*, Cd (0.01-10 μM) exposure for 30h significantly abolished estradiol-induced up-regulation of estrogen receptors in mRNA level. *In vivo*, Cd exposure (0.01-10 μM) for 72h significantly down-regulated the gene expression of esr1, esr2α, esr2β (encoding estrogen receptors) and cyp19a1b (encoding brain aromatase in fish) and efficiently inhibited the production of aromatase B in glial cells of zebrafish embryo (Chouchene et al. 2016). In neuroblastoma cells, Cd (25 μM) induced cell apoptosis after 6-24h exposure via ROS generation-mediated induction of GADD153, a member of the C/EBP (CAAT/enhancer-binding protein) family of bZIP transcription factors. This protein promoted C/EBP binding to Bak promoter and increased the intracellular level of Bak (a pro-apoptotic protein), thereby leading to cell apoptosis (Kim et al. 2013a). The data indicate that ROS production is the key causative of Cd-induced neurotoxicity.

3.4.5. Intestinal toxicity

Besides the adverse effects on these tissues above, gut was also considered as a new target of Cd recently (Tinkov et al. 2018). When human or animal are exposed to Cd by dietary food and water, the intestine can be the first target organ being subject to a high concentration of Cd.

3.4.5.1. Cd-induced morphological lesions in intestine

Cd consumption from food and water primarily retains in the mucosa, which causes intestinal histological changes, such as epithelial necrosis, apical villous damage, infiltration of inflammatory cells, leading to shorter and thicker villi (Zhao et al. 2006; Ninkov et al. 2015, 2016). The morphological changes on intestine result in reduction of nutrients absorption and decrease of body weight gain in animals (Teshfam et al. 2006).

3.4.5.2. Cd-induced modification of intestinal immune response

Cd is able to modulate the immune response on intestine characterized by induction of inflammatory cytokines. Several *in vivo* and *in vitro* studies have been carried out to investigate Cd-induced inflammatory response in intestine. Cadmium (5 and 10ppm) exposure for 30 days, induced a significant increase of inflammatory cytokines TNF, IL-1β, IFN-γ and IL-17 in the duodenum homogenates of rats. In addition, the level of high mobility group box1 (HMGB1), a potent mediator of inflammation as well as a suitable inflammatory biomarker (Palone et al. 2014) increased notably in the intestine (Ninkov et al. 2015, 2016). These results suggest that

Cd can regulate immune response in the intestine. The up-regulation of IFN-γ and IL-17 mRNA and the down-regulation of IL-10 were observed in the mesenteric lymph node (MLN), with the increased MLN cell proliferation suggesting immune activation in this lymphoid tissue, and emphasizing the important role of MLN immune response to oral Cd exposure. Nevertheless, in a mice model, Cd exposure (25, 100mg/kg body weight) for 3-24h did not affect the mRNA expression of IL-1β and TNF-α in duodenum and proximal jejunum. Whereas, the expression of MIP-2 (mouse homologue IL-8 in human) was markedly augmented at 3h post exposure, and was decreased from 12h, then returned to the normal level at 24h. Besides, the infiltration of neutrophils into the intestinal mucosa of mice was observed (Zhao et al. 2006). These results from Cd-exposed mice are consistent with *in vitro* experiment preformed with human Caco-2 cells. When treated with 50μM of Cd for 24h, only IL-8 cytokine level in the culture medium significantly increased, other cytokines TNF-α, IFN-γ and IL-1β were not stimulated by Cd. In this study, authors proved that the up-regulation of IL-8 gene and protein were driven by degradation of I-κBα and NF-κB activation in Cd-treated Caco-2 cells (Hyun et al. 2007).

Opposite to pro-inflammatory effects of Cd exposure, some studies demonstrated that Cd did not affect inflammatory response even anti-inflammation. Breton et al. induced colitis using DSS (dextran sodium sulfate) and TNBS (trinitrobenzene sulfonic acid) to mimic an inflammatory bowel disease (IBD) in mice, afterwards, the mice were exposed to Cd (20 and 68μM) for 6 weeks. The results showed that chronic Cd exposure suppressed the transcriptional activity of pro-inflammatory genes (IL-6, IL-1β, TNF-α, Nos2 and Cox-2) in the DSS-induced colitis, while in the TNBS-induced colitis, the expression of genes, such as IL-6, IL-1β, Nos-2 were down-regulated (Breton et al. 2016). In this case, Cd exposure exhibited a favorable side against intestinal inflammation. The data listed above suggest that the pro-inflammatory or anti-inflammatory effects caused by Cd depends on the species and the dose and duration of exposure.

3.4.5.3. Cd-induced oxidative stress on intestine

The capacity of Cd to induce ROS has been observed in different intestinal cell lines. Cadmium (50μM) efficiently induced ROS generation and high level of lipid peroxidation after 24h exposure in HT29 cell line (Zhai et al. 2016). Similar results were observed in the TC7 cells, a highly differentiated Caco-2 cell line exposed to 50μM of Cd, which showed a rapid formation of ROS within 30 min post exposure (Bolduc et al. 2004).

On the other hand, the effects of Cd on antioxidant system have been studied. Oral exposure of Cd (5 and 50 ppm) for 30 days, a dose-dependent increase of CAT activity was

observed in rat intestine, while the SOD activity was decreased notably by the high dose (50ppm) of Cd (Ninkov et al. 2015). An in vitro study using Caco-2 cells demonstrated that low dose of Cd (1-50μM) up-regulated the mRNA expression of NQO1 (an indicator of oxidative injury) and GR (glutathione reductase), whereas the high dose of Cd (100-300μM) down-regulated these genes (Rusanov et al. 2015). Another study on Caco-2 cells revealed that Cd (0.25-10mg/L) decreased the activity of SOD and GPx in a dose-dependent manner (Aziz et al. 2014). From these studies it could be concluded that high dose of Cd exposure inhibits the antioxidant system *in vivo* and *in vitro.*

3.4.5.4. Effects of Cd on the intestinal barrier function

Early studies showed that epithelial cells were able to absorb Cd, leading to decrease of monolayer TEER (transepithelial electrical resistance) and increase of permeability to mannitol (Rossi et al. 1996; Duizer et al. 1999). In Caco-2 cells, Cd (100 μM) exposure for 4h, altered the location of ZO-1, occludin and E-cadherin, which continued to develop after the intracellular Cd removing (Duizer et al. 1999). This study also showed that Caco-2 cells can transport Cd (5 μM) from the apical side to the basolateral side. These data suggest that intestinal cells are able to absorb Cd and the detained Cd in the body, which has a long biological half-life, would continue to affect human health even if the Cd sources are interrupted. This speculation was supported by a recent study, which reported that Cd absorption in Caco-2 cells was mediated by the ferrous iron transporter (DMT1) and zinc transporter (ZIP14) (Fujishiro et al. 2017). In HT-29 cells, Cd caused an irregular distribution of ZO-1 and claudin-1; in Caco-2 cells, Cd induced up-regulation of genes coding tight and adherens junction proteins, including ZO-1, claudin-4, E-cadherin, and p-120 catenin, among which the latter two genes are most sensitive to Cd influence (Rusanov et al. 2015; Zhai et al. 2016). These studies reveal an association between Cd exposure and the damage of intestinal barrier proteins. Both of ZO-1 and occludin involve cysteine group that contains thiol group (Itoh et al. 1993; Mitic and Anderson 1998). Cd has a high affinity to thiol group (Valko et al. 2005), therefore it may bind to ZO-1 and occludin in the thiol group to redistribute these junctional proteins in the intestinal cells, leading to loss of barrier function.

Table 4 Effect of Cd on intestinal barrier function

Model	Dose and time exposure	Effect on barrier function	References
In vitro			

Caco-2 cells	10-80 μM, 24h	Decrease in TEER values Increase in permeability of mannitol Disruption in integrity of tight junctions	(Rossi et al. 1996)
Caco-2 cells	100 μM, 4h	Decrease in TEER values Increase in permeability of mannitol and PEG-4000 Redistribution of ZO-1, occludin, E-cadherin	(Duizer et al. 1999)
Caco-2 cells	1-300 μM, 3h	Decrease in TEER values Increase in transcript level of E-cadherin, p120, Claudin4 and ZO-1	(Rusanov et al. 2015)
HT-29 cells	50 μM 24h	Redistribution of ZO-1 and Claudin1	(Zhai et al. 2016)
In vivo			
Mouse	100 mg/L water 8 weeks	Increase in permeability of DX-4000-FITC Decrease in transcript level of ZO-1, ZO-2, Claudin1 and occludin in jejunum and colon	(Zhai et al. 2016)

3.4.5.5. Effect of Cd on the intestinal microbiota

As a main actor of gut homeostasis, the microbiota has become a potential target of environmental chemicals (Joly Condette et al. 2014; Jin et al. 2015b). The adverse effects of Cd on the gut microbiota have attracted attention. A study performed on mice model showed that Cd exposure suppressed the growth of the resident gut microbiota and reduced the abundance of total bacteria (Li et al. 2016). The population of *Bifidobacterium* and *Lactobacillus* decreased significantly in Cd-exposed mice, as well as the gut microbiome SCFAs (short chain fatty acids) metabolism (Li et al. 2016), which positively regulate the TJ assembly and paracellular permeability (Wells et al. 2017). Cd exposure also reduced the ratio of *Bacteroidetes* (decrease) to *Firmicutes* (increase) in this study. It may be beneficial for resisting Cd as *Firmicutes* possesses a high content of peptidoglycan and teichoic acid. These elements could bind Cd ions to cell wall by ion exchange reaction (Vrieze et al. 2010). This is consistent with the study performed by Liu et al. (Liu et al. 2014). However, Ba et al and Zhang et al found that the amount of *Firmicutes* decreased in the Cd-exposed mice (Zhang et al. 2015; Ba et al. 2017). Ba et al. demonstrated that low-dose Cd increased plasma fat accumulation via microbiota alteration in male mice, but not in female (Ba et al. 2017). Zhang et al. proved that Cd increased the transcription of genes related to glucose and lipid metabolism in liver (Zhang et al. 2015). These data suggest that Cd-induced disorder of intestinal microbiota mediates dysregulation on energy metabolism. In Zhang's study, the increase of serum LPS level in the Cd-treated mice was also observed. Furthermore, serum LPS activity is closely associated with the concentration of blood Cd (Han et al. 2015). This imply that gut microbiota alteration may induce LPS production, which is related to systemic inflammation.

The conventional and germ-free mice models were used to detect the protective effects of gut microbiota against Cd toxicity. This research revealed that Cd accumulation in blood, kidney, liver and spleen of germ-free mice was significantly higher than that of conventional mice. The transcriptional level of metal transporter, such as metallothioneins (*MT1* and *MT2*) was considerably higher, whereas, the expression level of antioxidant enzyme *Nos2* was much lower in the duodenum and colon of germ-free mice compared to control mice (Breton et al. 2013). These data indicate that Cd impairs intestinal function not only through the alteration of junctional structure but also via the alteration of the composition of intestinal microbiota. In summary, Cd exposure could change the micro-ecological balance, which could affect the intestinal epithelial layer. While the alteration of epithelial layer, in turn, also affects the gut microbiota.

Experimental work

Generally, contaminants are not present alone but in mixture in food/feed. In recent decade years, the mixtures of different contaminants in food catch an increasing concern. Several studies showed that different heavy metals, like Cu, Pb, Cr and Cd accumulate in the edible parts of wheat, which was irrigated by contaminated water (Si et al. 2015; Zeng et al. 2015; Ran et al. 2016). Other studies revealed that different mycotoxins, including DON, ZEN, T-2 and HT-2 toxin are detected in the cereals or cereal-derived products (Mankevičienė et al. 2014; Vanheule et al. 2014; Yoshinari et al. 2014). Eskandari and Pakfetrat found that various mycotoxins and heavy metals in animal feed exceeded the permissible maximum levels in Iran (Eskandari and Pakfetrat 2014). The co-occurrence of mycotoxins and heavy metals was also observed in the corn flours in Turkey (Algül and Kara 2014). These studies indicate that mixed contamination of different toxicants in food is very common. Therefore, the co-occurrence of DON and Cd in food is possible. Indeed the last French Total Diet Studies have shown that several food products, such as bread and dried bread products, pasta, croissant-like pastries, sweet or savory biscuits, rice and wheat products were contaminated with both DON and Cd (Arnich et al. 2012; Sirot et al. 2013). Overall, the co-contamination of food products by DON and Cd raises the concern of consumers exposed to the cocktail of these contaminants.

The toxicity of a toxic mixture cannot be always predicted based on the effect of its individual toxicant, as exposure to mixtures could result in synergistic, antagonistic and additive effect (Alassane-Kpembi et al. 2017a; Tinkov et al. 2018). Some interactions in multi-contaminant have been reported. Synergy effects on Caco-2 cytotoxicity were observed between DON and its acetylated derivatives at low concentrations (Alassane-Kpembi et al. 2013) or between DON and aflatoxin B1 at higher doses (Sobral et al. 2018). In vivo study showed an additive and less than additive interaction between DON and aflatoxins on body weight gain (Grenier and Oswald 2011). Low dose of DON and nivalenol combination caused a synergistic interaction on inflammation induction in jejunal explants of pig (Alassane-Kpembi et al. 2017a). The genotoxicity of other toxicants such as colibactin and aristolochic acid, an *Escherichia coli*-derived and plant-derived genotoxin, can be aggravated by DON and ochratoxin A respectively in vivo and in vitro (Payros et al. 2017), implying a synergistic interaction between these toxins. The synergistic, additive and antagonistic effects were also reported between Cd and other chemicals, including lead, uranium, pesticide and benzo(a)pyrene (Roesijadi et al. 2009; Kim et al. 2013b; Margerit et al. 2015; Xu et al. 2017).

Therefore, it is important to determine whether DON and Cd interact with each other to cause a synergy or an antagonism.

Le et al. (Le et al. 2018) have studied the interaction types between DON and Cd using different *in vitro* models representing kidney (HEK293), intestine (Caco-2), blood (HL-60) and liver (HepG2). They found that the interaction type ranged from moderate antagonism to nearly additive in HEK293 and from nearly additive to antagonism in Caco-2 regardless of the ratios between DON and Cd into the mixture. In HL-60 and HepG2, interaction ranged from synergy to antagonism at ratio 1:1. This in vitro study has confirmed that DON and Cd affect each other on their toxic effect on intestine. These results give a sign that further in vitro and in vivo studies are needed to help understand the co-effects of the cocktail contaminants on the intestine and the underlying mechanisms.

Human and animals are exposed to both toxicants through ingestion of contaminated food and feed, especially cereals and cereal by-products (Arnich et al. 2012; Sirot et al. 2013). However, so far, the combined effects of DON and Cd are poorly studied. To the best of our knowledge, with the exception of one study (Le et al. 2018), no more investigations have been performed to explore the combined effects of these two contaminants. The aim of the present study was to assess the individual and combined effects of DON and Cd on the intestinal barrier using *in vitro, in vivo* and *ex vivo* models. The individual and combined effects of DON and $CdCl_2$ were analyzed *in vitro* on Caco-2 cells by measuring transepithelial electrical resistance (TEER), paracellular permeability and the abundance of junctional proteins. *In vivo* experiments were performed on rats to investigate the effects on intestinal histomorphology, abundance and localization of junctional proteins. *Ex vivo* trials were carried out on the jejunal explants of pig by measuring mRNA expression of inflammatory genes, TEER and paracellular permeability.

The results of this work are presented in the form of scientific articles:

Article 1

Su Luo, Chloe Terciolo, Ana Paula Bracarense, Delphine Payros, Philippe Pinton, Isabelle Oswald. In vitro and in vivo effects of a mycotoxin, deoxynivalenol, and a trace metal, cadmium, alone or in mixture on the intestinal barrier. Environment International, 132:105082, 2019. Available on line : https://www.ncbi.nlm.nih.gov/pubmed/31400600

Article 2

Su Luo, Sylvie Puel, Isabelle P. Oswald and Philippe Pinton. Specific intestinal toxicity of deoxynivalenol and cadmium: analysis on pig jejunal explant. Archiva Zootechnica, 20 :2, 39-50, 2017.

1. *In vitro* and *in vivo* effects of a mycotoxin, deoxynivalenol, and a trace metal, cadmium, alone or in mixture on the intestinal barrier

Su Luo[1], Chloe Terciolo[1], Ana Paula Bracarense[2], Delphine Payros[1], Philippe Pinton[1], Isabelle P. Oswald[1]

[1]: Toxalim (Research Center in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse, France

[2]: Depto Medicina Veterinária Preventiva, Laboratory of Animal Pathology, Universidade Estadual de Londrina, Londrina, Brazil

Corresponding author: Philippe Pinton

Philippe.Pinton@inra.fr

Abstract: Deoxynivalenol (DON), one of the most widespread mycotoxins in Europe, and cadmium (Cd), a widespread environmental pollutant, are common food contaminants. They exert adverse effects on different organs including kidney, liver, and intestine. The intestine is a common target of DON and Cd when they are ingested. Most studies have focused on their individual effects whereas their combined toxicity has rarely been studied. The aim of this study was thus to evaluate their individual and combined effects on the intestinal barrier function *in vitro* and *in vivo*. *In vitro*, Caco-2 cells were treated with increasing concentrations of DON and Cd (1-30 µM). *In vivo*, rats were used as controls or exposed to DON contaminated feed (8.2 mg/kg feed), Cd-contaminated water (5 mg/L) or both for four weeks. In Caco-2 cells, DON, Cd and the DON+Cd mixture reduced transepithelial electrical resistance (TEER) and increased paracellular permeability in a dose-dependent manner. Impairment of the barrier function was associated with a decrease in the amount of E-cadherin and occludin after exposure to the two contaminants alone or combined. A decrease in E-cadherin expression was observed in rats exposed to the two contaminants alone or combined, whereas occludin expression only decreased in animals exposed to DON and DON+Cd. Jejunal crypt depth was reduced in rats exposed to DON or Cd, whereas villi height was not affected. *In vitro* and *in vivo* results showed that the effects of exposure to combined DON and Cd on the intestinal barrier function in the jejunum of Wistar rats and in the colorectal cancer cell line (Caco-2) was similar to the effects of each individual contaminant. This suggests that regulations for each individual contaminant are sufficiently protective for consumers.

Keywords: Deoxynivalenol, cadmium, intestinal barrier function, mixture exposure, junctional proteins, mixture risk assessment

Highlights:

- Both DON and Cd alter the intestinal epithelial barrier function.
- Both DON and Cd disrupt the intercellular junctions (tight and adherens junctions).
- Both DON and Cd reduce the depth of jejunal crypts.
- Combined, DON and Cd have the same effects as each individual contaminant.

1.1 Introduction

Food safety is a major concern worldwide. Food and feedstuff are frequently contaminated by multiple contaminants (Silins and Högberg 2011a; Rather et al. 2017). The toxic effects of these mixtures cannot be predicted only based on the toxicity of individual contaminants, and simultaneous exposure to several contaminants can lead to synergistic, antagonistic or additive effects (Alassane-Kpembi et al. 2017b). In recent years, combined toxicity studies have been carried out to assess the effects of mixtures of food contaminants such as mycotoxins (Alassane-Kpembi et al. 2017a) trace metals elements (Claus Henn et al. 2014), pesticides (Lukowicz et al. 2018) or carcinogens (Miller et al. 2017). However, the toxicity of mixtures of contaminants from different families remains poorly documented (Payros et al. 2017; Le et al. 2018).

Mycotoxins are the most frequently occurring natural food contaminants in human and animal diets. Among mycotoxins, Deoxynivalenol (DON), mainly produced by *Fusarium graminearum* and *F. culmorum*, frequently contaminates cereals and cereal products. Almost half of 26,613 cereal samples collected from 21 European countries were found to be contaminated by DON, with the highest levels observed in wheat, maize and oat grains (EFSA 2013). A tolerable daily intake (TDI) for DON and its derivative was established at 1 µg/kg b.w./day (EFSA 2017). Analyses of adult urine samples in the United Kingdom revealed that 98% of them had been exposed to DON (Turner et al. 2008), while national and European surveys indicate that children exceed the health based guidance values (Sirot et al. 2013; Knutsen et al. 2017). DON interacts with the peptidyl transferase region of the 60S ribosomal subunit, inducing “ribotoxic stress,” resulting in the activation of mitogen-activated protein kinases and their downstream pathways (Pestka 2010a; Lucioli et al. 2013). Symptoms of intoxication in animals exposed to DON may include decreases food consumption and weight gain, neuro-endocrine changes as well as alteration of intestinal and immune functions. Following acute exposure, vomiting and bloody diarrhea have been observed (Pestka 2010a; Pinton and Oswald 2014; Payros et al. 2016).

Cadmium (Cd) is a heavy metal element naturally presented in the Earth’s crust. It is a common environmental pollutant occurring both naturally and as a result of industrial and agricultural activities (ATSDR 2012). Natural sources of Cd include volcanic activity, weathering of rocks, sea aerosols and forest fires. Anthropic origins of Cd include batteries, pigments, plastic stabilizers, pesticides and fertilizers, and photovoltaic devices, as well as rubber processing, galvanization, fossil combustion and waste incineration. Cadmium

compounds are soluble in water and can be taken up by plant roots and translocated to the edible parts where it accumulates. High amounts of Cd accumulate in tobacco leaves and tobacco smokers are exposed by inhalation (Ganguly et al. 2018). Among nonsmokers, ingestion of contaminated food is a major source of Cd exposure. Sixty-six per cent of the 137,200 food samples analyzed in 20 European countries contained 5% more than the maximum level of Cd (EFSA 2009). A tolerable weekly intake (TWI) for Cd is set at 2.5 µg/ kg b.w. (EFSA 2009). European adults' mean dietary exposure to Cd was close to this TWI, and subgroups, including vegetarians, children, and smokers, exceeded the TWI about 2-fold (EFSA 2009). Grain and cereal products, as well as fish and offal were the major contributors to human Cd exposure (Filipic et al. 2006; ATSDR 2012). Cd causes inflammation, apoptosis and oxidative stress in liver and kidney (Liu et al. 2015; Kim et al. 2015). Cadmium absorbed by inhalation and ingestion mainly accumulates in the liver and kidney, but the small intestine and lung may also be targets (Zhang et al. 2015; Tinkov et al. 2018). Cd increases the risk of cancer through oxidative stress, which damages DNA and inhibits DNA repair systems, and is consequently classified as a (group 1) human carcinogen (Bishak et al. 2015).

The intestinal epithelium is the first barrier against ingested chemicals and food contaminants. The gut barrier is formed to a large extent by intercellular junctions at the apical side of epithelial cells. These junctions seal the cells together and regulate the passage of ions and water across the epithelium (Suzuki 2013; Terciolo et al. 2019). Following ingestion of contaminated food, intestinal epithelial cells may be chronically exposed to contaminants such as DON and Cd. The effects of DON on the intestine are well described (Maresca 2013; Pinton and Oswald 2014). DON alters the intestinal structure, reduces the expression of several junctional proteins, reduces the barrier function, affects nutrient absorption, modulates intestinal microbiota and the local immune response (Pestka 2010a; Pinton and Oswald 2014; Payros et al. 2016). The effects of Cd on the intestine are less well documented, but recent studies show that it alters the gut microbiota, triggers a local inflammatory response and disrupts some tight junctions (Breton et al. 2016; Tinkov et al. 2018).

Human and animals are exposed to both compounds through ingestion of contaminated food and feed, especially cereals and cereal by-products (Arnich et al. 2012; Sirot et al. 2013). To the best of our knowledge, with the exception of one study (Le et al. 2018), the combined effects of these two contaminants have not been documented. The aim of the present study was to assess the individual and combined effects of DON and Cd on the intestinal barrier using *in vitro* and *in vivo* models. *In vitro,* the individual and combined effects of DON and Cd (0-30 µM) on Caco-2 cells were analyzed by measuring transepithelial electrical resistance (TEER),

paracellular permeability and the abundance of junctional proteins. *In vivo* experiments were performed to investigate the effects on intestinal histomorphology, the abundance and localization of junctional proteins in rats exposed to DON (8.2 mg/kg feed) and Cd (5ml/L of drinking water). Our *in vitro* and *in vivo* data on the parameters cited above demonstrated that the effects of different combinations of DON and Cd tested were comparable to those of the highest dose of each individual contaminant.

1.2 Materials and methods

1.2.1 Reagents

DON and $CdCl_2$ were purchased from Sigma (St Quentin Fallavier, France). For *in vitro* experiments, DON and $CdCl_2$ were dissolved in water and stock solutions (5 mM) were stored at -20 °C before dilution in complete cell culture medium. For *in vivo* experiments, DON was included in the rats' diet as previously described (Payros et al. 2016; Bracarense et al. 2017) while $CdCl_2$ was added to drinking water at a rate of 5 mg/L.

1.2.2 Cells

The Caco-2 cell line (Sigma, 86010202) was originally isolated from a primary colonic tumor in a 72-year-old Caucasian male. The cells were maintained in DMEM-Glutamax (Gibco, Life Technologies, Courtaboeuf, France) supplemented with 10% fetal calf serum (FCS) (Eurobio, Courtaboeuf, France), 1% non-essential amino acid (Sigma) and 0.5% gentamycin (Eurobio). Cells maintained in a humidified atmosphere at 37 °C with 5% CO_2 were passaged by trypsinization (0.5% trypsin in 0.5 mM EDTA) when they reached 80% confluency. Caco-2 cells were differentiated on 0.3 cm^2 polyethylene terephthalate membrane inserts with 0.4 µm pores (Corning Inc., Corning, NY, USA) for the assessment of transepithelial electrical resistance (TEER) and paracellular permeability, as previously described (Pinton et al. 2009; Pierron et al. 2016a).

1.2.3 Animals

The experimental protocol was carried out in accordance with European Guidelines for the Care and Use of Animals for Research Purposes and was validated by Toxcomethique Ethics Committee and the French Ministry of National Education Higher Education and

Research (TOXCOM/0142/PP). Three of the authors (DP, PP and IPO) have official authorization from the French Veterinary Services for animal experimentation.

Four-week-old male Wistar rats (140-170 g body weight), (Janvier Labs, Le Genest Saint Isle, France) were kept under the normal conditions with a 12-hour day/night cycle, 19-25 °C temperature, 50-70% humidity, at the Toxalim animal facility (INRA, UMR 1331, Toulouse) with *ad libitum* access to food and water throughout the study. After five days of acclimatization, the rats were divided into four groups of eight animals: control, exposed to DON contaminated-feed (8.2 mg DON/kg feed), exposed to Cd-contaminated water (5 mg $CdCl_2$/L) or exposed to both DON and Cd. No other mycotoxin was detected in the feed (supplementary table 1). Rats were weighed weekly. After four weeks, the animals were euthanized and the intestinal tissue was collected and fixed in 10% buffered formalin for histological assessment and immunohistochemical staining.

1.2.4 Transepithelial electrical resistance assay

Caco-2 cells grown on inserts differentiated and acquired an epithelial phenotype with polarity properties (apical and basolateral sides). They were treated apically with increasing concentrations of DON and Cd (0, 1, 3, 10, 30 μM) alone or combined. The TEER was measured every 4 h for 48 h using a cellZscope device (nanoAnalytics, Münster, Germany). Measurements were made on four replicates from four independent experiments.

1.2.5 Paracellular tracer flux assay

To assess paracellular flux, the 4-kDa fluorescein isothiocyanate-dextran (FITC-dextran) (Sigma), dissolved in cell culture medium, was added in the apical compartment (transwell final concentration 2.2 mg/mL) at 48 h post DON and/or Cd exposure. After 1 h of incubation, fluorescence was measured in the basal compartment (well of plate) with a microplate fluorimeter reader (Tecan, Lyon, France). The excitation and emission wavelengths were 490 and 520 nm, respectively. The background signal resulted from reagent-treated medium without cells. Measurements were made on four replicates from four independent experiments.

1.2.6 Western blot analysis of junctional proteins

Differentiated Caco-2 cells, cultured in 6-well plates, were treated with DON and Cd individually or in combination for 24 h to analyze junctional proteins. Three independent experiments were performed for each cell culture condition. After cell treatment, proteins were

extracted as previously described (Pinton et al. 2009) and separated on SDS-PAGE membranes probed with rabbit polyclonal Abs anti-occludin (#71-1500), -ZO-1 (#61-7300) (Thermo Fisher Scientific, Illkirch, France), -claudin-3 (#34-1700), -claudin-7 (#34-9100) (Invitrogen), rabbit monoclonal Ab anti-E-cadherin (Cell Signaling Technology, Leiden, The Netherlands), or mouse monoclonal Ab anti-claudin-4 (Invitrogen), diluted 1:250. Mouse monoclonal Ab or rabbit polyclonal anti-β-actin (Cell Signaling Technology, #3700 or #4967) was used as control (diluted 1:1000). Membranes were then washed and incubated with secondary antibodies CF680 goat anti rabbit IgG (#20067) or CF680 goat anti mouse IgG (#20065, diluted 1:10000) obtained from Biotium (Hayward, CA). Infrared fluorescence intensity of the specific bands was obtained with Odyssey Infrared Imaging System (LI-COR ScienceTec, Les Ulis, France). After correction for the background, the expression of the proteins was estimated after normalization calculated by the ratio of the intensity of the band of interest and of the β-actin band.

1.2.7 Immunofluorescence analysis

Differentiated Caco-2 cells cultured on glass cover slips were treated with DON and Cd individually or in combination for 24 h. The cells were fixed with 4% paraformaldehyde at 4 °C for 30 min and permeabilized with PBS containing 0.1% Triton X-100 for 10 min, then blocked with 10% goat serum at room temperature for 1 h. Occludin and E-cadherin were detected by incubation with rabbit polyclonal Abs anti-occludin (Thermo Fisher Scientific, #71-1500) and monoclonal anti-E-cadherin (Cell Signaling Technology, #3195) respectively. After three washes, the cells were incubated for 1 h with Alexa Fluor 546-conjugated goat immunoglobulin (1:500; Life Technologies, #A11035) raised against rabbit IgGs. Nuclei were stained with DAPI (Vector Laboratories) for 10 min. Cells were washed and mounted in Prolong® Gold antifade reagent (Invitrogen, Oregon, USA). Images were captured using an SP8 Leica confocal microscope and analyzed using FIJI software.

1.2.8 Histological and morphometric assessment of rat jejunum

The pieces of rat jejunum were fixed in 10% buffered formalin, embedded in paraffin and cut into 5 μm sections. The sections were stained with hematoxylin & eosin for histopathological evaluation. A lesional score, including the morphology of villi and enterocytes, interstitial edema, and lymph vessel dilation was designed to compare histological changes between different conditions as previously described (Pierron et al. 2018). Villus height

and crypt depth were measured randomly using a MOTIC Image Plus 2.0 ML analysis system (Motic Instruments, Richmond, Canada).

1.2.9 Immunohistochemical assessment of junctional proteins in the jejunum

After dewaxing and heat-induced antigen retrieval as described previously (Bracarense et al. 2012), paraffin-embedded 5 µm sections of rat jejunum were incubated overnight at 4 °C with the primary antibody anti-E-cadherin (Zymed, San Francisco, CA, USA) and anti-occludin *(Santa Cruz*. Biotechnology Inc., USA). The secondary antibody (SuperPicTure™ Polymer, Zymed) was applied followed by the addition of a chromogen (3,30-diaminobenzidine). Finally, tissue sections were counterstained with hematoxylin. The sections were then examined and the proportion of intestinal section expressing E-cadherin was estimated. Each sample was classified as having either normal or reduced staining.

1.2.10 Statistical analysis

The results are presented as the means ± standard error of mean (SEM) of independent experiments. Statistical analyses were performed using GraphPad Software (La Jolla, CA, USA). Significant differences between groups were analyzed by one-way ANOVA (non-parametric) with Bonferroni's multiple comparison test. $p < 0.05$ was considered statistically significant.

1.3 Results

1.3.1 Individual effects of DON and Cd on intestinal barrier function

Individual effects of DON and Cd were first assessed on two parameters that reveal the integrity of the intestinal epithelium: the TEER and the paracellular passage of dextran.

As shown in Figure 1A, both DON and Cd reduced the TEER of Caco-2 monolayers in a dose- and time-dependent manner. The TEER was not affected by 1 and 3 µM of either contaminants but was significantly decreased by 10 µM DON as early as 8 h after exposure (44% decrease, $p < 0.001$) and by 10 µM Cd after 12 h of exposure (40% decrease, $p < 0.001$). Treatment with 30 µM of DON or Cd reduced the TEER as early as 4 h (27%, $p < 0.05$), and the TEER decreased over time (82% and 87% decrease ($p < 0.001$) at 12 h respectively).

The permeability of Caco-2 monolayers to 4-kDa FITC-dextran was measured at the end of the experiment. As shown in Figure 1B, this parameter was not affected in cells exposed

to 1, 3 µM DON or 10 µM Cd. Higher doses of DON and/or Cd significantly increased the passage of FITC-dextran (18, 67 and 53-fold upon exposure to 10 µM DON, 30 µM DON and 30 µM Cd respectively).

These results show that both DON and Cd alter the intestinal barrier function as measured by decreased TEER and increased passage of dextran.

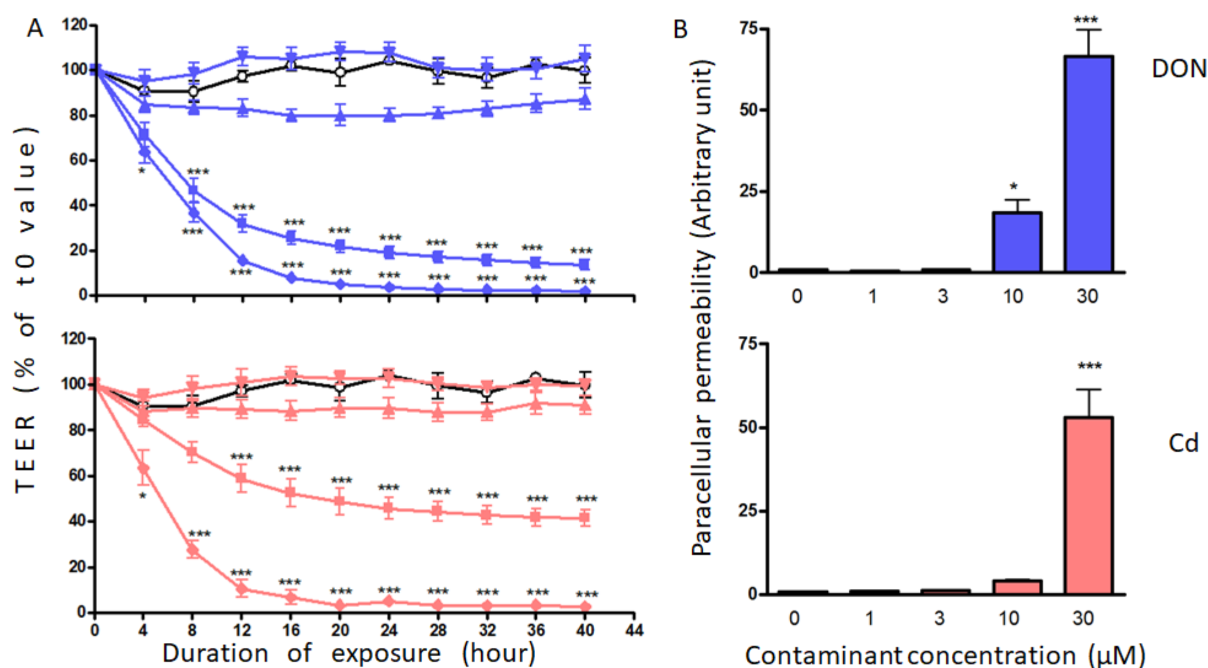


Figure 1. Effects of DON and Cd on TEER and paracellular permeability of differentiated Caco-2 cells.

(A) Effect on the TEER. Caco-2 cells were grown and differentiated on inserts and treated apically with DON (blue upper panel) or Cd (red lower panel) at 1 µM (inverted triangles), 3 µM (triangles), 10 µM (squares) or 30 µM (diamonds); control cells (circles, black line) were untreated. The TEER was recorded over a period of 40 h.

(B) Effect on paracellular permeability. At 48 h, 4-kDa FITC-dextran was added in the apical compartment and 1 h later, fluorescence was assessed in the basal compartment. Fluorescent intensity was measured and background levels subtracted.

Results, normalized to the controls, are expressed as the mean of 4 independent experiments ± SEM (*, $p < 0.05$; ***, $p < 0.001$). Data were analyzed by non-parametric one-way ANOVA

1.3.2 Individual effects of DON and Cd on the abundance of junctional proteins

Adherent and tight junctions belong to apical multiprotein complexes that link adjacent epithelial cells together and play an important role in the formation and the maintenance of the intestinal barrier (Suzuki 2013). Thus, the effect of DON and Cd on the abundance of several junctional proteins was examined.

As shown in Figure 2, DON induced a dose dependent decrease in the amount of occludin, E-cadherin and claudin-7. In the case of occludin, upon exposure to 10 and 30 µM DON, the decrease reached 54% ($p < 0.05$) and 66% ($p < 0.01$); for E-cadherin the reduction was 50% ($p < 0.05$) and 80% ($p < 0.001$) respectively. For claudin-7, a significant decrease was only observed at 30 µM DON (80%, $p < 0.05$). By contrast, exposure to DON at rates up to 30 µM, did not affect the amount of claudin-3, -4 and ZO-1.

Regarding Cd, a significant reduction in the abundance of occludin and E-cadherin was only observed upon exposure to 30 µM (58% ($p < 0.05$) and 70% ($p < 0.01$) decrease respectively), whereas claudin-3, -4, -7 and ZO-1 were not affected by the trace metal element.

These results suggest that DON and Cd impair the intestinal barrier function via a specific decrease in the abundance of E-cadherin and occludin.

1.3.3 Individual effects of DON and Cd on the distribution of junctional proteins

We next investigated the effect of DON and Cd on the distribution of junctional proteins in the Caco-2 cells. In control cultures, a well-defined band of membrane-associated immunofluorescence staining for E-cadherin and occludin was observed (Fig. 3A and B). Exposure to DON or Cd for 24 h caused significant changes in E-cadherin pattern as analyzed by fluorescence (white arrow) (Fig. 3A). The main modification caused by DON was reduced membranous expression of E-cadherin. At the same time, increased granular fluorescence was observed in the cytoplasm. After Cd exposure, the redistribution of E-cadherin into the cytoplasm was more visible. The loss of membranous expression with partial redistribution into the cytoplasm was also observed for occludin (white arrow) (Fig. 3B). The effects of DON and Cd on E-cadherin and occludin distribution were dose dependent.

Taken together, these results show that exposure to DON or Cd caused alterations in the cellular localization of E-cadherin and occludin.

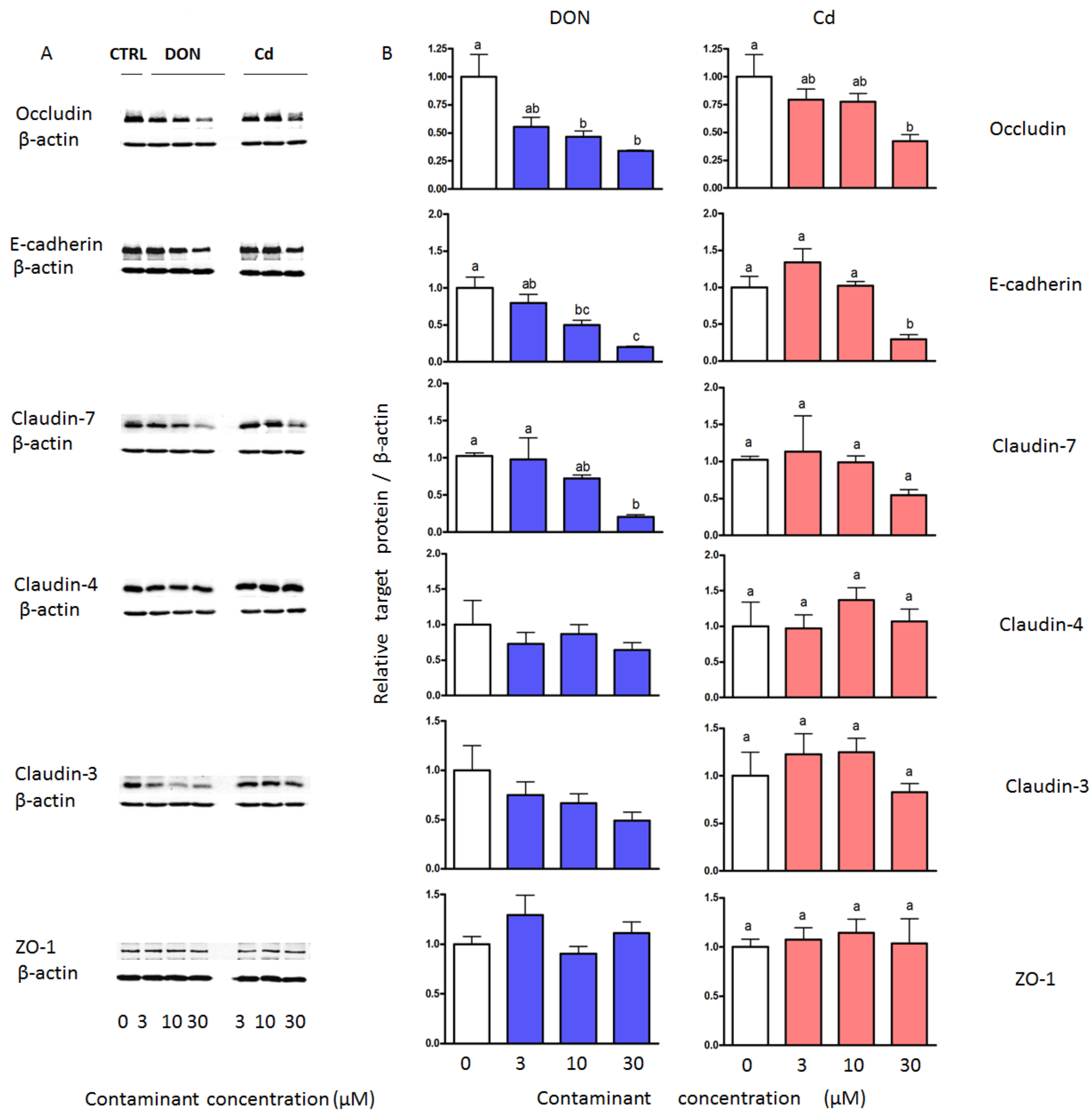


Figure 2. Effects of DON and Cd on the expression of junctional proteins in Caco-2 cells. Differentiated Caco-2 cells were exposed to different concentrations of DON and Cd for 24 h. (A) After extraction, proteins were analyzed by immunoblotting for claudin-3, 4, 7, occludin, E-cadherin and ZO-1.

(B) Protein expression was analyzed by densitometry and normalized to that of β-actin. Data are presented as means ± SEM of 3 independent experiments. Means with different letters differ ($p < 0.05$).). Results were analyzed by one-way ANOVA with Bonferroni's multiple comparison test.

A

CTRL | DON 3μM | DON 10μM | DON 30μM

E-cad

10 μm

10 μm

CTRL | **Cd 3μM** | **Cd 10μM** | **Cd 30μM**

B

CTRL | DON 3μM | DON 10μM | DON 30μM

Occludin

10 μm

10 μm

CTRL | **Cd 3μM** | **Cd 10μM** | **Cd 30μM**

Figure 3. Effect of DON and Cd on E-cadherin and occludin distribution in differentiated Caco-2 cells.

Differentiated Caco-2 cells were exposed to different concentrations of DON or Cd for 24 h. E-cadherin (A) and occludin (B) distribution (white arrow) was analyzed after immunostaining with specific antibody.

1.3.4 Combined effects of DON and Cd on intestinal barrier function

The data presented above indicated that individual exposure to DON or Cd has harmful effects on the intestinal barrier; however, in a mixture, their combined effects remain unknown.

The combined effects of DON and Cd on the TEER are summarized in Figure 4. In this heat-map, the first line and column show the individual effects of DON and Cd while the other cells show the effects of different combinations, with a color code indicating the strength of the effect. A time and dose dependent decrease in the TEER was observed when the toxins were present alone or in a mixture. Comparison of the combined effect with that of each individual compound revealed that after 8 h of exposure, the impact of Caco-2 cells exposed to 3 µM Cd combined with 10 µM DON was always stronger than that of 3 µM Cd alone ($p < 0.001$) but similar to that of DON alone. The same effects were observed for Cd 10 µM combined with DON (10 or 30 µM) ($p < 0.001$). Moreover, at 12 h of exposure, 10 µM Cd combined with DON (10 or 30 µM) also had a stronger impact than DON alone ($p > 0.05$). Conversely, 10 µM of Cd combined with 3 µM of DON led to a greater decrease in the TEER than DON alone ($p < 0.001$).These results were also observed when 30 µM Cd was combined with 10 µM DON ($p < 0.001$ from 8 h to 12 h and $p < 0.05$ from 24 h), except for 36 h. At 4 h, 24 h and 36 h, the effect of the combination of DON and Cd 30 µM was stronger than the effect observed with DON or 30 µM Cd alone ($p < 0.01$ at 4 h, 24 h and 36 h). These data show that, as far as the TEER is concerned, in most cases, the combination of DON and Cd had a similar effect to that of the individual contaminants.

The paracellular permeability to 4-kDa FITC-dextran was assessed on cell monolayers exposed to mixtures of DON and Cd for 48 h (Fig. 5). The results indicated that combinations of low doses of DON and Cd (1 to 3 µM) did not cause any significant changes in paracellular permeability except for the combination 3 µM DON and 10 µM Cd. In this case, the mixture had a stronger effect than the contaminant alone (DON, $p < 0.001$; Cd, $p < 0.05$). Moderate to high doses (10 to 30 µM) resulted in a marked increase in permeability to 4-kDa FITC-dextran. For example, the combination of DON and Cd at 10/10, 30/30, 10/30 and 30/10 µM increased the passage of dextran 46-, 82-, 76- and 81-fold, respectively, compared to untreated cells. Notably, 10 µM DON combined with 10 µM Cd resulted in a greater increase in paracellular permeability than 10 µM of Cd alone ($p < 0.001$). DON (10 µM) combined with 30 µM Cd had a stronger effect than 10 µM DON alone ($p < 0.001$). Similarly, 30 µM DON combined with 10 µM Cd led to a greater increase in paracellular permeability than 10 µM Cd alone ($p < 0.001$).

These data indicate that the combination of DON and Cd has a similar effect on the intestinal permeability to that of the individual contaminant.

DON (µM)

4h

Cd (µM)	0	1	3	10	30
0	100.0 ± 3.3	99.6 ± 3.9	93.3 ± 6.0	78.7 ± 8.7	70.3 ± 8.4
1	101.0 ± 4.8	103.0 ± 0.6	94.9 ± 10.0	--	--
3	97.5 ± 7.1	107.5 ± 7.1	89.2 ± 5.9	75.3 ± 6.1	--
10	93.2 ± 5.0	--	89.7 ± 6.9	# 71.6 ± 6.9	## 67.0 ± 6.2
30	77.0 ± 8.1	--	--	##/$$$ 41.3 ± 5.0	##/$ 41.6 ± 5.0

8h

Cd (µM)	0	1	3	10	30
0	100.0 ± 5.0	99.8 ± 1.9	88.7 ± 4.6	49.3 ± 9.5	39.0 ± 6.9
1	101.4 ± 9.1	104.3 ± 5.2	91. 0 ±6.0	--	--
3	95.4 ± 7.1	104.1 ± 3.5	79.5 ± 4.3	### 49.6 ± 5.7	--
10	78.5 ± 7.3	--	$$ 69.8 ± 4.4	### 37.6 ± 5.9	### 30.6 ± 3.1
30	29.4 ± 5.7	--	--	$$$ 8.1 ± 1.3	$$$ 10.7 ± 0.9

12h

Cd (µM)	0	1	3	10	30
0	100.0 ± 3.5	106.1 ± 4.4	85.6 ± 5.0	32.7 ± 6.3	15.8 ± 2.4
1	110.0 ± 1.3	105.5 ± 4.9	87.8 ± 5.4	--	--
3	92.0 ± 6.7	103.8 ± 3.2	# 74.0 ± 5.1	### 33.3 ± 4.0	--
10	60.5 ± 9.0	--	$$$ 54.5 ± 1.3	### 18.1 ± 4.8	### 10.2 ± 1.9
30	10.9 ± 6.9	--	--	$$$ 2.1 ± 0.5	$$$ 1.8 ± 0.6

24h

Cd (µM)	0	1	3	10	30
0	100.0 ± 2.4	102.0 ± 6.6	76.8 ± 3.8	18.0 ±3.3	3.4 ± 0.4
1	97.8 ± 3.7	103.6 ± 4.0	79.5 ± 1.6	--	--
3	86.0 ± 6.5	98.0 ± 7.1	### 65.0 ± 3.6	### 18.0 ± 3.3	--
10	43.9 ± 6.4	--	$$$ 38.7 ± 1.4	### 6.4 ± 3.1	### 1.6 ± 0.5
30	3.9 ± 2.7	--	--	$$ 1.5 ± 0.6	#/$$$ 1.0 ± 0.1

36h

Cd (µM)	0	1	3	10	30
0	100.0 ± 1.1	97.9 ± 6.7	82.6 ± 6.1	14.1 ± 3.0	1.97 ± 0.3
1	97.2 ± 4.0	102.6 ± 1.7	82.5 ± 3.4	--	--
3	89.2 ± 7.5	95.2 ± 8.8	### 68.2 ± 1.2	### 13.3 ± 2.8	--
10	40.6 ± 5.8	--	$$$ 34.0 ± 1.7	### 3.7 ± 1.9	### 1.1 ±0.3
30	2.7 ± 1.6	--	--	1.1 ±0.1	#/$ 1.0 ± 0.1

> 80 % | 60 % - 80 % | 40 % - 60 % | 20 % - 40 % | < 20 %

Figure 4 Heatmap showing the effects of single and binary combinations of DON and Cd on the TEER of Caco-2 monolayers after 4 h, 8 h, 12 h, 24 h and 36 h of exposure. At each time-point, the TEER of control untreated cells was considered as 100%. Results are expressed as the mean of 4 independent experiments ± SEM (#, combined effects VS effects of Cd alone, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$. $, combined effects VS effects of DON alone, $p < 0.05$; $$, $p < 0.01$; $$$, $p < 0.001$). Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test. The intensity of the color reflects the strength of effect on the TEER.

Cd (μM) \ DON (μM)	0	1	3	10	30
0	1.0 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	18.4 ± 6.1	66.7 ± 10.7
1	1.1 ± 0.2	0.6 ± 0.2	1.2 ± 0.2	--	--
3	1.3 ± 0.2	1.0 ± 0.1	$ 1.2 ± 0.1	18.8 ± 5.4	--
10	4.2 ± 0.8	--	##/$$$ 8.1 ± 0.7	### 46.2 ± 5.3	### 81.5 ± 18.5
30	53.0 ± 10.2	--	--	$$$ 75.8 ± 20.1	81.7 ± 17.9

< 20	20 - 40	40 - 60	60 - 80	> 80

Figure 5 Heatmap showing the effects of single compound and binary combinations of DON and Cd on the paracellular permeability of Caco-2 monolayers after 48 h of exposure. After 1 h of incubation with FITC-Dextran, the intensity of fluorescent was measured in the basal compartment. Results are expressed as fold increase in fluorescent intensity relative to controls. Results are expressed as the mean of 4 independent experiments ± SEM. (#, combined effects versus effects of Cd alone, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$. $, combined effects VS effects of DON alone, $p < 0.05$; $$, $p < 0.01$; $$$, $p < 0.001$). The depth of color reflects the strength of the effect on permeability. Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

1.3.5 Combined effects of DON and Cd on junctional protein abundance

To investigate the combined effect of DON and Cd on the intestinal barrier function in more detail, the abundance of two junctional proteins, occludin and E-cadherin, was analyzed. Figure 6 is a heatmap of the individual and combined effects of DON and Cd. The levels of the two proteins decreased continuously with increasing concentrations of combined compounds. The combination of DON and Cd at a rate of 3 µM induced a similar decrease in occludin and E-cadherin as 3 µM of DON or 3 µM of Cd alone. Only 10 µM Cd combined with 10 µM DON led to a bigger decrease in occludin and E-cadherin abundance than 10 µM of Cd alone ($p < 0.001$). When the cells were exposed to a combination of 30 µM DON and 30 µM Cd, the levels of occludin and E-cadherin were lower than the level observed in cells only exposed to DON or Cd alone (DON, $p < 0.001$; Cd, $p < 0.05$). The data show that except for very high concentrations, the effect of a combination of DON and Cd on the abundance of occludin and E-cadherin was similar to the effect of the individual compounds.

1.3.6 *In vivo* effects of DON and Cd alone or in combination on the histomorphometry of the jejunum

Given the impact of DON and Cd on intestinal barrier function on Caco-2 cells, experiments were also performed on animals. Rats were exposed to DON-contaminated feed (8.2 mg/kg), Cd-contaminated water (5 mg/L) or both. The individual and combined effects of these contaminants were assessed on the intestine after 4 weeks of exposure.

The animals' body weight gain was not affected in any of the conditions (Fig. 7). Histomorphometrical changes were analyzed in the jejunum of rats fed DON and Cd alone or in combination. These results showed moderate but significant lesions compared to the intestine of control rats. The main histological changes were atrophy and fusion of the villi. Interstitial edema and enterocyte apical flattening were also observed (Fig. 8A, B, C, D). Lesional scores were about 4-fold higher in rats exposed to DON, Cd or both than that in control rats (Fig. 8E). These results indicate that intestinal damage caused by the combination of DON and Cd were as severe as the damage caused by each contaminant individually.

The *in vivo* effects of the mycotoxin and the heavy metal on intestinal cell proliferation were also assessed by measuring villus height and crypt depth. A significant reduction in crypt depth was observed in the jejunum of rats exposed to the contaminants compared with control animals (Fig. 8F). The reduction in crypt depth in animals exposed to both DON and Cd was similar to the reduction observed in animals exposed to Cd alone but slightly greater than the

reduction observed in the animals only exposed to DON (−16%, $p < 0.01$). Villus heights did not differ significantly whatever the group of animals considered (Fig. 8G).

Taken together, these results indicate that ingestion of a combination of DON and Cd induces similar or slightly bigger lesions and has greater histomorphological effects on the intestine than ingestion of the individual contaminants.

1.3.7 *In vivo* effects of DON and Cd alone or in combination on the abundance of E-cadherin and occludin in the jejunum

As DON and Cd significantly reduced the amount of E-cadherin and occludin in Caco-2 cells, their abundance was also assessed in the jejunum of rats exposed to the same contaminants. Immunohistochemical staining revealed a significant reduction in E-cadherin in all exposed animals (Fig. 9). In control rats, strong homogeneous immunostaining was observed at the intercellular borders of epithelial cells (Fig. 9A). In rats exposed to either DON or Cd, the intensity of E-cadherin staining was significantly reduced (Fig. 9B, C). In rats exposed to the mixture, weak heterogeneous staining was also observed (Fig. 9D). The abundance of E-cadherin decreased by 57%, 49% and 71% in animals treated with DON, Cd, and DON+Cd respectively (Fig. 9E).

For occludin, strong homogeneous immunostaining at the cell membrane was observed in control rats, as well as in rats exposed to Cd (Fig. 10A, C). By contrast, in the jejunum of animals exposed to DON and to the combination of DON and Cd, weak heterogeneous staining was observed (Fig 10B, D) compared to that in control rats, the abundance of occludin decreased by 84% and 62% ($p < 0.001$), respectively (Fig. 10E).

These results indicate that exposure to either DON or Cd reduces the expression of the E-cadherin and occludin at the cell membrane. Exposure to the mixture induced the same reduction in junctional protein as ingestion of DON alone.

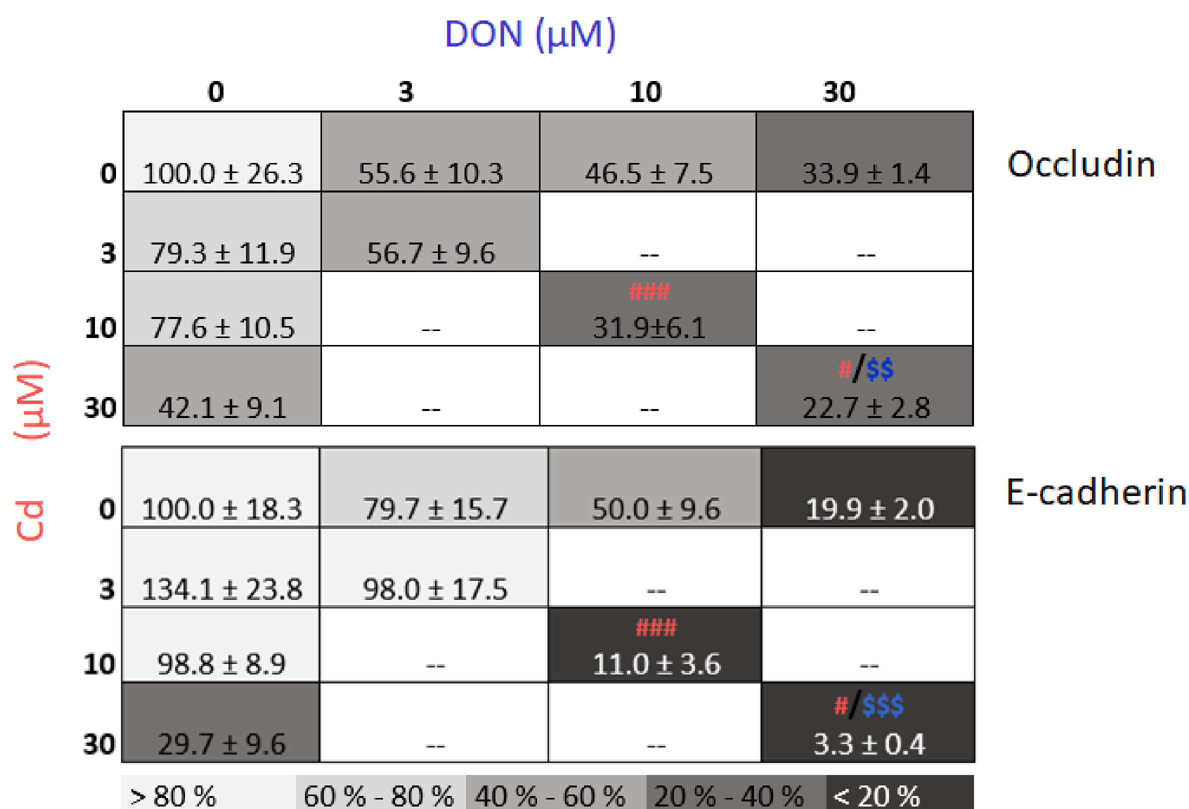


Figure 6. Heatmap showing the effects of individual compounds and binary combinations of DON and Cd on the expression of junctional proteins after 24 h of exposure. Results are expressed as means of 3 independent experiments ± SEM. (# combined effects versus effects of Cd alone, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$. $, combined effects versus effects of DON alone, $p < 0.05$; $$, $p < 0.01$; $$$, $p < 0.001$). The intensity of the color reflects the extent of the decrease in the junctional proteins. Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

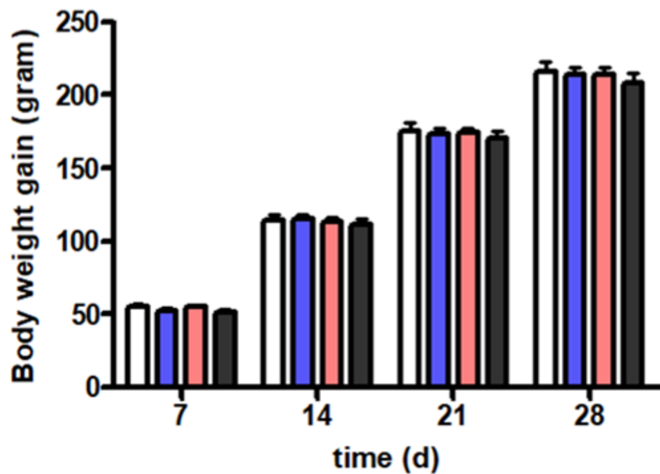


Figure 7. Individual and combined effects of DON and Cd on body weight gain.

Male Wistar rats were divided into four groups: control (white column), exposed to 10 mg/kg DON-contaminated feed (blue column), exposed to 5 mg/L Cd-contaminated feed (red column) or exposed to both DON and Cd (black column). Values are mean ± SEM (n=8 animals). Data were analyzed by non-parametric one-way ANOVA.

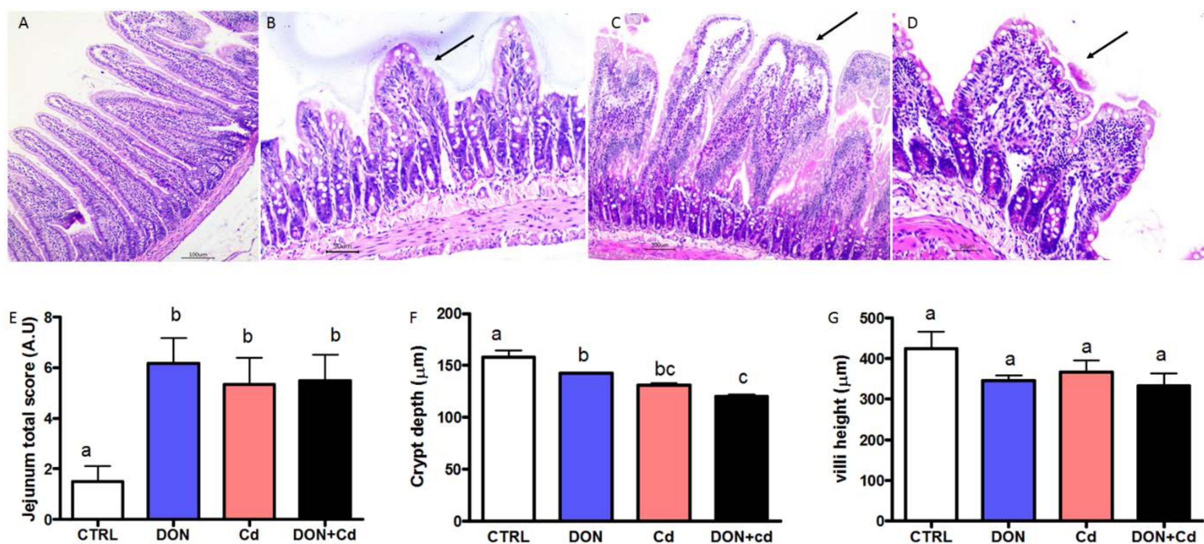


Figure 8. Individual and combined effects of DON and Cd on jejunum histology. Histology of the jejunum after hematoxylin–eosin staining: control rat (A), DON treated rat (B, villus atrophy and fusion, arrow; 20 X), Cd treated rat (C, moderate interstitial edema and enterocyte flattening, arrow; 10 X) and DON + Cd treated rat (D, villus atrophy and fusion, arrow; 20X). Lesional score (E); crypt depth (F) and villus height (G). Values are means ± SEM (n=8). Mean values with different letters differ ($p < 0.05$). Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

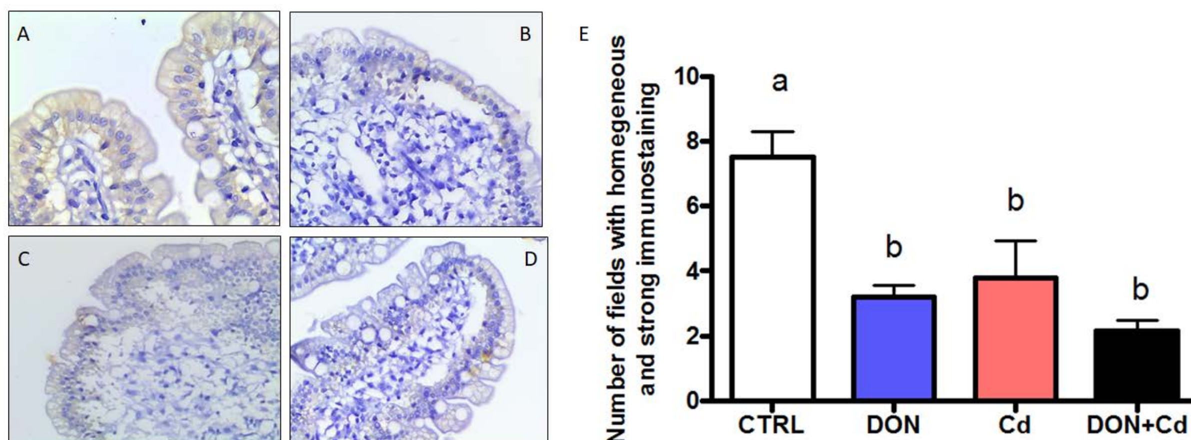


Figure 9. Individual and combined effects of DON and Cd on E-cadherin expression in jejunum. (A, 60x) Jejunum of rats in the control group showed strong homogeneous staining on E-cadherin; Less intense immunostaining on E-cadherin occurred in the jejunum of rats in the groups treated with DON (B, 40x), Cd (C, 40x) and DON + Cd (D, 40x). (E) The number of fields with strong homogeneous immunostaining. Values are mean ± SEM (n=8). Means with a different letter differ ($p < 0.05$). Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

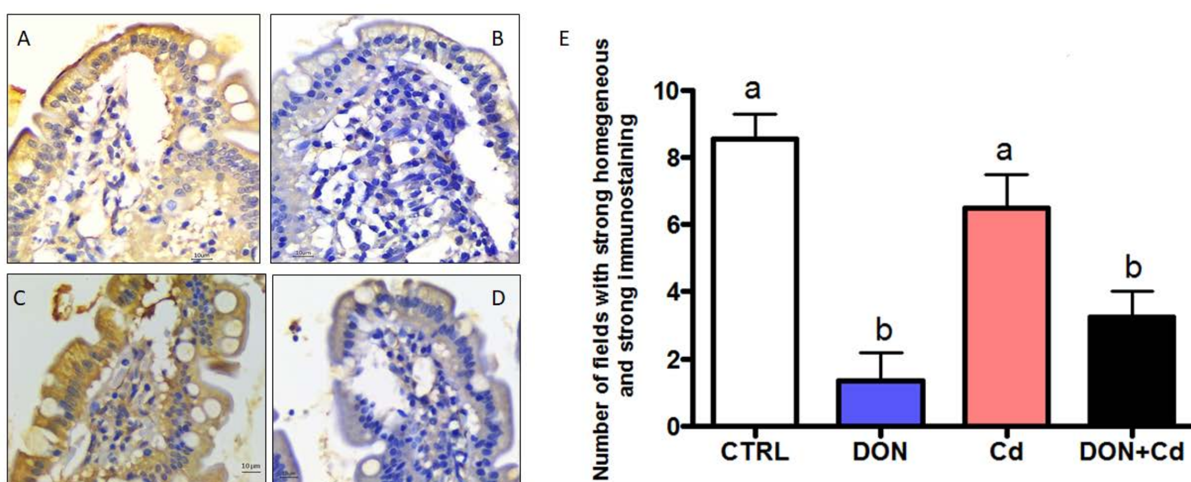


Figure 10. Individual and combined effect of DON and Cd on occludin expression in jejunum. (A, 60x) The jejunum of rats in the control group showed strong homogeneous staining on occludin; Less intense immunostaining on occludin occurred in the jejunum of rats in the groups treated with DON (B, 40x), Cd (C, 40x) and the combination of DON and Cd (D, 40x). (E) The number of fields with strong homogeneous immunostaining. Values are mean ± SEM (n=8). Means with a different letter differ ($p < 0.05$). Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

1.4 Discussion

The combined toxicity of pollutants is hard to predict based on the toxic effect of a single compound (Alassane-Kpembi et al. 2017b; More et al. 2019). In the last few years, the number of studies of the combined toxicity of pollutants has increased but knowledge of the impact on animal and human health of exposure to mixtures of pollutants is nevertheless still very poor (Silins and Högberg 2011b). The main objective of the present study was to investigate the effects of a mycotoxin, DON, and a heavy metal, Cadmium, alone and in combination, on the intestinal epithelial barrier function. Two experimental approaches were used: *in vitro* exposure of human intestinal epithelial cells (Caco-2) to DON and Cd alone or in combination, and the *in vivo* dietary exposure of rats to these contaminants. The range of concentrations tested *in vitro* (1 to 30 µM) was chosen to screen a large panel of plausible exposure and adverse effects. In human, assuming that DON consumption in one meal is diluted in 1 L of gastrointestinal fluid and is totally bioaccessible (Sergent et al. 2006). The doses used in this study correspond to 0.296, 0.89, 2.96 and 8.89 µg/ml, which lay in the range of the plausible intestinal concentrations of DON (0.25-10 µg/ml) (De Walle et al. 2008). Furthermore, as previous report indicates that the molar ratio of mean exposure to DON and Cd in food in Europe is 1:1 (Le et al. 2018), the doses chosen for Cd were in the same range of doses as DON. The recommendation for DON in feed intended for farm animals is 5 mg/kg feed (European Union 2006b). In our experiment, the concentration of DON in feed was 8.2 mg/kg. Indeed, the concentrations of 2-10 mg/kg feed of DON are the most frequently used in toxicological studies of farm and experimental animal models and demonstrate adverse effects in specific target organs (intestine, liver, kidney) (Pinton et al. 2008, 2009; Bracarense et al. 2012). The concentration of 5 mg/L CdCl2 in drinking water is an environmentally realistic low dose (Brzóska et al. 2003; Benoff et al. 2008). It led only to modification of the phenotype of innate immune cells in the mesenteric lymph nodes in a rat model (Ninkov et al. 2015). Our proposal was to investigate the potential increase of the adverse effects when cadmium was in combination with another toxic compound. We took into account that the molar ratio of mean exposure of European population to DON and Cd in food is estimated to be 1:1 (Le et al. 2018). Considering the daily ingestion of food and water by the rat and the content of DON in food, the concentration of 5 mg of Cd /L of drinking was in accordance to reach this realistic ratio.

To evaluate the effects of DON and Cd alone or in combination on the intestine *in vivo*, rats were exposed to the contaminants for four weeks. The body weight gain of animals was

not affected in any of the conditions tested. However, alterations in the histology and the morphology of the intestine were observed after exposure to DON and Cd alone or in combination. A significant decrease was observed in crypt depth in the jejunum of rats exposed to DON, Cd and DON+Cd. The effect of DON on crypt depth has already been reported in pigs (Gerez et al 2015) and might be due to the DON-induced ribotoxic stress that compromised protein synthesis and triggered apoptosis (Pestka 2010a; Pinton et al. 2010; Pierron et al. 2016b). In piglets exposed to DON, a decrease in the number of proliferating cells in the intestinal crypt in response to DON has been already observed (Bracarense et al. 2012; Gerez et al. 2015). A study on broiler chickens reported that Cd ingestion caused histological alterations such as a decrease in leaf-like villi and a reduction in crypt depth (Teshfam et al. 2006). Moreover, inhibition of proliferation and induction of apoptosis has been observed in human renal mesangial cells and subventricular neural stem cells exposed to Cd. These effects were mediated by activation of the JNK or p-38 pathway (Wang et al. 2017). It is thus tempting to hypothesize that Cd inhibits the proliferation of intestinal epithelial cells via the same MAPK-dependent mechanism. By contrast, villus height was not affected irrespective the contaminant to which the rats were exposed. This suggests proliferating cells are more sensitive to DON and/or Cd than differentiated ones, as already described *in vitro* for DON (Bony et al. 2006; Pierron et al. 2016b).

The histological and morphological alterations induced by the two contaminants tested here suggest that the intestinal barrier function may also be impaired. A dose-dependent reduction in the TEER and an increase in paracellular permeability were observed in DON and in Cd treated cells. The reduction in the barrier function caused by DON is mediated by MAPKs / ERK activation, as inhibition of this signaling pathway restored TEER in porcine and human intestinal epithelial cells (Pinton et al. 2010; Springler et al. 2016; Du et al. 2018). A MAPKs / ERK activation in the differentiated Caco-2 cells treated with Cd has also been reported (Mantha and Jumarie 2010), and may be involved in the disruption of the intestinal barrier by Cd.

The intestinal barrier function is closely linked to the junctional protein network and several studies have shown that DON or Cd alter some junctional proteins such as occludin or E-cadherin (Pinton et al. 2009; De Walle et al. 2010; Templeton and Liu 2013). However, the effects of combinations of DON and Cd on the junctional proteins have not previously been investigated. Here, we observed a decrease in E-cadherin expression in the three groups of exposed rats (DON, CD and DON+ Cd) while the decrease in occludin was only observed in animals exposed to DON (with or without Cd). By contrast, in Caco-2 cells, the amount of E-

cadherin and occludin decreased after exposure to the two contaminants (DON and Cd) alone or in combination. This was associated with redistribution of the junctional proteins from the membrane to the cytoplasm. A similar decrease in the expression of E-cadherin has already been observed in pigs fed a DON-contaminated diet (Bracarense et al. 2012; Pierron et al. 2018). As far as Cd is concerned, several studies have shown that, in intestinal cells, intercellular junctions, mainly E-cadherin and actin cytoskeleton, are sensitive to this contaminant (Duizer et al. 1999; Rusanov et al. 2015). In MCF-7 cells, Cd has been shown to disturb calcium homeostasis and cause a depletion of Ca^{2+} (Zhou et al. 2015); this induces the activation of Cdc42, a member of Rho family GTPases, and leads to the ubiquitination and degradation of E-cadherin via Src-mediated pathway (Shen et al. 2008). In a human airway tissue model, Cd induced redistribution of occludin by tyrosine phosphorylation through c-Src and PKC activation, resulting in TJ disruption (Cao et al. 2015). Whether these modes of action of Cd also occur in the intestine remains to be determined. Disruption of junctional adhesion associated with increased paracellular permeability can facilitate the passage of pathogens and/or harmful substances into the body. Therefore, animals or humans exposed to these toxins are likely to be more sensitive to intestinal pathogens. In addition, increased intestinal permeability may also favor permeability to other contaminants (Payros et al. 2017).

Humans and animals are exposed to multi-component mixtures in the environment and food, whereas chemical risk assessment generally concerns the toxicity of single compounds. The effects of combinations of several food contaminants are very poorly documented. In the case of DON and Cd, apart from a previous study by our team (Le et al. 2018), no study investigated their combined effect. The *in vitro* and *in vivo* data presented in this paper indicate that, whatever the parameter analyzed (TEER, paracellular permeability or the abundance of junction protein), the toxicity resulting from exposure to a combination of DON and Cd is similar to that caused by each individual contaminant. It is well known that chemical compounds have multiple modes of action, a range of targets and different degrees of affinity for these targets, and, conversely, that each mode of action can be used by a variety of chemical compounds (Thrupp et al. 2018). As described above, the mechanisms of action of DON and Cd share some pathways, one of which is the MAPkinase pathway. Indeed, DON and Cd induce the activation of ERK1/2 and p38 respectively, leading to disruption in tight junctions (Pinton et al. 2010; Rather et al. 2017). We hypothesize that, when combined, DON and Cd play competitive roles in the pathway, which would explain why the effect of a mixture of DON and Cd is no greater than the effect of each individual contaminant.

From the point of view of human health, our results suggest that existing regulation for DON and Cd are sufficient to protect consumers exposed to a mixture of these contaminants. Indeed, their combined effects on the intestine were identical to the effect of the most toxic compound alone, whatever the parameter analyzed. This points to a less than additive effect. More data are needed to determine if the results concerning the effect of DON and Cd on the intestine can be extended to other organs and/or to other mixtures.

Acknowledgments

We are grateful to the staff of the team EZOP for animal care. We thank the UPAE (*Unité de Preparation des Aliments Expérimentaux*, UE03000, Jouy-en-Josas, France) for the preparation of the diets. The authors thank D. Goodfellow for English editing.

Funding: the work was supported, in part, by the French National Research Agency (project CaDON 15-CE21-0001-02) and by the CAPES Cofecub SV 947 19.

Su Luo was supported by the China Scholarship Council.

2. Specific intestinal toxicity of deoxynivalenol and cadmium: analysis on pig jejunal explants

Specific intestinal toxicity of deoxynivalenol and cadmium: analysis on pig jejunal explants

Su Luo, Sylvie Puel, Isabelle P. Oswald and P. Pinton[†]

Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, 180 chemin de Tournefeuille, 31027 Toulouse cedex 3, France

SUMMARY

Deoxynivalenol (DON) is the most prevalent mycotoxin naturally present in grains and other commodities. It is produced by toxigenic fungus *Fusarium* species that are common pathogens of cereal crops under temperate climate. In pigs, chronic exposure to DON reduces feed consumption and weight gain, induces neuro-endocrine changes, and alters intestinal and immune functions. Cadmium (Cd) is one of common and widespread toxic heavy metals found naturally in the Earth's crust. Its presence in the environment is a consequence of both natural and anthropic processes. Cd can be released into soil, water and air. It is soluble in water and can be uptake by plant roots and accumulate into the edible parts especially cereal products. Chronic intoxication with Cd may result in various organs damage especially kidney. Through its high consumption of cereals, pigs can be exposed to both DON and Cd. Using pig intestinal explants this study was designed to analyze their intestinal toxicity when present alone or in combination. Jejunal explants were treated with increasing concentration of both contaminants for 4 hours and the expression of 17 genes targeting the immune response (IL-1β, IL-1α, IL-8, IL-10, IL-17a, IL-22, IFN-γ, TGF-β, TNF-α), and the oxidative stress (NF-κB, MT1A, MT2A, CCS, SOD1, SOD2, CAT, DUOX) was analyzed by RT-qPCR. Our data confirm the inflammatory effect of DON with induction of the expression of genes encoding for IL-1β, IL-1α, IL-8, and TNF-α. Exposure of pig explants to Cd induced the gene expression of metallothioneins (MT1A, MT2A) but did not have any effect on the inflammatory genes. When DON and Cd were present together, an increased expression of both inflammatory genes and metallothioneins genes was observed. Taken together our data demonstrate a specific intestinal effect of DON and Cd and suggest that these contaminants do not interact at the intestinal level.

[†] Corresponding author : philippe.pinton@tolouse.inra.fr

INTRODUCTION

The trichothecene deoxynivalenol (DON), known colloquially as "vomitoxin", is a secondary metabolite mainly produced by the *Fusarium* genus. DON and other trichothecene mycotoxins are commonly found worldwide in cereals including wheat, barley, oat, rye, and corn, which is an increasing food safety concern in the world [Pestka, 2010; Payros et al., 2016; Placinta et al., 1999]. Human and animals are exposed to these mycotoxins by consumption of contaminated cereals leading to health problems. DON causes gastroenteritis with some primary symptoms of nausea, diarrhea and vomiting. Apart from gastroenteritis, DON exposure induces growth effects and anorexia, immune dysregulation, reproductive and teratogenic effects in experimental animals [Pestka, 2010; Smith et al., 2012].

In recent decades, amount of researches carried out on swine have demonstrated that it is the most susceptible animal to DON. The reason maybe that DON absorption is very rapid in swine and it reaches peak plasma concentration rapidly [Pestka et al., 2005; Prelusky et al., 1998]. Chronic ingestion in piglets of DON at concentration of 3 mg/kg of feed induced morphological and histological changes in jejunum, altered the gene expression of inflammatory cytokines and the serum biochemical profile and simultaneously altered the integrity of the intestinal barrier [Bracarense et al., 2012; Wu et al., 2015]. At lower concentration (0.9 mg/kg feed), a short-term exposure to piglets negatively affected weight gain, induced histomorphological alterations in the jejunum and affected, the mRNA expression of different tight junction proteins, of inflammatory and of oxidative stress marker, along the intestine [Alizadeh et al., 2015].

Cadmium (Cd) is one of the most toxic heavy metal pollutants. Due to its high mobility in soil, plant, and water systems, it is widely distributed in the environment [Manquián-Cerda et al., 2016]. The soluble form of Cd in water can be uptaken by plant roots and accumulated into the edible parts especially of cereals. The Cd concentration in crops increases with the increasing Cd concentration in soil [Jones et al., 1992; Andersson et al., 1985], causing concern for the long-term dietary intake of Cd. In addition, Cd is carcinogen [Järup & Akesson, 2009; Joseph, 2009]. Exposure to Cd can damage various organs, such as heart, lung, liver, kidney and bone. Kidney is considered to be the main target of Cd-induced toxicity [Włostowski et al., 2008; Wang et al., 2011]. The well-known effect caused by Cd is "Itai-Itai" disease in Japan [Ishihara et al., 2001].

Swine is one of the main experimental animals for the study of cadmium toxicity. Some reports have shown that there was a marked correlation between cadmium levels in porcine kidney and cadmium in feed [Wu et al., 2012; Grawé et al., 1997]. Human is at the top of food chain, therefore an increase Cd concentration in pig might be an indicator of an

increase in human Cd exposure [Grawé et al., 1997]. Kidney and liver are the two dominating organs where Cd distributes to and accumulates in, and where it exerts toxicity [Grawé et al., 1997; Hoogenboom et al., 2015; Tomović, et al., 2011]. Ingestion of food and water contaminated by Cd reduced the number of lymphocytes, affected the serum biochemical profile [Du et al., 2013].

As described above, the main source of Cd and DON exposure is the diets. Hence the intestine could be the target organ for both ingested Cd and DON [Pestka, 2010; Smith et al., 2012; Jones et al., 1992; Ninkov et al., 2015]. In addition, swine is susceptible to these contaminants. Thus, it is a good way to use a pig intestinal explants model to explore the combined toxicity of Cd and DON. This *ex vivo* model helps to reduce the number of experimental animals and preserves the normal morphological structure of intestine [Nietfeld et al., 1991]. Previous studies have used the pig jejunal explants to explore the gastrointestinal toxicity of DON [Lucioli et al., 2013; Pierron et al., 2016], and to analyze the toxicity and the interaction types of mixtures of different mycotoxins [Basso et al., 2013; Alassane-Kpembi et al., 2017].

Our aim was to analyze for the first time the toxic effects of Cd and DON, alone or in combination, as well as the interaction types between them in the intestine. We assessed the dose-dependent effect of DON and Cd on the expression of different genes by quantitative real-time polymerase chain reactions.

MATERIAL AND METHODS

Animals

Six crossbred piglets (Landrace × Large White × Duroc), 5 week-old were housed in the animal facility of the INRA ToxAlim Laboratory (Toulouse, France) and used for explants experiments and. The experimental procedures were conducted in accordance with European Guidelines for the Care and Use of Animals for Research Purposes and were approved by the Ethics Committee of Pharmacology-Toxicology of Toulouse-Midi-Pyrénées (TOXCOM/0163 PP-APAFiS#6303). Animals were fed *ad libitum* prior to the experiment.

Toxins

DON and Cd were obtained from Sigma (St Quentin Fallavier, France). DON and Cd stock solutions (5 mM concentration) were prepared in sterile water and stored at −20 °C. Working dilutions were prepared in William's medium E (Sigma). The concentration range of DON and Cd were 0–24μM and 0–96 μM respectively. Explants were also exposed to a combination of DON and Cd at a ratio 1 DON/1 Cd in the range of doses 0/0 to 24/24 μM.

Culture of explants and exposure to DON and Cd

Jejunal explants were obtained immediately after euthanasia as previously described [25]. Fragments of 5 cm of middle jejunum were washed 4-6 times with phosphate buffered saline (PBS) and opened longitudinally with sterile scissors. Explants were collected by a biopsy punch (6 mm). Three explants were deposited, villi upward, on biopsy sponges in six-well plates containing control or DON-, Cd-, and DON/Cd-contaminated medium. All these operations were achieved in 1 h after the piglets were euthanized. Plates were incubated at 39 °C, 5% CO_2 for 4 h. Then explants were stored at -80 °C before transcriptional analysis

RNA extraction

Jejunal explants were lysed in 1 mL of Extract All reagent (Eurobio, Les Ulis, France) with ceramic beads (Bertin Technologies, St. Quentin en Yvelines, France). Total RNA was extracted as previously described [26,28]. The RNA concentration was measured by spectrophotometric analysis with Nanodrop ND1000 (Labtech International, Paris, France). The quality of RNA was determined by capillary electrophoresis with Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA).

Quantitative real-time polymerase chain reaction (RT-qPCR) analysis

The RT-qPCR were performed using total RNA samples (n = 6 per treatment group) as previously described[26]. Non-reverse-transcribed RNA was used as non-template control for verification of absence of genomic DNA amplification signal. The specificity of the qPCR products was assessed at the end of the reactions by analyzing the dissociation curves. Primers were purchased from Sigma. The list of primers and corresponding genes is presented in table 1. Amplification efficiency and initial fluorescence were determined by LinRegPCR(2014.x) for Real Time-PCR method [29]. Then values obtained were normalized by both house-keeping genes, β2-microglobulin and ribosomal protein L32(RPL32), which were chosen with NormFinder software [30]. Finally, gene expression was expressed relatively to the control group as already described [25].

Statistic analysis

All data was analyzed by R program (version 3.3.2, 31.10.2016). One-way ANOVA was used to analyze the significance when data exhibited normal distribution. Otherwise, Kruskal test was used, followed by Tukey HSD (multi-comparison). A p-value <0.05 was considered significant. Graphs were made by GraphPad Prism 4.

Table 1. Primer sequences

Gene symbol	Gene name	Primer sequence	Accession number and reference
IL-1β	Interleukin 1-beta	F : GAGCTGAAGGCTCTCCACCTC R :TTGTTGCTATCATCTCCTTGCAC	NM_001005149[28]
IL-1α	Interleukin 1-alpha	F :TCAGCCGCCCATCCA R :AGCCCCCGGTGCCATGT	NC_010445.3[31]
TNF-α	Tumor necrosis factor-alpha	F : ACTGCACTTCGAGGTTATCGG R : GGCGACGGGCTTATCTGA	NM_214022[31]
IL-8	Interleukin 8	F :GCTCTCTGTGAGGCTGCAGTTC R :AAGGTGTGGAATGCGTATTTATGC	NM_213867[28]
MT-1A	Metallothionein 1A	F :GCAAATGCACCTCCTGCAAG R:CAGCCCTGGGCACACTTG	ENSSSCT00000031974.1 (This study)
MT-2A	Metallothionein 2A	F:CTGCAAATGCAAAGAGTGCAAA R:ACACTTGGCACAGCCCACA	ENSSSCT00000029059.1 (This study)
RPL32	Ribosomal Protein L32	F :AGTTCATCCGGCACCAGTCA R :GAACCTTCTCCGCACCCTGT	NM_001001636[32]
β2-Microglob	β2-Microglobulin	F: TTCTACCTTCTGGTCCACACTGA R: TCATCCAACCCAGATGCA	NM_213978[32]

RESULTS

Individual or combined effect of DON and Cd on the expression of cytokine genes

Cytokines are very important in cell signaling and play a key role in immunomodulatory process. In order to determine the individual or combined effect of DON and Cd on the intestinal immunity, the mRNA expression of nine genes involved in inflammation (IL-1β, IL-1α, IL-8, IL-10, IL-17a, IL-22, IFN-γ, TGF-β, TNF-α) was analyzed by RT-qPCR.

The results showed that the expression levels of four inflammatory factors (IL-1β, IL-1α, IL-8, and TNF-α) were significantly elevated in the explant treated with DON at doses above 3μM (IL-1β, IL-8) or 24 μM (IL-1α, TNFα) (Fig.1). By contrast, in the Cd treatment groups, the expression levels of these genes were not affected (Fig. 1). In the explants exposed to the combination DON/Cd (ratio 1/1), the mRNA expression levels of these genes gradually increased in a dose-dependent manner, and the difference between treatment groups and control group became significant with doses higher than 6 μM, except IL-1α (Fig. 1).

In terms of genes associated with oxidative stress (NF-κB, MT1A, MT2A, CCS, SOD1, SOD2, CAT, DUOX), we only observed an increased expression of metallothioneins (MT1A, MT2A). The expression of these two genes increased with the increasing doses of Cd, and was statistically different from the control group at 24 μM. (Fig.2). By contrast, DON did not altered the expression of metallothioneins (Fig. 2). When explants were exposed to the two contaminants simultaneously, the gene expression levels increased in a dose-dependent manner, and the difference between

treatment and control groups occurred from a dose of 6 μM (ratio 1/1) (Fig.2).

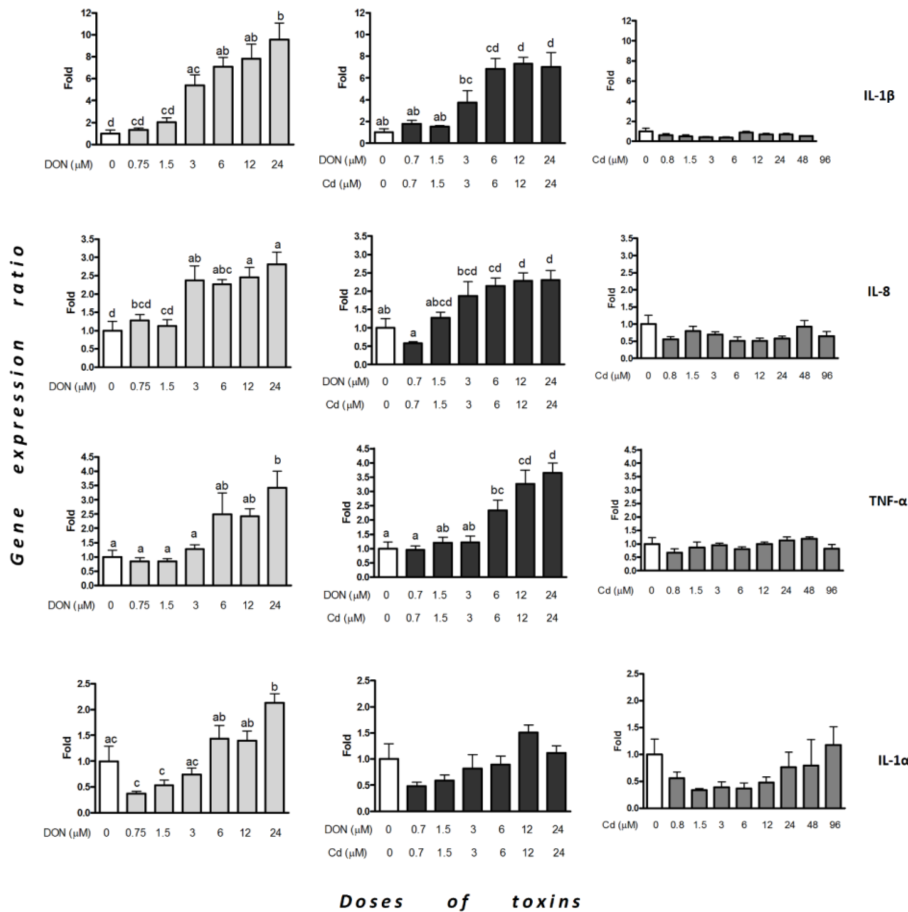


Fig.1 Dose-dependent regulation of inflammatory genes in the intestine upon exposure to DON, Cd and the co-contaminants. Jejunal explants from 6 piglets were exposed to graded levels of DON, Cd alone or in combination for 4 hours. The mRNA expression levels of IL-1β, IL-1α, IL-8, TNF-α, were measured by quantitative RT-PCR and analyzed using R program. RPL-32 and β2-microglobulin were used as the internal references. The data are the mean ± sem (n=6 animals). P-values <0.05 is considered significant.

DISCUSSION

In the present study, we explored the combined toxicity of DON and Cd in the intestine using pig jejunal explants. Previous studies have used this model of study of the intestine to analyze the effect of cocktail of mycotoxins [Basso et al., 2013; Kolf-Clauw et al., 2013]. So far, no study using explants to investigate the toxicity of Cd or Cd combined with mycotoxins have been reported.

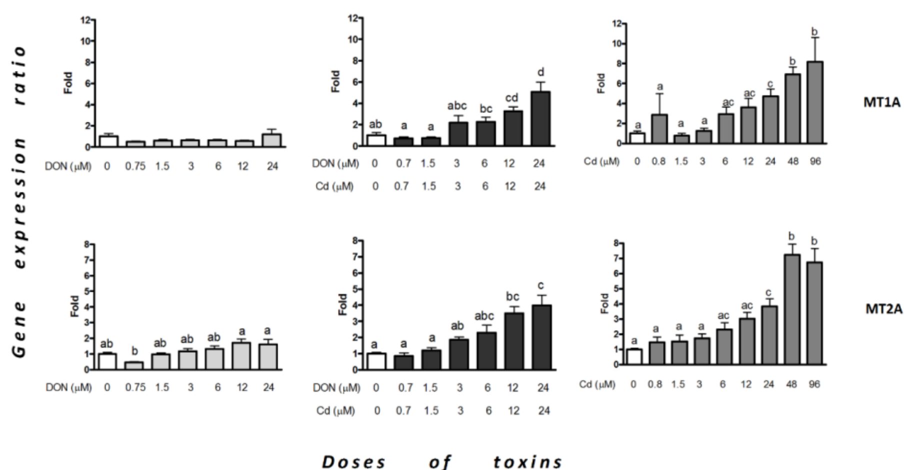


Fig.2 Dose-dependent regulation of MT genes in the intestine upon exposure to DON, Cd and the co-contaminants. Jejunal explants from 6 piglets were exposed to graded levels of DON, Cd alone or in combination for 4 hours. The mRNA expression levels of MT1A and MT2A were measured by quantitative RT-PCR and analyzed using R program. RPL-32 and β2-microglobulin were used as the internal references. The data are the mean ± sem (n=6 animals). P-values <0.05 is considered significant.

We used the RT-qPCR to detect the modulation of gene expression caused by the two contaminants. The elevation of four (IL-1β, IL-1α, IL-8, TNF-α) genes of inflammation were observed following DON exposure as already observed [Bracarense et al., 2012; Islam et al., 2006; Chung et al., 2003].

IL-8 is a chemokine, which recruits neutrophil cells and induces them to adhere to vascular endothelium and triggers extravasation into tissues [Linevsky et al., 2007]. TNF-α, IL-1β, and IL-1α are pro-inflammatory cytokines that play a key role in inflammation response. It has been reported that DON induces upregulation of pro-inflammatory genes including IL-1β, IL-6, TNF-α, MIP-2 and IL-8 through activation of MAPK [Islam et al., 2006; Chung et al., 2003]]. Therefore, we can hypothesize that the DON-induced MAPKs activation, already shown in explants [Lucioli et al., 2013] leads to an up-regulation of IL-1β, IL8, TNF-α and IL-1 α.

Metallothioneins are the markers of heavy metal toxicity and are necessary in Cd accumulation [Hoogenboom et al., 2015; Henry et al., 1994]. There are four isoforms of metallothioneins (MT-1 - MT-4), among which the MT-1 and MT-2 are the most common. They are ubiquitous in liver, intestine, pancreas and kidney [Sato & Kondoh, 2002; Werynska et al., 2015]. A decrease in metallothionein level in rat liver after exposure to moldy wheat contaminated by DON, zearalenone, T2-toxin and aflatoxins was observed. When feed was supplemented with vitamins, the levels

increased depending on the content of moldy wheat [Vasatkova et al., 2009]. To the best of our knowledge there is no report describing how DON affects the expression levels of metallothionein genes. In the present study, in the intestine exposed ex vivo, we did not observed any effect of DON on their expression at mRNA level.

MT levels and the mRNA expression levels of MT-1 and MT-2 have been found increased in pig kidney and rat mesenteric lymph node (MLN) after Cd exposure [Hoogenboom et al., 2015; Ninkov et al., 2015]. The present study corroborates the results obtained by Ninkov *et al.*

Cd exposure in rat for 30 days, could also induce mRNA up-regulation of pro-inflammatory cytokines in the intestine including TNF, IL-1β, IFN-γ, and IL-17 [Ninkov et al., 2015]. Nevertheless, in our study, Cd do not affect the expression of inflammatory cytokines genes. We can make the hypothesis that the duration of exposure of the explants was too short to observe any effect.

Conclusions

In summary, at the level of gene expression level in the jejunal explants model, the combined effects of DON and Cd correspond to the effects of the individual compounds suggesting that these contaminants do not interact with each other.

References

Pestka, J. J. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. *Arch. Toxicol.* 2010, *84*, 663–679, doi:10.1007/s00204-010-0579-8.

Payros, D.; Alassane-Kpembi, I.; Pierron, A.; Loiseau, N.; Pinton, P.; Oswald, I. P. Toxicology of deoxynivalenol and its acetylated and modified forms. *Arch. Toxicol.* 2016, *90*, 2931–2957, doi:10.1007/s00204-016-1826-4.

Placinta, C. M.; D'Mello, J. P. F.; Macdonald, A. M. C. A review of worldwide contamination of cereal grains and animal feed with Fusarium mycotoxins. *Anim. Feed Sci. Technol.* 1999, *78*, 21–37, doi:10.1016/S0377-8401(98)00278-8.

Smith, L. E.; Stoltzfus, R. J.; Prendergast, A. Food chain mycotoxin exposure, gut health, and impaired growth: a conceptual framework. *Adv. Nutr. Bethesda Md* 2012, *3*, 526–531, doi:10.3945/an.112.002188.

Pestka, J. J.; Smolinski, A. T. Deoxynivalenol: toxicology and potential effects on humans. *J. Toxicol. Environ. Health B Crit. Rev.* 2005, *8*, 39–69, doi:10.1080/10937400590889458.

Prelusky, D. B.; Hartin, K. E.; Trenholm, H. L.; Miller, J. D. Pharmacokinetic fate of 14C-labeled deoxynivalenol in swine. *Fundam. Appl. Toxicol. Off. J. Soc. Toxicol.* 1988, *10*, 276–286.

Bracarense, A.-P. F. L.; Lucioli, J.; Grenier, B.; Drociunas Pacheco, G.; Moll, W.-D.; Schatzmayr, G.; Oswald, I. P. Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets. *Br. J. Nutr.* 2012, *107*, 1776–1786, doi:10.1017/S0007114511004946.

Wu, L.; Liao, P.; He, L.; Ren, W.; Yin, J.; Duan, J.; Li, T. Growth performance, serum biochemical profile, jejunal morphology, and the expression of nutrients transporter genes in deoxynivalenol (DON)- challenged growing pigs. *BMC Vet. Res.* 2015, *11*, 144, doi:10.1186/s12917-015-0449-y.

Alizadeh, A.; Braber, S.; Akbari, P.; Garssen, J.; Fink-Gremmels, J. Deoxynivalenol Impairs Weight Gain and Affects Markers of Gut Health after Low-Dose, Short-Term Exposure of Growing Pigs. *Toxins* 2015, *7*, 2071–2095, doi:10.3390/toxins7062071.

Manquián-Cerda, K.; Escudey, M.; Zúñiga, G.; Arancibia-Miranda, N.; Molina, M.; Cruces, E. Effect of cadmium on phenolic compounds, antioxidant enzyme activity and oxidative stress in blueberry (Vaccinium corymbosum L.) plantlets grown in vitro. *Ecotoxicol. Environ. Saf.* 2016, *133*, 316–326, doi:10.1016/j.ecoenv.2016.07.029.

Jones, K. C.; Jackson, A.; Johnston, A. E. Evidence for an increase in the cadmium content of herbage since the 1860s. *Environ. Sci. Technol.* 1992, *26*, 834–836, doi:10.1021/es00028a026.

Andersson, A.; Bingefors, S. Trends and Annual Variations in Cd Concentrations in Grain of Winter Wheat. *Acta Agric. Scand.* 1985, *35*, 339–344, doi:10.1080/00015128509442044.

Järup, L.; Akesson, A. Current status of cadmium as an environmental health problem. *Toxicol. Appl. Pharmacol.* 2009, *238*, 201–208, doi:10.1016/j.taap.2009.04.020.

Joseph, P. Mechanisms of cadmium carcinogenesis. *Toxicol. Appl. Pharmacol.* 2009, *238*, 272–279, doi:10.1016/j.taap.2009.01.011.

Włostowski, T.; Krasowska, A.; Bonda, E. Joint effects of dietary cadmium and polychlorinated biphenyls on metallothionein induction, lipid peroxidation and histopathology in the kidneys and liver of bank voles. *Ecotoxicol. Environ. Saf.* 2008, *69*, 403–410, doi:10.1016/j.ecoenv.2007.03.014.

Wang, L.; Wang, H.; Li, J.; Chen, D.; Liu, Z. Simultaneous effects of lead and cadmium on primary cultures of rat proximal tubular cells: interaction of apoptosis and oxidative stress. *Arch. Environ. Contam. Toxicol.* 2011, *61*, 500–511, doi:10.1007/s00244-011-9644-4.

Ishihara, T.; Kobayashi, E.; Okubo, Y.; Suwazono, Y.; Kido, T.; Nishijyo, M.; Nakagawa, H.; Nogawa, K. Association between cadmium concentration in rice and mortality in the Jinzu River basin, Japan. *Toxicology* 2001, *163*, 23–28.

Wu, X.; Wei, S.; Wei, Y.; Guo, B.; Yang, M.; Zhao, D.; Liu, X.; Cai, X. The Reference Dose for Subchronic Exposure of Pigs to Cadmium Leading to Early Renal Damage by Benchmark Dose Method. *Toxicol. Sci.* 2012, *128*, 524–531, doi:10.1093/toxsci/kfs173.

Grawé, K. P.; Thierfelder, T.; Jorhem, L.; Oskarsson, A. Cadmium levels in kidneys from Swedish pigs in relation to environmental factors--temporal and spatial trends. *Sci. Total Environ.* 1997, *208*, 111–122.

Hoogenboom, R. L. A. P.; Hattink, J.; Polanen, A. van; Oostrom, S. van; Verbunt, J. T.; Traag, W. A.; Kan, K. A.; Eijkeren, J. C. H. van; Boeck, G. D.; Zeilmaker, M. J. Carryover of cadmium from feed in growing pigs. *Food Addit. Contam. Part A* 2015, *32*, 68–79, doi:10.1080/19440049.2014.979370.

Tomović, V. M.; Petrović, L. S.; Tomović, M. S.; Kevrešan, Ž. S.; Jokanović, M. R.; Džinić, N. R.; Despotović, A. R. Cadmium concentrations in the liver of 10 different pig genetic lines from Vojvodina, Serbia. *Food Addit. Contam. Part B Surveill.* 2011, *4*, 180–184, doi:10.1080/19393210.2011.589035.

Du, J.; Cheng, S. Y.; Hou, W. X.; Shi, B. M.; Shan, A. S. Effectiveness of Maifanite in Reducing the Detrimental Effects of Cadmium on Growth Performance, Cadmium Residue, Hematological Parameters, Serum Biochemistry, and the Activities of Antioxidant Enzymes in Pigs. *Biol. Trace Elem. Res.* 2013, *155*, 49–55, doi:10.1007/s12011-013-9769-6.

Ninkov, M.; Popov Aleksandrov, A.; Demenesku, J.; Mirkov, I.; Mileusnic, D.; Petrovic, A.; Grigorov, I.; Zolotarevski, L.; Tolinacki, M.; Kataranovski, D.; Brceski, I.; Kataranovski, M. Toxicity of oral cadmium intake: Impact on gut immunity. *Toxicol. Lett.* 2015, *237*, 89–99, doi:10.1016/j.toxlet.2015.06.002.

Nietfeld, J. C.; Tyler, D. E.; Harrison, L. R.; Cole, J. R.; Latimer, K. S.; Crowell, W. A. Culture and morphologic features of small intestinal mucosal explants from weaned pigs. *Am. J. Vet. Res.* 1991, *52*, 1142–1146.

Lucioli, J.; Pinton, P.; Callu, P.; Laffitte, J.; Grosjean, F.; Kolf-Clauw, M.; Oswald, I. P.; Bracarense, A. P. F. R. L. The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: interest of ex vivo models as an alternative to in vivo experiments. *Toxicon Off. J. Int. Soc. Toxinology* 2013, *66*, 31–36, doi:10.1016/j.toxicon.2013.01.024.

Pierron, A.; Mimoun, S.; Murate, L. S.; Loiseau, N.; Lippi, Y.; Bracarense, A.-P. F. L.; Liaubet, L.; Schatzmayr, G.; Berthiller, F.; Moll, W.-D.; Oswald, I. P. Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-

glucoside. *Arch. Toxicol.* 2016, *90*, 2037–2046, doi:10.1007/s00204-015-1592-8.

Basso, K.; Gomes, F.; Bracarense, A. P. L. Deoxynivanelol and fumonisin, alone or in combination, induce changes on intestinal junction complexes and in E-cadherin expression. *Toxins* 2013, *5*, 2341–2352, doi:10.3390/toxins5122341.

Alassane-Kpembi, I.; Puel, O.; Pinton, P.; Cossalter, A.-M.; Chou, T.-C.; Oswald, I. P. Co-exposure to low doses of the food contaminants deoxynivalenol and nivalenol has a synergistic inflammatory effect on intestinal explants. *Arch. Toxicol.* 2017, *91*, 2677–2687, doi:10.1007/s00204-016-1902-9.

Ruijter, J. M.; Ramakers, C.; Hoogaars, W. M. H.; Karlen, Y.; Bakker, O.; van den Hoff, M. J. B.; Moorman, A. F. M. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res.* 2009, *37*, e45, doi:10.1093/nar/gkp045.

Andersen, C. L.; Jensen, J. L.; Ørntoft, T. F. Normalization of Real-Time Quantitative Reverse Transcription-PCR Data: A Model-Based Variance Estimation Approach to Identify Genes Suited for Normalization, Applied to Bladder and Colon Cancer Data Sets. *Cancer Res.* 2004, *64*, 5245–5250, doi:10.1158/0008-5472.CAN-04-0496.

Cano, P. M.; Seeboth, J.; Meurens, F.; Cognie, J.; Abrami, R.; Oswald, I. P.; Guzylack-Piriou, L. Deoxynivalenol as a new factor in the persistence of intestinal inflammatory diseases: an emerging hypothesis through possible modulation of Th17-mediated response. *PloS One* 2013, *8*, e53647, doi:10.1371/journal.pone.0053647.

Grenier, B.; Loureiro-Bracarense, A.-P.; Lucioli, J.; Pacheco, G. D.; Cossalter, A.-M.; Moll, W.-D.; Schatzmayr, G.; Oswald, I. P. Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets. *Mol. Nutr. Food Res.* 2011, *55*, 761–771, doi:10.1002/mnfr.201000402.

Kolf-Clauw, M.; Sassahara, M.; Lucioli, J.; Rubira-Gerez, J.; Alassane-Kpembi, I.; Lyazhri, F.; Borin, C.; Oswald, I. P. The emerging mycotoxin, enniatin B1, down-modulates the gastrointestinal toxicity of T-2 toxin in vitro on intestinal epithelial cells and ex vivo on intestinal explants. *Arch. Toxicol.* 2013, *87*, 2233–2241, doi:10.1007/s00204-013-1067-8.

Islam, Z.; Gray, J. S.; Pestka, J. J. p38 Mitogen-activated protein kinase mediates IL-8 induction by the ribotoxin deoxynivalenol in human monocytes. *Toxicol. Appl. Pharmacol.* 2006, *213*, 235–244, doi:10.1016/j.taap.2005.11.001.

Chung, Y.-J.; Zhou, H.-R.; Pestka, J. J. Transcriptional and posttranscriptional roles for p38 mitogen-activated protein kinase in upregulation of TNF-alpha expression by deoxynivalenol (vomitoxin). *Toxicol. Appl. Pharmacol.* 2003, *193*, 188–201.

Linevsky, J. K.; Pothoulakis, C.; Keates, S.; Warny, M.; Keates, A. C.; Lamont, J. T.; Kelly, C. P. IL-8 release and neutrophil activation by Clostridium difficile toxin-exposed human monocytes. *Am. J. Physiol.* 1997, *273*, G1333-1340.

Henry, R. B.; Liu, J.; Choudhuri, S.; Klaassen, C. D. Species variation in hepatic metallothionein. *Toxicol. Lett.* 1994, *74*, 23–33.

Sato, M.; Kondoh, M. Recent studies on metallothionein: protection against toxicity of heavy metals and oxygen free radicals. *Tohoku J. Exp. Med.* 2002, *196*, 9–22.

Werynska, B.; Pula, B.; Kobierzycki, C.; Dziegiel, P.; Podhorska-Okolow, M. Metallothioneins in the lung cancer. *Folia Histochem. Cytobiol.* 2015, *53*, 1–10, doi:10.5603/FHC.a2015.0009.

Vasatkova, A.; Krizova, S.; Adam, V.; Zeman, L.; Kizek, R. Changes in metallothionein level in rat hepatic tissue after administration of natural mouldy wheat. *Int. J. Mol. Sci.* 2009, *10*, 1138–1160, doi:10.3390/ijms10031138.

General Discussion and conclusion

Co-occurrence of various chemicals including DON and Cd in food and feedstuffs has been frequently reported (Arnich et al. 2012; Sirot et al. 2013; Eskandari and Pakfetrat 2014; Algül and Kara 2014), which indicates that humans and animals can be exposed simultaneously to DON and Cd. This promotes us to speculate that there may exist an interaction between DON and Cd, such as synergy, antagonism or additive effects. So far, the regulations of DON and Cd in food and feed are based on their individual toxicity, which is not safe for the consumers exposed to the mixture of DON and Cd if there is synergistic or additive effect between them. Therefore, studying the combined effect of these two compounds is important for human and animal health and safety.

Caco-2 cells are a human epithelial cell line originally derived from colon carcinoma. This cell line has been widely used as human intestinal epithelial barrier model to study drug absorption or barrier disruption effect of toxic compounds (Breemen and Li 2005; Sun et al. 2008). We used it as an in vitro model, as Caco-2 cells spontaneously differentiate structurally and functionally into a monolayer with the typical properties of normal enterocytes in small intestine (Breemen and Li 2005; Sun et al. 2008). The differentiated Caco-2 monolayer has a higher transepithelial electric resistance than HT-29 cells, which is more similar to the in vivo situation (Lea 2015). And it also can express receptors, transporters and metabolic enzymes found in normal epithelium (Sun et al. 2008; Lea 2015). These properties of Caco-2 cells are important to make them closely mimic human intestinal epithelium. It is known that the biomarker of differentiated cells is the high activity of alkaline phosphatase, but we did not verify it in this study. It has been widely accepted that *in vitro* Caco-2 cells cultured for 3 weeks are considered as differentiated cells. For example, previouse study used 4 d- and 14 d-old cells as proliferating and differentiated cells (Aggarwal et al. 2011), and a lot of studies used 21 d-old cells as the differentiated cell model without verifying the activity of alkaline phosphatase (Pinton et al. 2009; De Walle et al. 2010; Akbari et al. 2014).

After selecting the model, we chose the concentrations to be considered on the base of the following assumption. The plausible intestinal concentration of DON in human is ranging from 0.25-10 μg/ml of gastrointestinal fluid (De Walle et al. 2008). In order to screen a large panel of plausible concentrations and adverse effects of DON, we selected the following doses: 1, 3, 10, and 30 μM. Assuming that DON consumption in one meal is diluted in 1 L of gastrointestinal fluid and is totally bio accessible (Sergent et al. 2006), the doses used in this

study correspond to 0.296, 0.89, 2.96 and 8.89 µg/ml. Namely, all the doses we used in vitro lay in the range of plausible intestinal concentration of DON. This approach has been used in the study of DON or other mycotoxins (Sergent et al. 2006; Pinton et al. 2009). From previous reports (EFSA 2017; Le et al. 2018), we got the knowledge that the molar ratio of the mean exposure of European consumers to DON and Cd is 1:1, thus the same doses of Cd were selected:1, 3, 10, and 30 µM), which corresponding to 0.112, 0.336, 1.12 and 3.36 µg/ml. These concentrations were also used in other studies of DON on intestinal cells (Maresca et al. 2002; Pinton et al. 2009; Rusanov et al. 2015).

Transepithelial electric resistance (TEER) is a quantitative measurement of the barrier function on *in vitro* culture models of epithelial/endothelial monolayers, which serves a strong indicator of the integrity of cellular barriers. This widely accepted technique has been applied to assess the permeability of different in vitro biological barriers with cells growing on semipermeable supports (Srinivasan et al. 2015). In this study, cellZscope device was used to assess the integrity of junctions by measuring the real-time alteration of TEER in Caco-2 cells caused by DON and Cd or the mixtures. Except TEER values, paracellular permeability indicating pore seize of junctions is also a good mark of the integrity of cellular barriers. This parameter is usually determined by the flux of paracellular tracer compounds with certain molecular weight (Arrieta et al. 2006; Srinivasan et al. 2015). A good tracer marker should be water soluble, non-toxic, uncharged and do not interfere with cells, such as fluorescein sodium salt, sucrose, mannitol or dextran (Arrieta et al. 2006; Srinivasan et al. 2015). Fluorescein isothiocyanate (FITC)-labeled dextran (4kD), one of the most common fluorescently labeled compounds was used in paracellular flux assay in this study.

Junctional proteins including tight junctions and adherens junctions play a central role in maintaining the normal function of intestinal barrier. These proteins bind to intracellular cytoskeleton proteins forming a number of transmembrane complexes, by which the adjacent cells are tightly linked (Suzuki 2013). These complexes contribute to generate transepithelial electric resistance when a continuous current pass through the cell monolayer (Anderson and Van Itallie 2009). Damage of these proteins causes decline of TEER and increase of paracellular permeability, in turn, alteration of TEER and paracellular permeability indicates injury on junctional proteins. Based on the results of TEER and FITC-dextran flux, western-blot technique was applied to analyze the changes of these junctional proteins. A main adherens junction, E-cadherin, and several tight junctions, ZO-1, occludin, claudin-3, claudin-4 and claudin-7 were selected in this study.

As described in literature, although Caco-2 cells provide a powerful in vitro epithelial model with many advantages, it still has some limits. For example, Caco-2 monolayer has only one cell type enterocyte and it cannot excrete mucins to build the mucus layer, which are not the same situation with *in vivo* (Lea 2015). In order not to limit our findings in *in vitro*, we selected rat as our in vivo model. Rat has been considered a standardized toxicological model with a more similar physiology with the corresponding human conditions than mouse (Iannaccone and Jacob 2009) and it required less toxic than pig. Although there are significant differences between rat and human, the use of rat models has provided a wealth of valuable insight into toxicology and pharmacology study on human. In this study, the effect of DON, Cd and their mixtures on body weight gain, morphological changes and junctional proteins in the intestine were investigated in rats. These results of *in vivo* model can further support the results generated by *in vitro* model.

For *in vivo* study, 8.2 mg DON /kg feed and 5 mg Cd /L water were chosen to induce a chronic effect on rat intestine. The regulation of DON for common animals like rat is 5 mg/kg feed that provide a complete protection against the toxicity of DON (European Union 2006b a). Therefore, the double of regulated concentration of DON (10 mg/kg feed) was initially selected to mix in feed. After mixing, high performance liquid chromatography (HPLC) analysis showed that the real concentration of DON in the feed was 8.2 mg/kg. Indeed, the concentration of 2-10 mg/kg feed of DON is the most used one in toxicological studies in animals and logical results are obtained (Pinton et al. 2008, 2009; Bracarense et al. 2012). The low concentration of $CdCl_2$ at 5 mg/L water is considered as equal to the dose of long-term exposure of Japanese women who suffer from Itai-itai disease. In rat, this concentration of Cd only affected the phenotype of innate immunity cells in the mesenteric lymph nodes without any clinical symptoms (Ninkov et al. 2015). Despite this, we were expecting that interactions between DON and Cd would induce stronger effects.

During the experiment, the food and water intake was noted weekly. The daily toxicant intake was calculated at the end of the experiment. The mean daily feed intake is 18 g/rat for animals fed DON- and DON+Cd-contaminated diet, thus the daily amount of DON taken up by rats is 147 μg/rat. The total daily water intake of animals fed Cd- and DON+Cd-contaminated diet is 21 and 22 ml/rat, which equals to 64 and 67 μg Cd /rat /day. In order to build a link between the doses used in vitro and in vivo, we calculate the mean exposure of DON and Cd by the following approach. It was reported that the volume of water in the gastrointestinal tract of female rats that normally get access to feed and water is 7.8±1.5 ml, the solid content is 2g

(McConnell et al. 2008). Assuming that the volume of solid content is 2 ml, plus the volume of water makes the total volume of gastrointestinal fluid around 10 ml. The mean intestinal concentration of DON in the rats fed DON- and DON+Cd-contaminated diet is 14.7 µg/ml, which is higher than the highest concentration in vitro (30 µM or 8.9 µg/ml). While the mean concentration of Cd is 6.4 and 6.7 µg/ml in the intestine of rats exposed to Cd and DON+Cd, which is also higher than the highest dose used in vitro (30 µM or 3.36 µg/ml). Despite this, we still expected to compare the results of *in vitro* and *in vivo*.

The results of TEER and FITC-dextran flux show as expected that DON, Cd and DON+Cd declined the barrier function in a time- and dose-dependent manner throughout the experiment. The results are consistent with previous studies (Duizer et al. 1999; Pinton et al. 2009, 2010; Akbari et al. 2014). At 1 µM, neither DON nor Cd affected the TEER values. At 3 µM, TEER was slightly decreased without statistical difference compared to control and 1 µM of contaminants. From 10 µM, TEER was significantly reduced by DON and Cd, where TEER decreased more by DON than by Cd. The decrease of TEER caused by 10 µM was also observed in other intestinal cell lines, such as HT-29-D4 and IPEC-1 (Maresca et al. 2002; Pinton et al. 2009). The extent of TEER decrease by DON and Cd is similar at 30 µM. However, from 24h post exposure, the TEER values did not change so much in any conditions. Since then, in the next experiment on proteins, cells are only exposed for 24h.

Lower TEER and higher permeability indicate the damage on the integrity of intestinal monolayer. Many cell signaling pathways are involved in regulation of intestinal barrier function, such as PKA (protein kinase A), PKC (protein kinase C), Rho GTPases, Src and MAPKs (González-Mariscal et al. 2008; Dörfel and Huber 2012). Conventional protein kinase C alpha (cPKCα) was demonstrated to disassembly tight junction, inhibit occludin incorporation into tight junctions and increase paracellular permeability, while novel protein kinase C epsilon (nPKCε) and nPKCη can enhance TEER and permeability of MDCK or Caco-2 monolayers (Andreeva et al. 2006; Suzuki et al. 2009). These data suggest that the effect of PKC on epithelial barrier is depending on specific isoforms. In addition, activation of c-Src, an isoform of Src kinase induced a reduction of occludin expression and of TEER values, delayed TJ assembly and increased inulin permeability in Caco-2 cells, which was recovered by inactivation of c-Src (Basuroy et al. 2003). Decrease of TEER and increase of FITC-4D permeability caused by ethanol was observed in Caco-2 monolayers, which were mediated by MAPKs activation icluding p-38, ERK and JNK (Elamin et al. 2014). The assessment of the

effect of DON and Cd on PKC, Src and MAPKs could provide useful information to understand their mode of action on induction of intestinal barrier disruption.

In vitro, the results showed that DON and Cd selectively affected junctional proteins. Both of them induced delocalization of E-cadherin and occludin from cell membrane into cytoplasm, which may be responsible for the decrease of barrier function. The abundance of E-cadherin, occludin and claudin-7 was significantly reduced by DON in a concentration-dependent manner. Cd only at the highest concentration 30 µM decreased the abundance of E-cadherin and occludin. Other analyzed proteins were not affected by DON and Cd. In the aspect of proteins, DON has a stronger effect than Cd. In most conditions, the combined effect is similar than the one with DON alone. *In vivo*, the intensity of immunostaining on E-cadherin was decreased by each condition, while for occludin it was negatively affected only by DON and DON+Cd. The combined effect is similar than DON or Cd alone. The results of these two proteins *in vivo* supported our in vitro results even the exposure doses of DON and Cd are slightly higher than in vitro. The results of DON on the protein expression of E-cadherin and occludin are consistent with previouse study performing on pigs, which also showed that DON decreased the abundance of these two junctional proteins (Bracarense et al. 2012). Although the alteration of E-cadherin and occludin in intestinal cells caused by DON and Cd was also observed in other studies (Duizer et al. 1999; Bracarense et al. 2012), the underlying mechanism has not been investigated.

Cell signaling molecules can directly or indirectly interact with junctional proteins to regulate the function of these proteins. Some studies reported that E-cadherin and occludin can be degraded by Src activation through ubiquitinatin-lysosomal or -proteasomal way. In MDCK and T84 cells (the immortalized human colon-derived epithelial cells), Src activation induced tyrosine phosphorylation and ubiquitination of E-cadherin resulting in E-cadherin endocytosis and degradation, which was restored by Src inhibition (Fujita et al. 2002; Smyth et al. 2012). *In vitro*, Src was observed to directly bind to C-terminal domain of occludin fusion protein which interrupted the linkage between occludin and ZO-1 (Kale et al. 2003). By contrast, the direct interaction of ERK/MAPKs with C-terminal region of occludin in differentiated Caco-2 extracts prevented occludin dissociation from ZO-1 (Basuroy et al. 2006). Degradation of other TJs including claudin-1, claudin-4 and ZO-1 by ubiquitation was also reported (Cai et al. 2018). These studies indicate that protein ubiquitination mediated by Src is an important regulator for TJs and AJs formation in epithelial/endothelial cells. Beside, inhibition of nPKCη increased paracellular permeability to inulin and decreased TEER by redistribution of occludin and ZO-

1 in MDCK and Caco-2 cell monolayers (Suzuki et al. 2009). ERK MAPKs activation induced redistribution and low expression of occludin, E-cadherin, ZO-1 and claudin-1 was also observed in Fas-transformed MDCK and endothelial cells (Chen et al. 2000; Fischer et al. 2005). To verify the effect of DON and Cd on Src, PKC and MAPKs would be helpful to understand their mechanism of action involved in deficiency of TJs and AJs.

The common effect of DON on animals is a reduction of their body weight gain. Low dose level of DON at 0.9 mg/kg feed negatively affected the body weight gain of pigs after 10 d exposure (Alizadeh et al. 2015). However, exposed to DON at 1.75 mg /kg feed for 30 d or 11.4 mg/kg feed for 7-14 d did not alter the body weight gain of rats, while DON at 11.4 mg/kg feed exposure for 30 d significantly decreased animals weight gain (Bracarense et al. 2017). In our study, the body weight gain of rat was not affected by any treatments during 4 weeks. This classical effect of DON in relation to body weight gain was not observed either in another unpublished study, in which the same concentration of DON was used in rat feed (Payros *et al*, submitted). These data suggest that the concentration of DON (8.2 mg/kg feed), slightly higher than the regulation (5 mg/kg feed), used in our study has no effect on the body weight gain of rat. These data indicate that the effect of DON on body weight gain depends upon the time and dose of exposure, as well as species used. In mice, intraperitoneal exposure to 0.25 and 1 mg/kg b.w. of Cd for 24h induced a significant decrease of body weight gain (Braga et al. 2015). Nevertheless, Cd exposure at 50 mg/L water or at 1.1 mg/kg feed for 12 weeks (Jurczuk et al. 2003; Haouem and El Hani 2013), or 6.6 mg/kg b.w. exposure for 20 d did not significantly alter the body weight gain of rats. These results are consistent with ours indicating that Cd is not a main inducer for decrease of body weight gain on rat. Compared to these studies, the dose of Cd used in our study is quite low, which is corresponding to 0.25-0.27 mg/kg b.w after calculation. This possibly induces no effect on body weight gain.

The main cause of loss of body weight gain is malnutrition caused by reduction of food intake and damage of absorptive enterocytes. However, in this study, the enterocytes in villi are only slightly injured and food intake is not changed by any conditions. For instance, several slight lesions in small intestine of treated animals like cell vacuolation and low frequency of villus fusion and atrophy were observed without any necrosis on the top of villi. These mild injuries do not affect the villi height as showed *in vivo*. These data are consistent with the results on unaffected body weight gain. Surprisingly, the crypt depth of jejunum was slightly but significantly decreased by DON, Cd and DON+Cd. This result indicates that cells in the crypt are more sensitive to these contaminants than the cells in the villi. This is because the cells at

the crypt are proliferating cells, while the cells at the villi are differentiated cells. Differentiated cells are more resistant to food contaminants than the proliferating cells (Pierron et al. 2016a; Broekaert et al. 2016). This may be the reason why we observed decreased crypt depth and unaffected villi height.

In order to investigate the direct effect of DON, Cd and their mixture on the intestine, an *ex vivo* model of pig jejunal explants was used. Pig is one of the most sensitive species to the toxicity of DON (Pestka 2007). Furthermore, similarities on intestinal physiology and immune system with human make pig an idea model to study the toxic effect of DON. Amounts of interesting results and valuable conclusions on the toxicity of DON are obtained from pig models. Explants application helps to reduce the number of experimental animals and meanwhile can provide large number of replications in scientific procedures. In addition, *in vitro* tissue culture is able to preserve the normal morphological structure of intestine (Kolf-Clauw et al. 2009). Although the investigation of toxic effects of food contaminants is limited by the short cultural time of *ex vivo* model, the effects on jejunum explants reflect the early events caused by DON and Cd after ingestion.

DON is capable of modifying immune response. Induction of chemokines and pro-inflammatory cytokines by DON has been frequently reported. The pro-inflammatory effect of Cd is still in dispute. As pro-inflammatory cytokines are able to interfere with tight junction proteins resulting in opening of the intestinal barrier, we analyzed the effect of DON, Cd and DON+Cd on mRNA level of these cytokines. We found that DON up-regulated several cytokine genes (IL-1β, IL-1α, IL-8, TNF-α). DON+Cd upregulated these gene expression at the same level than DON alone. However, none of the selected inflammatory genes was impacted by Cd whatever the doses tested. The results of DON are consistent with previous study, which also showed an up-regulation effect on the mRNA of IL-1β, IL-1α, IL-8 (Alassane-Kpembi et al. 2017a). While the results of Cd are inconsistent with some reports (Ninkov et al. 2015) but consistent with other ones (Zhao et al. 2006; Hyun et al. 2007). Ninkov *et al* found the protein level of IL-1β, IFN-γ and IL-17 but not TNF-α was slightly increased in the duodenum of rat exposed to 5 mg/ L Cd for 30d, and all of these cytokines were increased by 50 mg/ L Cd (Ninkov et al. 2015). However, the mRNA transcript level was not analyzed in this study. In mice exposed to 25 and 100 mg Cd /kg b.w., or in Caco-2 cells exposed to 50 μM for 24h, the secretion of IL-1β and TNF-α was not affected (Zhao et al. 2006; Hyun et al. 2007). A similar result was observed on Cd-treated airway epithelial cells (Cormet-Boyaka et al. 2012). One common point in these studies is that the secretion level of IL-8 was significantly augmented

in vivo and *in vitro* (Zhao et al. 2006; Hyun et al. 2007; Cormet-Boyaka et al. 2012). In our study, the non-effect of Cd on cytokine expression in pig explants is possibly due to the short-term exposure (4h). On the other hand, to the best of our knowledge, this is the first study on the pro-inflammatory effect of Cd on pig intestine. The pro-inflammatory effect of Cd may also be affected by the species used. If Cd could induce inflammatory response in intestine, our results indicate that intestinal immunity is more sensitive to DON than to Cd.

Metallothioneins are small intracellular cysteine-rich proteins with four isoforms (MT1-MT4) in mammals. MT1 and MT2 are expressed in most organs, while MT3 and MT 4 mainly presents in brain or skin (Ruttkay-Nedecky et al. 2013). The sulphydryl group of cysteine has a high affinity for metals that makes them strongly bind to metal ions by covalent binding, thereby regulating metal homeostasis and detoxifying heavy metals (Ruttkay-Nedecky et al. 2013; Schulkens et al. 2014). The correlation between MTs level and resistance to heavy metal poisoning has been confirmed. For example, cells with amount of MTs are resistant to Cd toxicity, while cells lacking of MTs are sensitive to Cd. In addition, MT1/MT2 knockdown mice are more susceptible to Cd (Ruttkay-Nedecky et al. 2013; Schulkens et al. 2014). The relation between DON and the level of MTs is rarely reported. There are two studies performed on rat demonstrated that DON (3500 ± 5 µg/kg feed) or DON (80 ± 5 µg/kg feed) mixed with other mycotoxins did not affecte or decreased MTs level in rat liver (Vasatkova et al. 2009; Sobrova et al. 2012). Thus, we hypothesized that DON and Cd may affect MT level and induce MTs expression in intestine. The results in pig explants showed that the mRNA transcript of MT1A and MT2A was only upregulated by Cd and DON+Cd, but not by DON alone. The effect of DON+Cd on MT1A and MT2A is similar than Cd alone. This indicate that MTs may not be the main detoxification mechanism for DON in intestine.

Surprisingly, other cytokine and antioxidant enzyme genes were not affected by DON or Cd in pig explants. NF-kB is a transcription factor and the changes of its mRNA expression by DON was not observed in this study. Transcription factors are expressed in cells prior to pro-inflammatory cytokines (Zhou et al. 2003a). Previous study pointed out that DON induces IL-8 expression through NF-κB pathway in Caco-2 cells. The gene expression of IL-8 in our study indicates that the increase of NF-kB gene expression may occur earlier and then return to control level at 4 h post-exposure.

IL-10 is an anti-inflammatory cytokine, which is predominantly produced by macrophages residing in the subepithelial lamina propria (Krause et al. 2015; Morhardt et al. 2019). TGF-β mainly produced by immune cells such as dendrical cells (DCs), is an immunosuppressive

cytokine involved in inhibition of inflammatory response in intestinal immunity (Bauché and Marie 2017; Ihara et al. 2017). IL-17A is an inflammatory cytokine produced by T cells and macrophages, DCs and neutrophils (Geha et al. 2017). IFNγ is mainly produced by T helper cells (Schuhmann et al. 2011). These cytokines play an important role in regulation of immune response, composition of microbiota and integrity of epithelial barrier in intestine (Al-Sadi et al. 2009). The producers of these cytokines reside in the intestinal laminal propria. Exposure time of 4 h might be not long enough for these cells migrating from lamina propria to the sites of injury to release corresponding cytokines.

The reason for that DON did not induce the expression of antioxidant enzymes maybe due to the short exposure time or the low doses used in this study. In previous studies, 40 µM DON for 24 h or 0.5 µM DON for 72 h were shown to induce oxidative damage in intestinal cells (Kouadio et al. 2005; Bony et al. 2006). In our study, the explants were exposed to DON up to 24 µM only for 24 h that might be too short to induce oxidative stress.

Cd significantly increased the transcription of MTs, indicating the synthesis of metallothionein proteins. The Cd in the medium might be bound to MT proteins and could not induce inflammation and oxidative stress. This hypothesis needs to be confirmed in further studies. On the other hand, the short exposure period may also be the reason for no effects of Cd on the gene expression of cytokines and antioxidant enzymes.

Overall, the results in *in vitro*, *in vivo* and *ex vivo* indicate the combined effects of DON and Cd on intestinal barrier function are equivalent to the most toxic compound alone whatever the parameter analyzed. These results indicate a less than additive effect. It is not clear yet how this less than additive effect happened.

DON and Cd exhibit some common toxic effects on intestinal cells through the same or alternative mechanisms (Fig. 7, 8). Abundant studies demonstrated that DON induces MAPKs activation in intestine or intestinal cell lines (Pinton et al. 2010; Cano et al. 2013; Alassane-Kpembi et al. 2017a). Only few reports focus on Cd-induced MAPKs activation in intestinal cells. Mantha and Jumarie verified that Cd exposure enhanced ERK and p-38 activation in differentiated Caco-2 cells, which is likely to increase protein synthesis (Mantha and Jumarie 2010). Furthermore, DON and Cd can cause cell apoptosis through a mitochondrial pathway, in which MAPKs are involved (Pestka 2010b; Yuan et al. 2015). In addition, both of them trigger oxidative stress, DNA damage, inflammation in intestinal cells (Fig. 7, 8)(Hyun et al. 2007; Krishnaswamy et al. 2010; Kadota et al. 2013; Ninkov et al. 2015; Lynch et al. 2017). With regard to intestinal microbiota, DON and Cd can decrease the number of *Bacteroidetes*

and increase that of *Firmicutes* and *Odoribacter*. Based on these facts, we speculate that DON and Cd have some similar mode of actions, which indicates that they may have common targets in cells. For instance, both of DON and Cd were reported to induce degradation of the same molecule inhibitor of κB, leading to NF-κB activation and to increase IL-8 transcription in Caco-2 cells (Hyun et al. 2007; De Walle et al. 2008). We hypothesized there may be a competition for the same molecular targets between DON and Cd. When present in mixture, they would compete for the same receptors and finally only the effect of the compound with a stronger effect could be displayed. To confirm this hypothesis, further studies are needed. On the other hand, there are also some dissimilar mode of actions between DON and Cd. For example, the well-known toxicity of DON is its ability to bind ribosome and induce inhibition of protein synthesis (Fig. 7). While, Cd is famous for its interference with other divalent metals in cells or in proteins, thereby inducing oxidative stress or dysfunction of proteins. Although a few studies provide evidence for mixture effect of dissimilarly acting toxicants, there is still more studies demonstrating that no mixture effect was observed between compounds with dissimilar actions (Kortenkamp et al. 2007). In summary, the less than additive effect between DON and Cd may be caused by their competition for the same targets or by their different mode of actions.

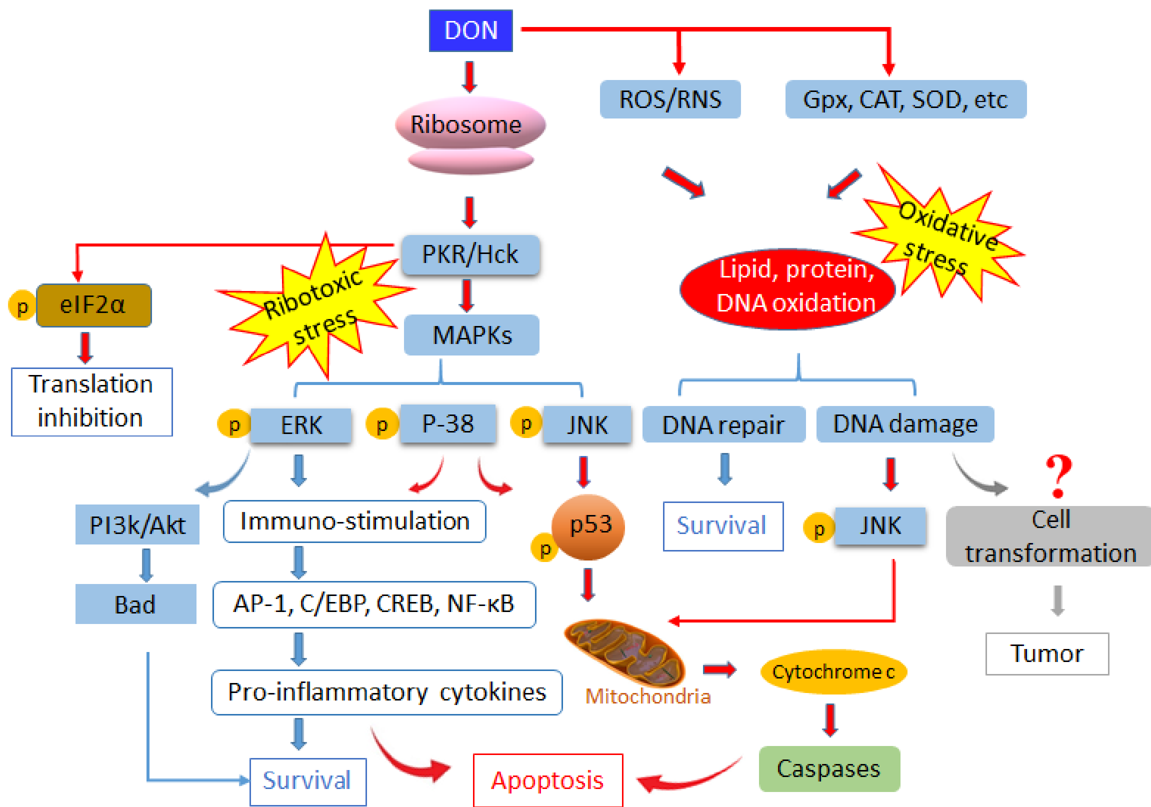


Figure 7 Mode of action of DON in cells. Blue arrows indicate cell survival pathways, red arrows indicate cell apoptosis or death pathways, grey arrows indicat possible pathway of the effect of DON.

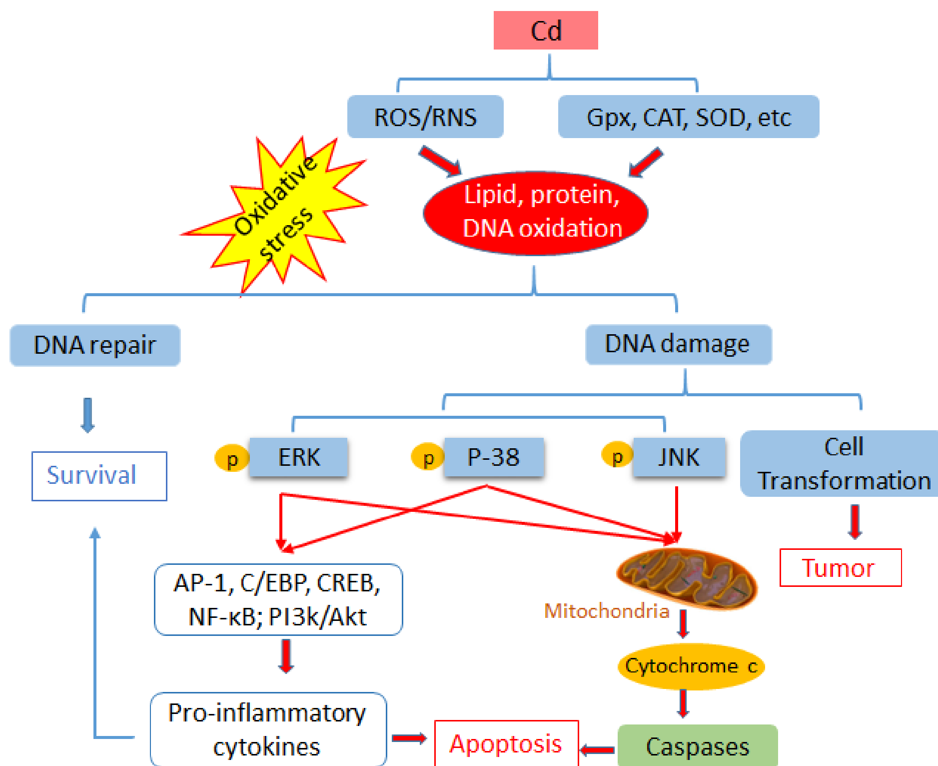


Figure 8 Mode of action of Cd. Blue arrows indicate cell survival pathways, red arrows indicate cell apoptosis or death pathways.

In conclusion, we demonstrated that DON and Cd disrupted intestinal barrier characterized by lower TEER and higher paracellular permeability via breaking and redistributing junctional proteins *in vitro* (Caco-2). In *in vivo*, DON and Cd did not affect villi height but significantly decreased crypt depth and the expression of junctional proteins in jejunum of rats, which further supported our *in vitro* results. In *ex vivo*, DON and Cd stimulated the gene expression of inflammatory cytokines and metallothioneins respectively in pig jejunal explants. The combined effect of DON and Cd on the intestinal parameters tested in this study is always similar than the individual compound with a stronger effect whatever *in vitro*, *in vivo* or *ex vivo*. These results suggest that the current regulation concerning each individual compound should provide sufficient protection for the consumers exposed to the mixtures of DON and Cd. However, if the results of this study can be extended to other target organs of DON and Cd such as liver and kidney need to be verified.

Perspectives

Understand the mechanism of action of DON and Cd on intestinal barrier

This study showed that DON, Cd and their combinations disrupt intestinal barrier function through damaging the integrity of tight and adherens junctions by decreasing or relocalization of these proteins. The combination DON+Cd induced similar effects to the most toxic individual compound *in vitro*, *in vivo* and *ex vivo*. So far, how the junctional proteins are injured by DON and Cd is unclear. Generally, protein levels in cells are regulated by the balance of protein synthesis and degradation. Therefore, protein degradation and synthesis inhibition could be the main causes for the decreased abundance of junctions. Although DON is able to inhibit protein synthesis (Pestka 2007), it cannot completely explain the decrease of junctional proteins as changes of specific junction proteins were observed in different cell lines (Pinton et al. 2010; Gu et al. 2014). This indicates alternative mechanisms of DON-induced junction reduction may exist. Cd-induced decrease of junctional proteins was studied *in vitro* and *in vivo* (Rusanov et al. 2015; Zhai et al. 2016), however the mechanisms of this effect has not been reported. That makes it difficult to explain the less than additive effect between DON and Cd. Thus, it is necessary to investigate the underlying mechanisms of disruption of intestinal barrier caused by DON and Cd.

Although other protein kinases like Src or PKC are of interest to consider, here we are going to talk about MAPKs as they seem to play a more important role in regulation of biological barrier function including intestinal barrier. For instance, p-38 and JNK activation induced by LPS decreased occludin expression in human cerebral microvascular endothelial cell, which decreased the blood-brain barrier function (BBB) (Qin et al. 2015). MAPKs (p-38, ERK and JNK) activation was associated with ethanol-induced delocalization of TJs and AJs, decrease of TEER and increase of permeability in Caco-2 monolayers (Elamin et al. 2014). In rat testes and sertoli cells, p-38 MAPK activation induced disruption of blood-testis barrier (BTB) characterized by TEER decrease and ubiquitin-degradation of junctional proteins (Jia et al. 2017). These data reveal that MAPKs pathways play a crucial role in regulation of the integrity of BBB, BTB and intestinal barrier.

DON is able to active MAPKs pathways in epithelial cells. DON (30 μM) reduced the expression of Claudin-4 resulting in decrease of TEER and increase of paraellualr permeability by ERK/MAPKs activation in IPEC-1 cells, which was inhibited by ERK inhibitor U0126 (Pinton et al. 2010). In IPEC-J2 cells, ERK activation by DON reduced TEER and the

expression of claudin-1 and claudin-3, which were partially recovered by ERK inhibitor (Springler et al. 2016). The data show that MAPKs pathways play an important role in DON-induced damage on intestinal claudins. However, the relation between MAPKs and intestinal E-cadherin and occludin has not been established. ERK, p-38 and JNK activation caused by Cd in Caco-2 cells was observed (Mantha and Jumarie 2010). Cd induced disruption of intestinal barrier function and impairment on TJs and AJs was reported (Duizer et al. 1999; Rusanov et al. 2015; Zhai et al. 2016). Nevertheless, the link between MAPKs activation and dysfunction of intestinal barrier has never been investigated.

Since both DON and Cd can activate MAPKs, which are involved in regulation of TJs and AJs, the next step of this work is to investigate the relation between MAPKs activation and DON- and Cd-induced reduction and redistribution of intestinal junctions. Such a study would improve the understanding on the mechanisms that involved in intestinal barrier destruction by DON and Cd, which may also be helpful in explaining the less than additive effect between them observed in the present study.

To further understand the underlying molecular basis of DON and Cd induced intestinal barrier dysfunction, transcriptomics and proteomics research of the impacts of DON and Cd on gene and protein expression levels would be helpful.

Assess interactions of DON and Cd on other organs

Besides intestine, liver and kidney are the other two major targets of DON and Cd. Interaction between DON and Cd on the intestine was not observed *in vitro*, neither *in vivo* in this study. As the nature of the interaction between them may depend on organs (Le et al. 2018), it is interesting to explore the combined effects of DON and Cd on these two organs in future studies. It would be helpful to understand better the effects of these two toxic compounds.

Liver is the main detoxification organ of the body, which makes it the first target organ of food contaminants after absorption by intestine. DON firstly distributes to liver reaching to a maximum level rapidly, where it triggers histopathological changes such as cytoplasmic vacuolisation and hepatocelular megalocytosis (Bracarense et al. 2016; Bernhoft et al. 2017). HepG2 cells exposed to 0.9-3.5 μM/L of DON showed a decrease of cell viability after 24h exposure (Mayer et al. 2017). Similar results were observed in rat liver clone-9 cells (Sahu et al. 2008). *In vitro*, Cd decreased viability of liver cells HL-7702 and HepG2 (Aziz et al. 2014; Le et al. 2018). The interaction between DON and Cd on the cytotoxicity to HepG2 is synergy and antagonism for the high (60-80%) and low (20-30%) cytotoxicity levels (Le et al. 2018).

More *in vitro* and *in vivo* studies are needed to assess how DON and Cd interact when they are presented together in the liver.

Kidney is the main excretory organ of the body and can be damaged by DON and Cd when they entered into this tissue. In female mice, 1.5-2.5 μM of DON induced oxidative stress-mediated apoptosis of kidney cells resulting in renal dysfunction after 12d exposure (Liang et al. 2015). In HEK-293 cell line, 2.5-15 μM of DON exposure for 12h reduced cell viability, induced lipid peroxidation and cell apoptosis (Dinu et al. 2011). The nephrotoxicity of Cd is due to its efficient accumulation in kidney (Rana et al. 2018). *In viv*o study revealed that 5 mg/kg b.w. of Cd induced histopathological changes, oxidative stress and kidney dysfunction in rats (Gabr et al. 2019). *In vitro*, significant cytotoxicity and ROS production were observed in bovine kidney cells exposed to 50 μM Cd for 24h (Zhang et al. 2014). The interaction between DON and Cd on the cytotoxicity to kidney cells HEK-293 is synergy and antagonism at high and low affected fraction respectively (Le et al. 2018). It is necessary to conduct *in vivo* experiments to confirm these *in vitro* results.

Assess interactions between other mixtures of heavy metals and mycotoxins

Although the interaction between DON and Cd on intestine was not observed, the interaction is still expected between other heavy metals such as lead (Pb), mercury (Hg) and other mycotoxins like ochratoxins, aflatoxins, and zearalenone.

Ochratoxin A (OTA) is a widely distributed and chemically stable mycotoxin. The main target organ of OTA is kidney particularly the proximal tubules (PT) (Bui-Klimke and Wu 2015). OTA is associated with the human diseases chronic interstitial nephropathy (CIN), and Balkan endemic nephropathy (BEN) characterized by progressive renal fibrosis (Bui-Klimke and Wu 2015). OTA exposure induced cytotoxicity and oxidative damage on rat primary PT cells and the LLC-PK1 cell line (Schaaf et al. 2002). Lead (Pb) is the most pervasive nephrotoxic metal. Acute exposure of Pb impairs the morphology of PT such as tubular necrosis while chronic exposure induces glomerular atrophy, tubular degeneration and interstitial fibrosis (Lentini et al. 2017; Orr and Bridges 2017). Pb (0.25-1 μM) decreased the cell viability and increased cell apoptosis and necrosis in rat primary PT cells (Wang et al. 2009). Since kidney is the common target of OTA and Pb, it is interesting to investigate the combined effect of them on this organ.

Aflatoxins are mycotoxins mainly produced by *Aspergillus spp*. The most common and toxic aflatoxin is aflatoxin B1 (AFB1) that mainly targets on liver. AFB1 is a hepatocarcinogenic compound due to its oxidative damage to DNA (Verma 2004). It induces lipid peroxidation and

MDA production in rat liver (Shen et al. 1994; Abdel-Wahhab et al. 2006). Pb and Cd mainly target on liver and their hepatotoxicity is primarily mediated by oxidative stress. They induce ROS production that may result in histological damage and dysfunction of liver (Jarrar and Taib 2012; Matović et al. 2015). In addition, Pb and Cd have a high affinity for sulfhydryl (SH) groups. They can block the function of antioxidant enzymes by binding to their SH groups, including SOD, CAT, GPx (Patra et al. 2011). Due to the same mode of action of Pb or Cd with AFB1 in the same target organ, it is of great value to explore the co-effect of Pb + AFB1 or Cd + AFB1 on liver.

Zearalenone (ZEA) is a worldwide spread mycotoxin with estrogenic effect. It has a similar structure to estrogen thus it can bind to estrogen receptors (ERs) and impact the growth of estrogen-dependent cells, which may induce reproductive dysfunction (Minervini and Dell'Aquila 2008). ZEA was demonstrated to stimulate T47D cell (human breast cancer cells) growth and MCF-7 cell (human breast cancer cells) proliferation by binding to ERs (Khosrokhavar et al. 2009; Tatay et al. 2018). Cd is able to mimic the estrogenic effect that causes reproductive disorders (Byrne et al. 2009). Cd exposure increased proliferation of MCF-7 cells through the ERα-dependent mechanism (Brama et al. 2007). Cd also enhanced DNA synthesis caused by estrogen on T47D cells (Zang et al. 2009). Due to their estrogenic effects, Cd and ZEA are toxic to reproductive organs and could increase the incidence of breast, ovarian, testicular and prostate cancers (Tatay et al. 2018). Even much work has been done on the individual estrogenic effects of Cd and ZEA, their combined effect has never been considered. Therefore, to investigate the mixture effect is helpful to reveal the interactions between Cd and ZEA, which is also beneficial for evaluating the existed individual regulation of them in food and feed.

Fumonisins are mycotoxins produced by *Fusarium verticilloides* and naturally occur in various crops especially in maize. Fumonisin B1 (FB1) is the most common and most toxic fumonisins (Voss and Riley 2013). The structure of FB1 is very similar to sphingolipids thus it can disrupt the metabolism of sphingolipids (Domijan 2012). FB1 is known to induce equine leukoencephalomalacia (ELEM) characterized by focal necrosis of cerebral white. Neuronal degeneration and inhibition of sphingolipid metabolism in the cortex of brain were observed in FB1-exposed mice (Doi and Uetsuka 2011; Domijan 2012). The neurotoxic metal mercury interrupts the central nervous system by induction of ROS. For example, inorganic mercury ($HgCl_2$) induced oxidative stress resulting in neurodegeneration in motor cortex (Teixeira et al. 2018). Methylmercury induced neurotoxicity on PC-12 cells was efficiently restored by

antioxidant Pyrroloquinoline quinone (Zhang et al. 2009a). These toxic effects on central nervous system indicate that the mixture effect may exist between FB1 and Hg, which is worth to investigate.

Except the compounds mentioned above, other toxic food contaminants may also co-occur in food/feed and induce mixture effect in human and animals. For example, most organochlorine pesticides, such as chlordane, DDT (dichlorodiphenyl trichloroethane), BHC (benzene hexachloride) and mirex target on liver, reproductive organs, central nervous system, thyroid and kidney (Jayaraj et al. 2016), as well as other chemicals like dioxins and bisphenol A (White and Birnbaum 2009; Michałowicz 2014). It is also necessary to study whether there are some interactions between these compounds and mycotoxins or heavy metals with same target organs or similar mode of action.

References

Abdel-Wahhab MA, Ahmed H H, Hagazi MM (2006) Prevention of aflatoxin B1-initiated hepatotoxicity in rat by marine algae extracts. Journal of Applied Toxicology 26:229–238. https://doi.org/10.1002/jat.1127

Achanzar WE, Achanzar KB, Lewis JG, et al (2000) Cadmium induces c-myc, p53, and c-jun expression in normal human prostate epithelial cells as a prelude to apoptosis. Toxicol Appl Pharmacol 164:291–300. https://doi.org/10.1006/taap.1999.8907

Achanzar WE, Diwan BA, Liu J, et al (2001) Cadmium-induced malignant transformation of human prostate epithelial cells. Cancer Res 61:455–458

Aggarwal S, Suzuki T, Taylor WL, et al (2011) Contrasting effects of ERK on tight junction integrity in differentiated and under-differentiated Caco-2 cell monolayers. Biochem J 433:51–63. https://doi.org/10.1042/BJ20100249

Aimola P, Carmignani M, Volpe AR, et al (2012) Cadmium induces p53-dependent apoptosis in human prostate epithelial cells. PLoS ONE 7:e33647. https://doi.org/10.1371/journal.pone.0033647

Akar Y, Ahmad N, Khalıd M (2018) The effect of cadmium on the bovine in vitro oocyte maturation and early embryo development. Int J Vet Sci Med 6:S73–S77. https://doi.org/10.1016/j.ijvsm.2018.03.001

Akbari P, Braber S, Gremmels H, et al (2014) Deoxynivalenol: a trigger for intestinal integrity breakdown. FASEB J 28:2414–2429. https://doi.org/10.1096/fj.13-238717

Al-Assaf AH, Alqahtani AM, Alshatwi AA, et al (2013) Mechanism of cadmium induced apoptosis in human peripheral blood lymphocytes: the role of p53, Fas and Caspase-3. Environ Toxicol Pharmacol 36:1033–1039. https://doi.org/10.1016/j.etap.2013.09.006

Alassane-Kpembi I, Kolf-Clauw M, Gauthier T, et al (2013) New insights into mycotoxin mixtures: the toxicity of low doses of Type B trichothecenes on intestinal epithelial cells is synergistic. Toxicol Appl Pharmacol 272:191–198. https://doi.org/10.1016/j.taap.2013.05.023

Alassane-Kpembi I, Puel O, Pinton P, et al (2017a) Co-exposure to low doses of the food contaminants deoxynivalenol and nivalenol has a synergistic inflammatory effect on intestinal explants. Arch Toxicol 91:2677–2687. https://doi.org/10.1007/s00204-016-1902-9

Alassane-Kpembi I, Schatzmayr G, Taranu I, et al (2017b) Mycotoxins co-contamination: Methodological aspects and biological relevance of combined toxicity studies. Critical Reviews in Food Science and Nutrition 57:3489–3507. https://doi.org/10.1080/10408398.2016.1140632

Algül I, Kara D (2014) Determination and chemometric evaluation of total aflatoxin, aflatoxin B1, ochratoxin A and heavy metals content in corn flours from Turkey. Food Chem 157:70–76. https://doi.org/10.1016/j.foodchem.2014.02.004

Alizadeh A, Braber S, Akbari P, et al (2015) Deoxynivalenol Impairs Weight Gain and Affects Markers of Gut Health after Low-Dose, Short-Term Exposure of Growing Pigs. Toxins (Basel) 7:2071–2095. https://doi.org/10.3390/toxins7062071

Alm H, Greising T, Brüssow K-P, et al (2002) The influence of the mycotoxins deoxynivalenol and zearalenol on in vitro maturation of pig oocytes and in vitro culture of pig zygotes. Toxicology in Vitro 16:643–648. https://doi.org/10.1016/S0887-2333(02)00059-0

Al-Sadi R, Boivin M, Ma T (2009) Mechanism of cytokine modulation of epithelial tight junction barrier. Front Biosci 14:2765–2778

Al-Sadi R, Guo S, Ye D, Ma TY (2013) TNF-α modulation of intestinal epithelial tight junction barrier is regulated by ERK1/2 activation of Elk-1. Am J Pathol 183:1871–1884. https://doi.org/10.1016/j.ajpath.2013.09.001

Al-Sadi RM, Ma TY (2007) IL-1β Causes an Increase in Intestinal Epithelial Tight Junction Permeability. J Immunol 178:4641–4649

Alzoghaibi MA (2013) Concepts of oxidative stress and antioxidant defense in Crohn's disease. World J Gastroenterol 19:6540–6547. https://doi.org/10.3748/wjg.v19.i39.6540

Anderson JM, Van Itallie CM (2009) Physiology and Function of the Tight Junction. Cold Spring Harb Perspect Biol 1:. https://doi.org/10.1101/cshperspect.a002584

Andreeva AY, Piontek J, Blasig IE, Utepbergenov DI (2006) Assembly of tight junction is regulated by the antagonism of conventional and novel protein kinase C isoforms. Int J Biochem Cell Biol 38:222–233. https://doi.org/10.1016/j.biocel.2005.09.001

Arnich N, Sirot V, Rivière G, et al (2012) Dietary exposure to trace elements and health risk assessment in the 2nd French Total Diet Study. Food Chem Toxicol 50:2432–2449. https://doi.org/10.1016/j.fct.2012.04.016

Arrieta MC, Bistritz L, Meddings JB (2006) Alterations in intestinal permeability. Gut 55:1512–1520. https://doi.org/10.1136/gut.2005.085373

ATSDR (2012) Toxicological Profile for Cadmium. https://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=48&tid=15. Accessed 11 Mar 2019

Avantaggiato G, Havenaar R, Visconti A (2004) Evaluation of the intestinal absorption of deoxynivalenol and nivalenol by an in vitro gastrointestinal model, and the binding efficacy of activated carbon and other adsorbent materials. Food and Chemical Toxicology 42:817–824. https://doi.org/10.1016/j.fct.2004.01.004

Aziz R, Rafiq MT, Yang J, et al (2014) Impact assessment of cadmium toxicity and its bioavailability in human cell lines (Caco-2 and HL-7702). Biomed Res Int 2014:839538. https://doi.org/10.1155/2014/839538

Ba Q, Li M, Chen P, et al (2017) Sex-Dependent Effects of Cadmium Exposure in Early Life on Gut Microbiota and Fat Accumulation in Mice. Environ Health Perspect 125:437–446. https://doi.org/10.1289/EHP360

Bannon DI, Abounader R, Lees PSJ, Bressler JP (2003) Effect of DMT1 knockdown on iron, cadmium, and lead uptake in Caco-2 cells. American Journal of Physiology-Cell Physiology 284:C44–C50. https://doi.org/10.1152/ajpcell.00184.2002

Basso K, Gomes F, Bracarense APL (2013) Deoxynivanelol and fumonisin, alone or in combination, induce changes on intestinal junction complexes and in E-cadherin expression. Toxins (Basel) 5:2341–2352. https://doi.org/10.3390/toxins5122341

Basuroy S, Seth A, Elias B, et al (2006) MAPK interacts with occludin and mediates EGF-induced prevention of tight junction disruption by hydrogen peroxide. Biochem J 393:69–77. https://doi.org/10.1042/BJ20050959

Basuroy S, Sheth P, Kuppuswamy D, et al (2003) Expression of kinase-inactive c-Src delays oxidative stress-induced disassembly and accelerates calcium-mediated reassembly of tight junctions in the Caco-2 cell monolayer. J Biol Chem 278:11916–11924. https://doi.org/10.1074/jbc.M211710200

Bauché D, Marie JC (2017) Transforming growth factor β: a master regulator of the gut microbiota and immune cell interactions. Clin Transl Immunology 6:e136. https://doi.org/10.1038/cti.2017.9

Behrens M, Hüwel S, Galla H-J, Humpf H-U (2015) Blood-Brain Barrier Effects of the Fusarium Mycotoxins Deoxynivalenol, 3 Acetyldeoxynivalenol, and Moniliformin and Their Transfer to the Brain. PLOS ONE 10:e0143640. https://doi.org/10.1371/journal.pone.0143640

Benoff S, Auborn K, Marmar JL, Hurley IR (2008) Link between low-dose environmentally relevant cadmium exposures and asthenozoospermia in a rat model. Fertil Steril 89:e73-79. https://doi.org/10.1016/j.fertnstert.2007.12.035

Bernhoft A, Høgåsen HR, Rosenlund G, et al (2017) Tissue distribution and elimination of deoxynivalenol and ochratoxin A in dietary-exposed Atlantic salmon (Salmo salar). Food Additives & Contaminants: Part A 34:1211–1224. https://doi.org/10.1080/19440049.2017.1321149

Beyersmann D, Hartwig A (2008) Carcinogenic metal compounds: recent insight into molecular and cellular mechanisms. Arch Toxicol 82:493–512. https://doi.org/10.1007/s00204-008-0313-y

BIOMIN (2019) BIOMIN World Mycotoxin Survey Report 2018. https://www.biomin.net/en/articles/biomin-world-mycotoxin-survey-report-2018/. Accessed 11 Apr 2019

Bishak YK, Payahoo L, Osatdrahimi A, Nourazarian A (2015) Mechanisms of cadmium carcinogenicity in the gastrointestinal tract. Asian Pac J Cancer Prev 16:9–21

Bolduc J-S, Denizeau F, Jumarie C (2004) Cadmium-Induced Mitochondrial Membrane-Potential Dissipation Does Not Necessarily Require Cytosolic Oxidative Stress: Studies Using Rhodamine-123 Fluorescence Unquenching. Toxicol Sci 77:299–306. https://doi.org/10.1093/toxsci/kfh015

Bol-Schoenmakers M, Braber S, Akbari P, et al (2016) The mycotoxin deoxynivalenol facilitates allergic sensitization to whey in mice. Mucosal Immunol 9:1477–1486. https://doi.org/10.1038/mi.2016.13

Bony S, Carcelen M, Olivier L, Devaux A (2006) Genotoxicity assessment of deoxynivalenol in the Caco-2 cell line model using the Comet assay. Toxicology Letters 166:67–76. https://doi.org/10.1016/j.toxlet.2006.04.010

Bracarense A p. f. l., Basso K m., Da Silva E o., et al (2016) Deoxynivalenol in the liver and lymphoid organs of rats: effects of dose and duration on immunohistological changes. World Mycotoxin Journal 10:89–96. https://doi.org/10.3920/WMJ2016.2094

Bracarense APFL, Basso KM, Da Silva EO, et al (2017) Deoxynivalenol in the liver and lymphoid organs of rats: effects of dose and duration on immunohistological changes. World Mycotoxin Journal 10:89–96. https://doi.org/10.3920/wmj2016.2094

Bracarense A-PFL, Lucioli J, Grenier B, et al (2012) Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets. Br J Nutr 107:1776–1786. https://doi.org/10.1017/S0007114511004946

Braga MM, Dick T, de Oliveira DL, et al (2015) Evaluation of zinc effect on cadmium action in lipid peroxidation and metallothionein levels in the brain. Toxicol Rep 2:858–863. https://doi.org/10.1016/j.toxrep.2015.05.014

Brama M, Gnessi L, Basciani S, et al (2007) Cadmium induces mitogenic signaling in breast cancer cell by an ERα-dependent mechanism. Molecular and Cellular Endocrinology 264:102–108. https://doi.org/10.1016/j.mce.2006.10.013

Breemen RB van, Li Y (2005) Caco-2 cell permeability assays to measure drug absorption. Expert Opinion on Drug Metabolism & Toxicology 1:175–185. https://doi.org/10.1517/17425255.1.2.175

Breton J, Daniel C, Dewulf J, et al (2013) Gut microbiota limits heavy metals burden caused by chronic oral exposure. Toxicol Lett 222:132–138. https://doi.org/10.1016/j.toxlet.2013.07.021

Breton J, Daniel C, Vignal C, et al (2016) Does oral exposure to cadmium and lead mediate susceptibility to colitis? The dark-and-bright sides of heavy metals in gut ecology. Sci Rep 6:. https://doi.org/10.1038/srep19200

Broekaert N, Devreese M, Demeyere K, et al (2016) Comparative in vitro cytotoxicity of modified deoxynivalenol on porcine intestinal epithelial cells. Food Chem Toxicol 95:103–109. https://doi.org/10.1016/j.fct.2016.06.012

Brzóska MM, Kamiński M, Supernak-Bobko D, et al (2003) Changes in the structure and function of the kidney of rats chronically exposed to cadmium. I. Biochemical and histopathological studies. Arch Toxicol 77:344–352. https://doi.org/10.1007/s00204-003-0451-1

Bui-Klimke TR, Wu F (2015) Ochratoxin A and human health risk: A review of the evidence. Crit Rev Food Sci Nutr 55:1860–1869. https://doi.org/10.1080/10408398.2012.724480

Byrne C, Divekar SD, Storchan GB, et al (2009) Cadmium - a metallohormone? Toxicol Appl Pharmacol 238:266–271. https://doi.org/10.1016/j.taap.2009.03.025

Cadet J, Wagner JR (2013) DNA base damage by reactive oxygen species, oxidizing agents, and UV radiation. Cold Spring Harb Perspect Biol 5:. https://doi.org/10.1101/cshperspect.a012559

Cai J, Culley MK, Zhao Y, Zhao J (2018) The role of ubiquitination and deubiquitination in the regulation of cell junctions. Protein Cell 9:754–769. https://doi.org/10.1007/s13238-017-0486-3

Cano PM, Seeboth J, Meurens F, et al (2013) Deoxynivalenol as a new factor in the persistence of intestinal inflammatory diseases: an emerging hypothesis through possible modulation of Th17-mediated response. PLoS ONE 8:e53647. https://doi.org/10.1371/journal.pone.0053647

Cao X, Lin H, Muskhelishvili L, et al (2015) Tight junction disruption by cadmium in an in vitro human airway tissue model. Respiratory Research 16:30. https://doi.org/10.1186/s12931-015-0191-9

Chang K-C, Hsu C-C, Liu S-H, et al (2013) Cadmium induces apoptosis in pancreatic β-cells through a mitochondria-dependent pathway: the role of oxidative stress-mediated c-Jun N-terminal kinase activation. PLoS ONE 8:e54374. https://doi.org/10.1371/journal.pone.0054374

Chen Y h, Lu Q, Schneeberger EE, Goodenough DA (2000) Restoration of tight junction structure and barrier function by down-regulation of the mitogen-activated protein kinase pathway in ras-transformed Madin-Darby canine kidney cells. Mol Biol Cell 11:849–862. https://doi.org/10.1091/mbc.11.3.849

Chouchene L, Pellegrini E, Gueguen M-M, et al (2016) Inhibitory effect of cadmium on estrogen signaling in zebrafish brain and protection by zinc. J Appl Toxicol 36:863–871. https://doi.org/10.1002/jat.3285

Chung Y-J, Zhou H-R, Pestka JJ (2003) Transcriptional and posttranscriptional roles for p38 mitogen-activated protein kinase in upregulation of TNF-alpha expression by deoxynivalenol (vomitoxin). Toxicol Appl Pharmacol 193:188–201

Claus Henn B, Coull BA, Wright RO (2014) Chemical mixtures and children's health. Current opinion in pediatrics 26:223–9. https://doi.org/10.1097/MOP.0000000000000067

Collins TFX, Sprando RL, Black TN, et al (2006) Effects of deoxynivalenol (DON, vomitoxin) on in utero development in rats. Food Chem Toxicol 44:747–757. https://doi.org/10.1016/j.fct.2005.10.007

Cormet-Boyaka E, Jolivette K, Bonnegarde-Bernard A, et al (2012) An NF-κB–Independent and Erk1/2-Dependent Mechanism Controls CXCL8/IL-8 Responses of Airway Epithelial Cells to Cadmium. Toxicol Sci 125:418–429. https://doi.org/10.1093/toxsci/kfr310

Da Silva EO, Gerez JR, Hohmann MSN, et al (2019) Phytic Acid Decreases Oxidative Stress and Intestinal Lesions Induced by Fumonisin B1 and Deoxynivalenol in Intestinal Explants of Pigs. Toxins 11:18. https://doi.org/10.3390/toxins11010018

Dänicke S, Valenta H, Döll S (2004) On the toxicokinetics and the metabolism of deoxynivalenol (don) in the pig. Archives of Animal Nutrition 58:169–180. https://doi.org/10.1080/00039420410001667548

De Walle JV, Romier B, Larondelle Y, Schneider Y-J (2008) Influence of deoxynivalenol on NF-kappaB activation and IL-8 secretion in human intestinal Caco-2 cells. Toxicol Lett 177:205–214. https://doi.org/10.1016/j.toxlet.2008.01.018

De Walle JV, Sergent T, Piront N, et al (2010) Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis. Toxicol Appl Pharmacol 245:291–298. https://doi.org/10.1016/j.taap.2010.03.012

Del Regno M, Adesso S, Popolo A, et al (2015) Nivalenol induces oxidative stress and increases deoxynivalenol pro-oxidant effect in intestinal epithelial cells. Toxicol Appl Pharmacol 285:118–127. https://doi.org/10.1016/j.taap.2015.04.002

Desurmont M (1983) Carcinogenic effect of metals. Sem Hop 59:2097–2099

Diesing A-K, Nossol C, Dänicke S, et al (2011a) Vulnerability of polarised intestinal porcine epithelial cells to mycotoxin deoxynivalenol depends on the route of application. PLoS ONE 6:e17472. https://doi.org/10.1371/journal.pone.0017472

Diesing A-K, Nossol C, Panther P, et al (2011b) Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and IPEC-J2. Toxicol Lett 200:8–18. https://doi.org/10.1016/j.toxlet.2010.10.006

Dinu D, Bodea GO, Ceapa CD, et al (2011) Adapted response of the antioxidant defense system to oxidative stress induced by deoxynivalenol in Hek-293 cells. Toxicon 57:1023–1032. https://doi.org/10.1016/j.toxicon.2011.04.006

Dizdaroglu M, Jaruga P (2012) Mechanisms of free radical-induced damage to DNA. Free Radic Res 46:382–419. https://doi.org/10.3109/10715762.2011.653969

Dkhil MA, Al-Quraishy S, Diab MMS, et al (2014) The potential protective role of Physalis peruviana L. fruit in cadmium-induced hepatotoxicity and nephrotoxicity. Food Chem Toxicol 74:98–106. https://doi.org/10.1016/j.fct.2014.09.013

Doi K, Uetsuka K (2011) Mechanisms of Mycotoxin-Induced Neurotoxicity through Oxidative Stress-Associated Pathways. Int J Mol Sci 12:5213–5237. https://doi.org/10.3390/ijms12085213

Domijan A-M (2012) Fumonisin B(1): a neurotoxic mycotoxin. Arh Hig Rada Toksikol 63:531–544. https://doi.org/10.2478/10004-1254-63-2012-2239

Dong F, Guo F, Li L, et al (2014) Cadmium induces vascular permeability via activation of the p38 MAPK pathway. Biochemical and Biophysical Research Communications 450:447–452. https://doi.org/10.1016/j.bbrc.2014.05.140

Dörfel MJ, Huber O (2012) Modulation of Tight Junction Structure and Function by Kinases and Phosphatases Targeting Occludin. In: BioMed Research International. https://www.hindawi.com/journals/bmri/2012/807356/. Accessed 22 May 2019

Dorta DJ, Leite S, DeMarco KC, et al (2003) A proposed sequence of events for cadmium-induced mitochondrial impairment. J Inorg Biochem 97:251–257

Du Z, Kim KH, Kim J, Moon Y (2018) Fungal Deoxynivalenol-Induced Enterocyte Distress Is Attenuated by Adulterated Adlay: In Vitro Evidences for Mucoactive Counteraction. Frontiers in immunology 9:186. https://doi.org/10.3389/fimmu.2018.00186

Duizer E, Gilde AJ, Versantvoort CH, Groten JP (1999) Effects of cadmium chloride on the paracellular barrier function of intestinal epithelial cell lines. Toxicol Appl Pharmacol 155:117–126. https://doi.org/10.1006/taap.1998.8589

EFSA (2013) Deoxynivalenol in food and feed: occurrence and exposure. EFSA Journal 11:3379. https://doi.org/10.2903/j.efsa.2013.3379

EFSA (2017) Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. EFSA Journal 15:. https://doi.org/10.2903/j.efsa.2017.4718

EFSA (2009) Cadmium in food - Scientific opinion of the Panel on Contaminants in the Food Chain. EFSA Journal 7:980. https://doi.org/10.2903/j.efsa.2009.980

EFSA (2011) Statement on tolerable weekly intake for cadmium. EFSA Journal 9:1975. https://doi.org/10.2903/j.efsa.2011.1975

Elamin E, Masclee A, Troost F, et al (2014) Ethanol impairs intestinal barrier function in humans through mitogen activated protein kinase signaling: a combined in vivo and in vitro approach. PLoS ONE 9:e107421. https://doi.org/10.1371/journal.pone.0107421

El-Boshy ME, Risha EF, Abdelhamid FM, et al (2015) Protective effects of selenium against cadmium induced hematological disturbances, immunosuppressive, oxidative stress and hepatorenal damage in rats. J Trace Elem Med Biol 29:104–110. https://doi.org/10.1016/j.jtemb.2014.05.009

Eskandari MH, Pakfetrat S (2014) Aflatoxins and heavy metals in animal feed in Iran. Food Addit Contam Part B Surveill 7:202–207. https://doi.org/10.1080/19393210.2013.876675

European Union (2006a) Commission Regulation (EC) No 1881 / 2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance). https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=celex%3A32006R1881. Accessed 8 Mar 2019

European Union (2006b) Commission Recommendation of 17 August 2006 on the Presence of Deoxynivalenol, Zearalenone, Ochratoxin A, T-2 and HT-2 and Fumonisins in Products Intended for Animal Feeding. https://eur-lex.europa.eu/eli/reco/2006/576/oj. Accessed 7 Mar 2019

European Union (2013) Commission Regulation (EU) No 1275/2013 of 6 December 2013 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum level for arsenic, cadmium, lead, nitrites, volatile mustard oil and harmful botanical impurities (Text with EEA relevance). https://eur-lex.europa.eu/eli/reg/2013/1275/oj. Accessed 12 Mar 2019

European Union (2014) Commission Regulation (EU) No 488/2014 of 12 May 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of cadmium in foodstuffs (Text with EEA relevance). https://eur-lex.europa.eu/eli/reg/2014/488/oj. Accessed 12 Mar 2019

Fahmy MA, Aly FAE (2000) In vivo and in vitro studies on the genotoxicity of cadmium chloride in mice. Journal of Applied Toxicology 20:231–238.

https://doi.org/10.1002/(SICI)1099-1263(200005/06)20:3<231::AID-JAT653>3.0.CO;2-T

Federico A, Morgillo F, Tuccillo C, et al (2007) Chronic inflammation and oxidative stress in human carcinogenesis. Int J Cancer 121:2381–2386. https://doi.org/10.1002/ijc.23192

Filipic M, Fatur T, Vudrag M (2006) Molecular mechanisms of cadmium induced mutagenicity. Hum Exp Toxicol 25:67–77. https://doi.org/10.1191/0960327106ht590oa

Fischer S, Wiesnet M, Renz D, Schaper W (2005) H2O2 induces paracellular permeability of porcine brain-derived microvascular endothelial cells by activation of the p44/42 MAP kinase pathway. Eur J Cell Biol 84:687–697. https://doi.org/10.1016/j.ejcb.2005.03.002

Food and Drug Administration (2010) Guidance for Industry and FDA: Advisory Levels for Deoxynivalenol (DON) in Finished Wheat Products for Human Consumption and Grains and Grain By-Products used for Animal Feed. https://www.fda.gov/Food/GuidanceRegulation/ucm120184.htm. Accessed 8 Mar 2019

Fujishiro H, Hamao S, Tanaka R, et al (2017) Concentration-dependent roles of DMT1 and ZIP14 in cadmium absorption in Caco-2 cells. J Toxicol Sci 42:559–567. https://doi.org/10.2131/jts.42.559

Fujishiro H, Yano Y, Takada Y, et al (2012) Roles of ZIP8, ZIP14, and DMT1 in transport of cadmium and manganese in mouse kidney proximal tubule cells. Metallomics 4:700–708. https://doi.org/10.1039/C2MT20024D

Fujita Y, Krause G, Scheffner M, et al (2002) Hakai, a c-Cbl-like protein, ubiquitinates and induces endocytosis of the E-cadherin complex. Nat Cell Biol 4:222–231. https://doi.org/10.1038/ncb758

Gabr SA, Alghadir AH, Ghoniem GA (2019) Biological activities of ginger against cadmium-induced renal toxicity. Saudi Journal of Biological Sciences 26:382–389. https://doi.org/10.1016/j.sjbs.2017.08.008

Gafter-Gvili A, Zingerman B, Rozen-Zvi B, et al (2013) Oxidative stress-induced DNA damage and repair in human peripheral blood mononuclear cells: protective role of hemoglobin. PLoS ONE 8:e68341. https://doi.org/10.1371/journal.pone.0068341

Ganguly K, Levänen B, Palmberg L, et al (2018) Cadmium in tobacco smokers: a neglected link to lung disease? European Respiratory Review 27:170122. https://doi.org/10.1183/16000617.0122-2017

García GR, Payros D, Pinton P, et al (2018) Intestinal toxicity of deoxynivalenol is limited by Lactobacillus rhamnosus RC007 in pig jejunum explants. Arch Toxicol 92:983–993. https://doi.org/10.1007/s00204-017-2083-x

Garcia MA, Nelson WJ, Chavez N (2018) Cell-Cell Junctions Organize Structural and Signaling Networks. Cold Spring Harb Perspect Biol 10:. https://doi.org/10.1101/cshperspect.a029181

Gauthier T, Waché Y, Laffitte J, et al (2013) Deoxynivalenol impairs the immune functions of neutrophils. Mol Nutr Food Res 57:1026–1036. https://doi.org/10.1002/mnfr.201200755

Geha M, Tsokos MG, Bosse RE, et al (2017) IL-17A Produced by Innate Lymphoid Cells Is Essential for Intestinal Ischemia-Reperfusion Injury. The Journal of Immunology 199:2921–2929. https://doi.org/10.4049/jimmunol.1700655

Gerez JR, Pinton P, Callu P, et al (2015) Deoxynivalenol alone or in combination with nivalenol and zearalenone induce systemic histological changes in pigs. Exp Toxicol Pathol 67:89–98. https://doi.org/10.1016/j.etp.2014.10.001

Ghosh K, N I (2018) Cadmium treatment induces echinocytosis, DNA damage, inflammation, and apoptosis in cardiac tissue of albino Wistar rats. Environ Toxicol Pharmacol 59:43–52. https://doi.org/10.1016/j.etap.2018.02.009

Giaginis C, Gatzidou E, Theocharis S (2006) DNA repair systems as targets of cadmium toxicity. Toxicol Appl Pharmacol 213:282–290. https://doi.org/10.1016/j.taap.2006.03.008

Godt J, Scheidig F, Grosse-Siestrup C, et al (2006) The toxicity of cadmium and resulting hazards for human health. J Occup Med Toxicol 1:22. https://doi.org/10.1186/1745-6673-1-22

González-Mariscal L, Tapia R, Chamorro D (2008) Crosstalk of tight junction components with signaling pathways. Biochim Biophys Acta 1778:729–756. https://doi.org/10.1016/j.bbamem.2007.08.018

Goossens J, Pasmans F, Verbrugghe E, et al (2012) Porcine intestinal epithelial barrier disruption by the Fusarium mycotoxins deoxynivalenol and T-2 toxin promotes transepithelial passage of doxycycline and paromomycin. BMC Vet Res 8:245. https://doi.org/10.1186/1746-6148-8-245

Goyarts T, Brüssow K-P, Valenta H, et al (2010) On the effects of the Fusarium toxin deoxynivalenol (DON) administered per os or intraperitoneal infusion to sows during days 63 to 70 of gestation. Mycotoxin Res 26:119–131. https://doi.org/10.1007/s12550-010-0047-6

Goyarts T, Dänicke S (2006) Bioavailability of the Fusarium toxin deoxynivalenol (DON) from naturally contaminated wheat for the pig. Toxicology Letters 163:171–182. https://doi.org/10.1016/j.toxlet.2005.10.007

Graziani F, Pujol A, Nicoletti C, et al (2015) The Food-Associated Ribotoxin Deoxynivalenol Modulates Inducible NO Synthase in Human Intestinal Cell Model. Toxicol Sci 145:372–382. https://doi.org/10.1093/toxsci/kfv058

Grenier B, Oswald I (2011) Mycotoxin co-contamination of food and feed: meta-analysis of publications describing toxicological interactions. World Mycotoxin Journal 4:285–313. https://doi.org/10.3920/WMJ2011.1281

Groschwitz KR, Hogan SP (2009) Intestinal Barrier Function: Molecular Regulation and Disease Pathogenesis. J Allergy Clin Immunol 124:3–22. https://doi.org/10.1016/j.jaci.2009.05.038

Gu MJ, Song SK, Park SM, et al (2014) Bacillus subtilis Protects Porcine Intestinal Barrier from Deoxynivalenol via Improved Zonula Occludens-1 Expression. Asian-Australas J Anim Sci 27:580–586. https://doi.org/10.5713/ajas.2013.13744

Guinane CM, Cotter PD (2013) Role of the gut microbiota in health and chronic gastrointestinal disease: understanding a hidden metabolic organ. Therap Adv Gastroenterol 6:295–308. https://doi.org/10.1177/1756283X13482996

Gundacker C, Hengstschläger M (2012) The role of the placenta in fetal exposure to heavy metals. Wien Med Wochenschr 162:201–206. https://doi.org/10.1007/s10354-012-0074-3

Han SJ, Ha KH, Jeon JY, et al (2015) Impact of Cadmium Exposure on the Association between Lipopolysaccharide and Metabolic Syndrome. Int J Environ Res Public Health 12:11396–11409. https://doi.org/10.3390/ijerph120911396

Haouem S, El Hani A (2013) Effect of Cadmium on Lipid Peroxidation and on Some Antioxidants in the Liver, Kidneys and Testes of Rats Given Diet Containing Cadmium-polluted Radish Bulbs. J Toxicol Pathol 26:359–364. https://doi.org/10.1293/tox.2013-0025

He K, Zhou H-R, Pestka JJ (2012) Targets and intracellular signaling mechanisms for deoxynivalenol-induced ribosomal RNA cleavage. Toxicol Sci 127:382–390. https://doi.org/10.1093/toxsci/kfs134

Hyun JS, Satsu H, Shimizu M (2007) Cadmium induces Interleukin-8 production via NF-κB activation in the human intestinal epithelial cell, Caco-2. Cytokine 37:26–34. https://doi.org/10.1016/j.cyto.2007.02.011

Iannaccone PM, Jacob HJ (2009) Rats! Dis Model Mech 2:206–210. https://doi.org/10.1242/dmm.002733

IARC (ed) (1993) Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins: views and expert opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, which met in Lyon, 9 - 16 June 1992. Lyon

Ihara S, Hirata Y, Koike K (2017) TGF-β in inflammatory bowel disease: a key regulator of immune cells, epithelium, and the intestinal microbiota. J Gastroenterol 52:777–787. https://doi.org/10.1007/s00535-017-1350-1

Islam MR, Roh YS, Kim J, et al (2013) Differential immune modulation by deoxynivalenol (vomitoxin) in mice. Toxicology Letters 221:152–163. https://doi.org/10.1016/j.toxlet.2013.05.656

Islam Z, Gray JS, Pestka JJ (2006) p38 Mitogen-activated protein kinase mediates IL-8 induction by the ribotoxin deoxynivalenol in human monocytes. Toxicol Appl Pharmacol 213:235–244. https://doi.org/10.1016/j.taap.2005.11.001

Itoh M, Nagafuchi A, Yonemura S, et al (1993) The 220-kD protein colocalizing with cadherins in non-epithelial cells is identical to ZO-1, a tight junction-associated protein in epithelial cells: cDNA cloning and immunoelectron microscopy. J Cell Biol 121:491–502

Jarrar BM, Taib NT (2012) Histological and histochemical alterations in the liver induced by lead chronic toxicity. Saudi J Biol Sci 19:203–210. https://doi.org/10.1016/j.sjbs.2011.12.005

Jayaraj R, Megha P, Sreedev P (2016) Organochlorine pesticides, their toxic effects on living organisms and their fate in the environment. Interdiscip Toxicol 9:90–100. https://doi.org/10.1515/intox-2016-0012

JECFA (2011) Evaluation of certain food additives and contaminants: seventy-third report of the Joint FAO/WHO Expert Committee on Food Additives. World Health Organization, Geneva, Switzerland

Jia X, Xu Y, Wu W, et al (2017) Aroclor1254 disrupts the blood–testis barrier by promoting endocytosis and degradation of junction proteins via p38 MAPK pathway. Cell Death & Disease 8:e2823. https://doi.org/10.1038/cddis.2017.224

Jiang C, Yuan Y, Hu F, et al (2014) Cadmium induces PC12 cells apoptosis via an extracellular signal-regulated kinase and c-Jun N-terminal kinase-mediated mitochondrial apoptotic pathway. Biol Trace Elem Res 158:249–258. https://doi.org/10.1007/s12011-014-9918-6

Jin Y, Liu Z, Liu F, et al (2015a) Embryonic exposure to cadmium (II) and chromium (VI) induce behavioral alterations, oxidative stress and immunotoxicity in zebrafish (Danio

rerio). Neurotoxicology and Teratology 48:9–17. https://doi.org/10.1016/j.ntt.2015.01.002

Jin Y, Zeng Z, Wu Y, et al (2015b) Oral Exposure of Mice to Carbendazim Induces Hepatic Lipid Metabolism Disorder and Gut Microbiota Dysbiosis. Toxicol Sci 147:116–126. https://doi.org/10.1093/toxsci/kfv115

Joly Condette C, Khorsi-Cauet H, Morlière P, et al (2014) Increased gut permeability and bacterial translocation after chronic chlorpyrifos exposure in rats. PLoS ONE 9:e102217. https://doi.org/10.1371/journal.pone.0102217

Jurczuk M, Brzóska MM, Rogalska J, Moniuszko-Jakoniuk J (2003) IRON BODY STATUS OF RATS CHRONICALLY EXPOSED TO CADMIUM AND ETHANOL. Alcohol Alcohol 38:202–207. https://doi.org/10.1093/alcalc/agg057

Kadota T, Furusawa H, Hirano S, et al (2013) Comparative study of deoxynivalenol, 3-acetyldeoxynivalenol, and 15-acetyldeoxynivalenol on intestinal transport and IL-8 secretion in the human cell line Caco-2. Toxicol In Vitro 27:1888–1895. https://doi.org/10.1016/j.tiv.2013.06.003

Kale G, Naren AP, Sheth P, Rao RK (2003) Tyrosine phosphorylation of occludin attenuates its interactions with ZO-1, ZO-2, and ZO-3. Biochemical and Biophysical Research Communications 302:324–329. https://doi.org/10.1016/S0006-291X(03)00167-0

Khosrokhavar R, Rahimifard N, Shoeibi S, et al (2009) Effects of zearalenone and α-Zearalenol in comparison with Raloxifene on T47D cells. Toxicol Mech Methods 19:246–250. https://doi.org/10.1080/15376510802455347

Kim HS, Kim YJ, Seo YR (2015) An Overview of Carcinogenic Heavy Metal: Molecular Toxicity Mechanism and Prevention. Journal of cancer prevention 20:232–40. https://doi.org/10.15430/JCP.2015.20.4.232

Kim S, Cheon H-S, Kim S-Y, et al (2013a) Cadmium induces neuronal cell death through reactive oxygen species activated by GADD153. BMC Cell Biol 14:4. https://doi.org/10.1186/1471-2121-14-4

Kim Y, Ha E-H, Park H, et al (2013b) Prenatal lead and cadmium co-exposure and infant neurodevelopment at 6 months of age: the Mothers and Children's Environmental Health (MOCEH) study. Neurotoxicology 35:15–22. https://doi.org/10.1016/j.neuro.2012.11.006

Knutsen HK, Alexander J, Barregård L, et al (2017) Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. EFSA J 15:e04718. https://doi.org/10.2903/j.efsa.2017.4718

Kolf-Clauw M, Castellote J, Joly B, et al (2009) Development of a pig jejunal explant culture for studying the gastrointestinal toxicity of the mycotoxin deoxynivalenol: histopathological analysis. Toxicol In Vitro 23:1580–1584. https://doi.org/10.1016/j.tiv.2009.07.015

Korhonen R, Lahti A, Kankaanranta H, Moilanen E (2005) Nitric oxide production and signaling in inflammation. Curr Drug Targets Inflamm Allergy 4:471–479

Kortenkamp A, Faust M, Scholze M, Backhaus T (2007) Low-level exposure to multiple chemicals: reason for human health concerns? Environ Health Perspect 115 Suppl 1:106–114. https://doi.org/10.1289/ehp.9358

Kouadio JH, Mobio TA, Baudrimont I, et al (2005) Comparative study of cytotoxicity and oxidative stress induced by deoxynivalenol, zearalenone or fumonisin B1 in human intestinal cell line Caco-2. Toxicology 213:56–65. https://doi.org/10.1016/j.tox.2005.05.010

Krause P, Morris V, Greenbaum JA, et al (2015) IL-10-producing intestinal macrophages prevent excessive antibacterial innate immunity by limiting IL-23 synthesis. Nat Commun 6:1–12. https://doi.org/10.1038/ncomms8055

Krishnaswamy R, Devaraj SN, Padma VV (2010) Lutein protects HT-29 cells against Deoxynivalenol-induced oxidative stress and apoptosis: prevention of NF-kappaB nuclear localization and down regulation of NF-kappaB and Cyclo-Oxygenase-2 expression. Free Radic Biol Med 49:50–60. https://doi.org/10.1016/j.freeradbiomed.2010.03.016

Låg M, Rodionov D, Øvrevik J, et al (2010) Cadmium-induced inflammatory responses in cells relevant for lung toxicity: Expression and release of cytokines in fibroblasts, epithelial cells and macrophages. Toxicology Letters 193:252–260. https://doi.org/10.1016/j.toxlet.2010.01.015

Lautert C, Ferreiro L, Wolkmer P, et al (2014) Individual in vitro effects of ochratoxin A, deoxynivalenol and zearalenone on oxidative stress and acetylcholinesterase in lymphocytes of broiler chickens. Springerplus 3:506. https://doi.org/10.1186/2193-1801-3-506

Le T-H, Alassane-Kpembi I, Oswald IP, Pinton P (2018) Analysis of the interactions between environmental and food contaminants, cadmium and deoxynivalenol, in different target organs. The Science of the total environment 622–623:841–848. https://doi.org/10.1016/j.scitotenv.2017.12.014

Lea T (2015) Caco-2 Cell Line. In: Verhoeckx K, Cotter P, López-Expósito I, et al. (eds) The Impact of Food Bioactives on Health: in vitro and ex vivo models. Springer International Publishing, Cham, pp 103–111

Lee J-C, Son Y-O, Pratheeshkumar P, Shi X (2012) Oxidative stress and metal carcinogenesis. Free Radic Biol Med 53:742–757. https://doi.org/10.1016/j.freeradbiomed.2012.06.002

Lee J-Y, Tokumoto M, Hattori Y, et al (2016) Different Regulation of p53 Expression by Cadmium Exposure in Kidney, Liver, Intestine, Vasculature, and Brain Astrocytes. Toxicol Res 32:73–80. https://doi.org/10.5487/TR.2016.32.1.073

Lei S, Cheng T, Guo Y, et al (2014) Somatostatin ameliorates lipopolysaccharide-induced tight junction damage via the ERK-MAPK pathway in Caco2 cells. Eur J Cell Biol 93:299–307. https://doi.org/10.1016/j.ejcb.2014.05.003

Lei YX, Lu Q, Shao C, et al (2015) Expression profiles of DNA repair-related genes in rat target organs under subchronic cadmium exposure. Genet Mol Res 14:515–524. https://doi.org/10.4238/2015.January.26.5

Lentini P, Zanoli L, Granata A, et al (2017) Kidney and heavy metals - The role of environmental exposure (Review). Molecular Medicine Reports 15:3413–3419. https://doi.org/10.3892/mmr.2017.6389

Lessard M, Savard C, Deschene K, et al (2015) Impact of deoxynivalenol (DON) contaminated feed on intestinal integrity and immune response in swine. Food Chem Toxicol 80:7–16. https://doi.org/10.1016/j.fct.2015.02.013

Li M, Pestka JJ (2008) Comparative induction of 28S ribosomal RNA cleavage by ricin and the trichothecenes deoxynivalenol and T-2 toxin in the macrophage. Toxicol Sci 105:67–78. https://doi.org/10.1093/toxsci/kfn111

Li W, Gu X, Zhang X, et al (2015) Cadmium delays non-homologous end joining (NHEJ) repair via inhibition of DNA-PKcs phosphorylation and downregulation of XRCC4 and Ligase IV. Mutat Res 779:112–123. https://doi.org/10.1016/j.mrfmmm.2015.07.002

Li Y, Liu K, Shen J, Liu Y (2016) Wheat bran intake can attenuate chronic cadmium toxicity in mice gut microbiota. Food Funct 7:3524–3530. https://doi.org/10.1039/c6fo00233a

Liang Z, Ren Z, Gao S, et al (2015) Individual and combined effects of deoxynivalenol and zearalenone on mouse kidney. Environmental Toxicology and Pharmacology 40:686–691. https://doi.org/10.1016/j.etap.2015.08.029

Liao P, Liao M, Li L, et al (2017) Effect of deoxynivalenol on apoptosis, barrier function, and expression levels of genes involved in nutrient transport, mitochondrial biogenesis and function in IPEC-J2 cells. Toxicol Res (Camb) 6:866–877. https://doi.org/10.1039/c7tx00202e

Liao Y, Peng Z, Chen L, et al (2018) Deoxynivalenol, gut microbiota and immunotoxicity: A potential approach? Food Chem Toxicol 112:342–354. https://doi.org/10.1016/j.fct.2018.01.013

Ling K-H, Wan MLY, El-Nezami H, Wang M (2016) Protective Capacity of Resveratrol, a Natural Polyphenolic Compound, against Deoxynivalenol-Induced Intestinal Barrier Dysfunction and Bacterial Translocation. Chem Res Toxicol 29:823–833. https://doi.org/10.1021/acs.chemrestox.6b00001

Liu J, Qu W, Kadiiska MB (2009) Role of oxidative stress in cadmium toxicity and carcinogenesis. Toxicol Appl Pharmacol 238:209–214. https://doi.org/10.1016/j.taap.2009.01.029

Liu L, Tao R, Huang J, et al (2015) Hepatic oxidative stress and inflammatory responses with cadmium exposure in male mice. Environ Toxicol Pharmacol 39:229–236. https://doi.org/10.1016/j.etap.2014.11.029

Liu T, He W, Yan C, et al (2011) Roles of reactive oxygen species and mitochondria in cadmium-induced injury of liver cells. Toxicol Ind Health 27:249–256. https://doi.org/10.1177/0748233710386408

Liu Y, Li Y, Liu K, Shen J (2014) Exposing to cadmium stress cause profound toxic effect on microbiota of the mice intestinal tract. PLoS ONE 9:e85323. https://doi.org/10.1371/journal.pone.0085323

Lu Z, Ding L, Lu Q, Chen Y-H (2013) Claudins in intestines: Distribution and functional significance in health and diseases. Tissue Barriers 1:e24978. https://doi.org/10.4161/tisb.24978

Lucioli J, Pinton P, Callu P, et al (2013) The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: interest of ex vivo models as an alternative to in vivo experiments. Toxicon 66:31–36. https://doi.org/10.1016/j.toxicon.2013.01.024

Lucke A, Böhm J, Zebeli Q, Metzler-Zebeli BU (2018) Dietary Deoxynivalenol Contamination and Oral Lipopolysaccharide Challenge Alters the Cecal Microbiota of Broiler Chickens. Front Microbiol 9:804. https://doi.org/10.3389/fmicb.2018.00804

Lukowicz C, Ellero-Simatos S, Régnier M, et al (2018) Metabolic Effects of a Chronic Dietary Exposure to a Low-Dose Pesticide Cocktail in Mice: Sexual Dimorphism and Role of the Constitutive Androstane Receptor. Environmental health perspectives 126:067007. https://doi.org/10.1289/EHP2877

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

Madara JL, Stafford J (1989) Interferon-gamma directly affects barrier function of cultured intestinal epithelial monolayers. J Clin Invest 83:724–727

Manda G, Mocanu MA, Marin DE, Taranu I (2015) Dual effects exerted in vitro by micromolar concentrations of deoxynivalenol on undifferentiated caco-2 cells. Toxins (Basel) 7:593–603. https://doi.org/10.3390/toxins7020593

Mankevičienė A, Jablonskytė-Raščė D, Maikštėnienė S (2014) Occurrence of mycotoxins in spelt and common wheat grain and their products. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 31:132–138. https://doi.org/10.1080/19440049.2013.861614

Mantha M, Jumarie C (2010) Cadmium-induced hormetic effect in differentiated Caco-2 cells: ERK and p38 activation without cell proliferation stimulation. J Cell Physiol 224:250–261. https://doi.org/10.1002/jcp.22128

Maresca M (2013) From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. Toxins (Basel) 5:784–820. https://doi.org/10.3390/toxins5040784

Maresca M, Mahfoud R, Garmy N, Fantini J (2002) The mycotoxin deoxynivalenol affects nutrient absorption in human intestinal epithelial cells. J Nutr 132:2723–2731. https://doi.org/10.1093/jn/132.9.2723

Maresca M, Yahi N, Younès-Sakr L, et al (2008) Both direct and indirect effects account for the pro-inflammatory activity of enteropathogenic mycotoxins on the human intestinal epithelium: stimulation of interleukin-8 secretion, potentiation of interleukin-1beta effect and increase in the transepithelial passage of commensal bacteria. Toxicol Appl Pharmacol 228:84–92. https://doi.org/10.1016/j.taap.2007.11.013

Margerit A, Lecomte-Pradines C, Svendsen C, et al (2015) Nested interactions in the combined toxicity of uranium and cadmium to the nematode Caenorhabditis elegans. Ecotoxicol Environ Saf 118:139–148. https://doi.org/10.1016/j.ecoenv.2015.04.023

Matović V, Buha A, Đukić-Ćosić D, Bulat Z (2015) Insight into the oxidative stress induced by lead and/or cadmium in blood, liver and kidneys. Food and Chemical Toxicology 78:130–140. https://doi.org/10.1016/j.fct.2015.02.011

Mayer E, Novak B, Springler A, et al (2017) Effects of deoxynivalenol (DON) and its microbial biotransformation product deepoxy-deoxynivalenol (DOM-1) on a trout, pig, mouse,

and human cell line. Mycotoxin Res 33:297–308. https://doi.org/10.1007/s12550-017-0289-7

McConnell EL, Basit AW, Murdan S (2008) Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments. J Pharm Pharmacol 60:63–70. https://doi.org/10.1211/jpp.60.1.0008

McCormick SP, Stanley AM, Stover NA, Alexander NJ (2011) Trichothecenes: From Simple to Complex Mycotoxins. Toxins (Basel) 3:802–814. https://doi.org/10.3390/toxins3070802

Michałowicz J (2014) Bisphenol A – Sources, toxicity and biotransformation. Environmental Toxicology and Pharmacology 37:738–758. https://doi.org/10.1016/j.etap.2014.02.003

Miller MF, Goodson WH, Manjili MH, et al (2017) Low-Dose Mixture Hypothesis of Carcinogenesis Workshop: Scientific Underpinnings and Research Recommendations. Environmental health perspectives 125:163–169. https://doi.org/10.1289/EHP411

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

Mishra S, Dwivedi PD, Pandey HP, Das M (2014) Role of oxidative stress in Deoxynivalenol induced toxicity. Food Chem Toxicol 72:20–29. https://doi.org/10.1016/j.fct.2014.06.027

Mitic LL, Anderson JM (1998) Molecular architecture of tight junctions. Annu Rev Physiol 60:121–142. https://doi.org/10.1146/annurev.physiol.60.1.121

Moldal T, Bernhoft A, Rosenlund G, et al (2018) Dietary Deoxynivalenol (DON) May Impair the Epithelial Barrier and Modulate the Cytokine Signaling in the Intestine of Atlantic Salmon (Salmo salar). Toxins 10:376. https://doi.org/10.3390/toxins10090376

Moon Y, Yang H, Lee SH (2007) Modulation of early growth response gene 1 and interleukin-8 expression by ribotoxin deoxynivalenol (vomitoxin) via ERK1/2 in human epithelial intestine 407 cells. Biochem Biophys Res Commun 362:256–262. https://doi.org/10.1016/j.bbrc.2007.07.168

More SJ, Hardy A, Bampidis V, et al (2019) Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. EFSA Journal 17:. https://doi.org/10.2903/j.efsa.2019.5634

Moret I, Cerrillo E, Navarro-Puche A, et al (2014) [Oxidative stress in Crohn's disease]. Gastroenterol Hepatol 37:28–34. https://doi.org/10.1016/j.gastrohep.2013.01.008

Morhardt TL, Hayashi A, Ochi T, et al (2019) IL-10 produced by macrophages regulates epithelial integrity in the small intestine. Sci Rep 9:1–10. https://doi.org/10.1038/s41598-018-38125-x

Ninkov M, Popov Aleksandrov A, Demenesku J, et al (2015) Toxicity of oral cadmium intake: Impact on gut immunity. Toxicol Lett 237:89–99. https://doi.org/10.1016/j.toxlet.2015.06.002

Ninkov M, Popov Aleksandrov A, Mirkov I, et al (2016) Strain differences in toxicity of oral cadmium intake in rats. Food Chem Toxicol 96:11–23. https://doi.org/10.1016/j.fct.2016.07.021

Olszowski T, Baranowska-Bosiacka I, Gutowska I, Chlubek D (2012) Pro-inflammatory properties of cadmium. Acta Biochim Pol 59:475–482

Orr SE, Bridges CC (2017) Chronic Kidney Disease and Exposure to Nephrotoxic Metals. Int J Mol Sci 18:. https://doi.org/10.3390/ijms18051039

Osselaere A, Santos R, Hautekiet V, et al (2013) Deoxynivalenol impairs hepatic and intestinal gene expression of selected oxidative stress, tight junction and inflammation proteins in broiler chickens, but addition of an adsorbing agent shifts the effects to the distal parts of the small intestine. PLoS ONE 8:e69014. https://doi.org/10.1371/journal.pone.0069014

Oyinloye BE, Adenowo AF, Kappo AP (2015) Reactive oxygen species, apoptosis, antimicrobial peptides and human inflammatory diseases. Pharmaceuticals (Basel) 8:151–175. https://doi.org/10.3390/ph8020151

Palone F, Vitali R, Cucchiara S, et al (2014) Role of HMGB1 as a suitable biomarker of subclinical intestinal inflammation and mucosal healing in patients with inflammatory

bowel disease. Inflamm Bowel Dis 20:1448–1457. https://doi.org/10.1097/MIB.0000000000000113

Palus J, Rydzynski K, Dziubaltowska E, et al (2003) Genotoxic effects of occupational exposure to lead and cadmium. Mutation Research/Genetic Toxicology and Environmental Mutagenesis 540:19–28. https://doi.org/10.1016/S1383-5718(03)00167-0

Park JD, Cherrington NJ, Klaassen CD (2002) Intestinal absorption of cadmium is associated with divalent metal transporter 1 in rats. Toxicol Sci 68:288–294. https://doi.org/10.1093/toxsci/68.2.288

Paszti-Gere E, Csibrik-Nemeth E, Szeker K, et al (2012) Acute oxidative stress affects IL-8 and TNF-α expression in IPEC-J2 porcine epithelial cells. Inflammation 35:994–1004. https://doi.org/10.1007/s10753-011-9403-8

Patra RC, Rautray AK, Swarup D (2011) Oxidative Stress in Lead and Cadmium Toxicity and Its Amelioration. Vet Med Int 2011:. https://doi.org/10.4061/2011/457327

Payros D, Alassane-Kpembi I, Pierron A, et al (2016) Toxicology of deoxynivalenol and its acetylated and modified forms. Arch Toxicol 90:2931–2957. https://doi.org/10.1007/s00204-016-1826-4

Payros D, Dobrindt U, Martin P, et al (2017) The Food Contaminant Deoxynivalenol Exacerbates the Genotoxicity of Gut Microbiota. MBio 8:. https://doi.org/10.1128/mBio.00007-17

Pestka JJ (2008) Mechanisms of deoxynivalenol-induced gene expression and apoptosis. Food Addit Contam Part A Chem Anal Control Expo Risk Assess 25:1128–1140

Pestka JJ (2010a) Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Arch Toxicol 84:663–679. https://doi.org/10.1007/s00204-010-0579-8

Pestka JJ (2007) Deoxynivalenol: Toxicity, mechanisms and animal health risks. Animal Feed Science and Technology 137:283–298. https://doi.org/10.1016/j.anifeedsci.2007.06.006

Pestka JJ (2010b) Deoxynivalenol-induced proinflammatory gene expression: mechanisms and pathological sequelae. Toxins (Basel) 2:1300–1317. https://doi.org/10.3390/toxins2061300

Pestka JJ, Zhou H-R, Moon Y, Chung YJ (2004) Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. Toxicol Lett 153:61–73. https://doi.org/10.1016/j.toxlet.2004.04.023

Pierron A, Bracarense APFL, Cossalter A-M, et al (2018) Deepoxy-deoxynivalenol retains some immune-modulatory properties of the parent molecule deoxynivalenol in piglets. Archives of Toxicology 92:3381–3389. https://doi.org/10.1007/s00204-018-2293-x

Pierron A, Mimoun S, Murate LS, et al (2016a) Microbial biotransformation of DON: molecular basis for reduced toxicity. Sci Rep 6:29105. https://doi.org/10.1038/srep29105

Pierron A, Mimoun S, Murate LS, et al (2016b) Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-glucoside. Arch Toxicol 90:2037–2046. https://doi.org/10.1007/s00204-015-1592-8

Pinton P, Accensi F, Beauchamp E, et al (2008) Ingestion of deoxynivalenol (DON) contaminated feed alters the pig vaccinal immune responses. Toxicol Lett 177:215–222. https://doi.org/10.1016/j.toxlet.2008.01.015

Pinton P, Braicu C, Nougayrede J-P, et al (2010) Deoxynivalenol impairs porcine intestinal barrier function and decreases the protein expression of claudin-4 through a mitogen-activated protein kinase-dependent mechanism. J Nutr 140:1956–1962. https://doi.org/10.3945/jn.110.123919

Pinton P, Nougayrède J-P, Del Rio J-C, et al (2009) The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression. Toxicol Appl Pharmacol 237:41–48. https://doi.org/10.1016/j.taap.2009.03.003

Pinton P, Oswald IP (2014) Effect of deoxynivalenol and other Type B trichothecenes on the intestine: a review. Toxins (Basel) 6:1615–1643. https://doi.org/10.3390/toxins6051615

Pinton P, Tsybulskyy D, Lucioli J, et al (2012) Toxicity of deoxynivalenol and its acetylated derivatives on the intestine: differential effects on morphology, barrier function, tight junction proteins, and mitogen-activated protein kinases. Toxicol Sci 130:180–190. https://doi.org/10.1093/toxsci/kfs239

Proctor RH, McCormick SP, Kim H-S, et al (2018) Evolution of structural diversity of trichothecenes, a family of toxins produced by plant pathogenic and entomopathogenic fungi. PLOS Pathogens 14:e1006946. https://doi.org/10.1371/journal.ppat.1006946

Qin L, Huang W, Mo X, et al (2015) LPS Induces Occludin Dysregulation in Cerebral Microvascular Endothelial Cells via MAPK Signaling and Augmenting MMP-2 Levels. Oxid Med Cell Longev 2015:. https://doi.org/10.1155/2015/120641

Qin W, Bazeille N, Henry E, et al (2016) Mechanistic insight into cadmium-induced inactivation of the Bloom protein. Scientific Reports 6:26225. https://doi.org/10.1038/srep26225

Qu W, Fuquay R, Sakurai T, Waalkes MP (2006) Acquisition of apoptotic resistance in cadmium-induced malignant transformation: specific perturbation of JNK signal transduction pathway and associated metallothionein overexpression. Mol Carcinog 45:561–571. https://doi.org/10.1002/mc.20185

Qu W, Ke H, Pi J, et al (2007) Acquisition of apoptotic resistance in cadmium-transformed human prostate epithelial cells: Bcl-2 overexpression blocks the activation of JNK signal transduction pathway. Environ Health Perspect 115:1094–1100. https://doi.org/10.1289/ehp.10075

Ran J, Wang D, Wang C, et al (2016) Heavy metal contents, distribution, and prediction in a regional soil-wheat system. Sci Total Environ 544:422–431. https://doi.org/10.1016/j.scitotenv.2015.11.105

Rana MN, Tangpong J, Rahman MdM (2018) Toxicodynamics of Lead, Cadmium, Mercury and Arsenic- induced kidney toxicity and treatment strategy: A mini review. Toxicology Reports 5:704–713. https://doi.org/10.1016/j.toxrep.2018.05.012

Rani A, Kumar A, Lal A, Pant M (2014) Cellular mechanisms of cadmium-induced toxicity: a review. Int J Environ Health Res 24:378–399. https://doi.org/10.1080/09603123.2013.835032

Rao JN, Wang J-Y (2010) Intestinal Architecture and Development. Morgan & Claypool Life Sciences

Rao RK, Basuroy S, Rao VU, et al (2002) Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. Biochem J 368:471–481. https://doi.org/10.1042/BJ20011804

Rather IA, Koh WY, Paek WK, Lim J (2017) The Sources of Chemical Contaminants in Food and Their Health Implications. Frontiers in Pharmacology 8:830. https://doi.org/10.3389/fphar.2017.00830

Razafimanjato H, Benzaria A, Taïeb N, et al (2011) The ribotoxin deoxynivalenol affects the viability and functions of glial cells. Glia 59:1672–1683. https://doi.org/10.1002/glia.21214

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

Roesijadi G, Rezvankhah S, Perez-Matus A, et al (2009) Dietary cadmium and benzo(a)pyrene increased intestinal metallothionein expression in the fish Fundulus heteroclitus. Mar Environ Res 67:25–30. https://doi.org/10.1016/j.marenvres.2008.10.002

Rossi A, Poverini R, Di Lullo G, et al (1996) Heavy metal toxicity following apical and basolateral exposure in the human intestinal cell line Caco-2. Toxicol In Vitro 10:27–36

Rusanov AL, Smirnova AV, Poromov AA, et al (2015) Effects of cadmium chloride on the functional state of human intestinal cells. Toxicol In Vitro 29:1006–1011. https://doi.org/10.1016/j.tiv.2015.03.018

Ruttkay-Nedecky B, Nejdl L, Gumulec J, et al (2013) The role of metallothionein in oxidative stress. Int J Mol Sci 14:6044–6066. https://doi.org/10.3390/ijms14036044

Ryu D-Y, Lee S-J, Park DW, et al (2004) Dietary iron regulates intestinal cadmium absorption through iron transporters in rats. Toxicology Letters 152:19–25. https://doi.org/10.1016/j.toxlet.2004.03.015

Sahu SC, Garthoff LH, Robl MG, et al (2008) Rat liver clone-9 cells in culture as a model for screening hepatotoxic potential of food-related products: hepatotoxicity of deoxynivalenol. J Appl Toxicol 28:765–772. https://doi.org/10.1002/jat.1337

Saint-Cyr MJ, Perrin-Guyomard A, Houée P, et al (2013) Evaluation of an oral subchronic exposure of deoxynivalenol on the composition of human gut microbiota in a model of human microbiota-associated rats. PLoS ONE 8:e80578. https://doi.org/10.1371/journal.pone.0080578

Schaaf GJ, Nijmeijer SM, Maas RFM, et al (2002) The role of oxidative stress in the ochratoxin A-mediated toxicity in proximal tubular cells. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 1588:149–158. https://doi.org/10.1016/S0925-4439(02)00159-X

Schneeberger EE, Lynch RD (2004) The tight junction: a multifunctional complex. American Journal of Physiology-Cell Physiology 286:C1213–C1228. https://doi.org/10.1152/ajpcell.00558.2003

Schuhmann D, Godoy P, Weiß C, et al (2011) Interfering with interferon-γ signalling in intestinal epithelial cells: selective inhibition of apoptosis-maintained secretion of anti-inflammatory interleukin-18 binding protein. Clin Exp Immunol 163:65–76. https://doi.org/10.1111/j.1365-2249.2010.04250.x

Schulkens IA, Castricum KCM, Weijers EM, et al (2014) Expression, regulation and function of human metallothioneins in endothelial cells. J Vasc Res 51:231–238. https://doi.org/10.1159/000365550

Schumann M, Günzel D, Buergel N, et al (2012) Cell polarity-determining proteins Par-3 and PP-1 are involved in epithelial tight junction defects in coeliac disease. Gut 61:220–228. https://doi.org/10.1136/gutjnl-2011-300123

Sergent T, Parys M, Garsou S, et al (2006) Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. Toxicol Lett 164:167–176. https://doi.org/10.1016/j.toxlet.2005.12.006

Shen HM, Shi CY, Lee HP, Ong CN (1994) Aflatoxin B1-Induced Lipid Peroxidation in Rat Liver. Toxicology and Applied Pharmacology 127:145–150. https://doi.org/10.1006/taap.1994.1148

Shen Y, Hirsch DS, Sasiela CA, Wu WJ (2008) Cdc42 regulates E-cadherin ubiquitination and degradation through an epidermal growth factor receptor to Src-mediated pathway. The Journal of biological chemistry 283:5127–37. https://doi.org/10.1074/jbc.M703300200

Shi Y, Porter K, Parameswaran N, et al (2009) Role of GRP78/BiP degradation and ER stress in deoxynivalenol-induced interleukin-6 upregulation in the macrophage. Toxicol Sci 109:247–255. https://doi.org/10.1093/toxsci/kfp060

Si W, Liu J, Cai L, et al (2015) Health risks of metals in contaminated farmland soils and spring wheat irrigated with Yellow River water in Baotou, China. Bull Environ Contam Toxicol 94:214–219. https://doi.org/10.1007/s00128-014-1435-y

Silins I, Högberg J (2011a) Combined toxic exposures and human health: biomarkers of exposure and effect. International journal of environmental research and public health 8:629–47. https://doi.org/10.3390/ijerph8030629

Silins I, Högberg J (2011b) Combined toxic exposures and human health: biomarkers of exposure and effect. International journal of environmental research and public health 8:629–47. https://doi.org/10.3390/ijerph8030629

Sirot V, Fremy J-M, Leblanc J-C (2013) Dietary exposure to mycotoxins and health risk assessment in the second French total diet study. Food Chem Toxicol 52:1–11. https://doi.org/10.1016/j.fct.2012.10.036

Smyth D, Leung G, Fernando M, McKay DM (2012) Reduced surface expression of epithelial E-cadherin evoked by interferon-gamma is Fyn kinase-dependent. PLoS ONE 7:e38441. https://doi.org/10.1371/journal.pone.0038441

Sobral MMC, Faria MA, Cunha SC, Ferreira IMPLVO (2018) Toxicological interactions between mycotoxins from ubiquitous fungi: Impact on hepatic and intestinal human epithelial cells. Chemosphere 202:538–548. https://doi.org/10.1016/j.chemosphere.2018.03.122

Sobrova P, Vasatkova A, Skladanka J, et al (2012) Study of deoxynivalenol effect on metallothionein and glutathione levels, antioxidant capacity, and glutathione-S-transferase and liver enzymes activity in rats. Chem Pap 66:1092–1102. https://doi.org/10.2478/s11696-012-0229-0

Springler A, Hessenberger S, Schatzmayr G, Mayer E (2016) Early Activation of MAPK p44/42 Is Partially Involved in DON-Induced Disruption of the Intestinal Barrier Function and Tight Junction Network. Toxins 8:264. https://doi.org/10.3390/toxins8090264

Srinivasan B, Kolli AR, Esch MB, et al (2015) TEER measurement techniques for in vitro barrier model systems. J Lab Autom 20:107–126. https://doi.org/10.1177/2211068214561025

Sun H, Chow EC, Liu S, et al (2008) The Caco-2 cell monolayer: usefulness and limitations. Expert Opin Drug Metab Toxicol 4:395–411. https://doi.org/10.1517/17425255.4.4.395

Suzuki T (2013) Regulation of intestinal epithelial permeability by tight junctions. Cell Mol Life Sci 70:631–659. https://doi.org/10.1007/s00018-012-1070-x

Suzuki T, Elias BC, Seth A, et al (2009) PKC eta regulates occludin phosphorylation and epithelial tight junction integrity. Proc Natl Acad Sci USA 106:61–66. https://doi.org/10.1073/pnas.0802741106

Tatay E, Espín S, García-Fernández A-J, Ruiz M-J (2018) Estrogenic activity of zearalenone, α-zearalenol and β-zearalenol assessed using the E-screen assay in MCF-7 cells. Toxicol Mech Methods 28:239–242. https://doi.org/10.1080/15376516.2017.1395501

Teixeira FB, de Oliveira ACA, Leão LKR, et al (2018) Exposure to Inorganic Mercury Causes Oxidative Stress, Cell Death, and Functional Deficits in the Motor Cortex. Front Mol Neurosci 11:. https://doi.org/10.3389/fnmol.2018.00125

Templeton DM, Liu Y (2013) Effects of cadmium on the actin cytoskeleton in renal mesangial cells. Canadian Journal of Physiology and Pharmacology 91:1–7. https://doi.org/10.1139/cjpp-2012-0229

Terciolo C, Dapoigny M, Andre F (2019) Beneficial effects of Saccharomyces boulardii CNCM I-745 on clinical disorders associated with intestinal barrier disruption. Clinical and Experimental Gastroenterology Volume 12:67–82. https://doi.org/10.2147/CEG.S181590

Teshfam M, Gharagozlou MJ, Salaramoli J, Hassanpour H (2006) Morphological alterations of the small intestine mucosa following oral administration of cadmium in broiler chickens. J Appl Anim Res 29:65–68. https://doi.org/10.1080/09712119.2006.9706573

Thompson J, Bannigan J (2008) Cadmium: toxic effects on the reproductive system and the embryo. Reprod Toxicol 25:304–315. https://doi.org/10.1016/j.reprotox.2008.02.001

Thrupp TJ, Runnalls TJ, Scholze M, et al (2018) The consequences of exposure to mixtures of chemicals: Something from 'nothing' and 'a lot from a little' when fish are exposed to steroid hormones. Science of The Total Environment 619–620:1482–1492. https://doi.org/10.1016/J.SCITOTENV.2017.11.081

Thursby E, Juge N (2017) Introduction to the human gut microbiota. Biochem J 474:1823–1836. https://doi.org/10.1042/BCJ20160510

Tinkov AA, Gritsenko VA, Skalnaya MG, et al (2018) Gut as a target for cadmium toxicity. Environ Pollut 235:429–434. https://doi.org/10.1016/j.envpol.2017.12.114

Tobwala S, Wang H-J, Carey JW, et al (2014) Effects of Lead and Cadmium on Brain Endothelial Cell Survival, Monolayer Permeability, and Crucial Oxidative Stress Markers in an in Vitro Model of the Blood-Brain Barrier. Toxics 2:258–275. https://doi.org/10.3390/toxics2020258

Tsuru A, Imai Y, Saito M, Kohno K (2016) Novel mechanism of enhancing IRE1α-XBP1 signalling via the PERK-ATF4 pathway. Sci Rep 6:24217. https://doi.org/10.1038/srep24217

Turner PC, Rothwell JA, White KLM, et al (2008) Urinary deoxynivalenol is correlated with cereal intake in individuals from the United kingdom. Environmental health perspectives 116:21–5. https://doi.org/10.1289/ehp.10663

Valko M, Morris H, Cronin MTD (2005) Metals, toxicity and oxidative stress. Curr Med Chem 12:1161–1208

Valverde M, Trejo C, Rojas E (2001) Is the capacity of lead acetate and cadmium chloride to induce genotoxic damage due to direct DNA-metal interaction? Mutagenesis 16:265–270

Vandenbroucke V, Croubels S, Martel A, et al (2011) The Mycotoxin Deoxynivalenol Potentiates Intestinal Inflammation by Salmonella Typhimurium in Porcine Ileal Loops. PLOS ONE 6:e23871. https://doi.org/10.1371/journal.pone.0023871

Vanheule A, Audenaert K, De Boevre M, et al (2014) The compositional mosaic of Fusarium species and their mycotoxins in unprocessed cereals, food and feed products in Belgium. Int J Food Microbiol 181:28–36. https://doi.org/10.1016/j.ijfoodmicro.2014.04.012

Vasatkova A, Krizova S, Adam V, et al (2009) Changes in metallothionein level in rat hepatic tissue after administration of natural mouldy wheat. Int J Mol Sci 10:1138–1160. https://doi.org/10.3390/ijms10031138

Verma RJ (2004) Aflatoxin Cause DNA Damage. International Journal of Human Genetics 4:231–236. https://doi.org/10.1080/09723757.2004.11885899

Vesey DA (2010) Transport pathways for cadmium in the intestine and kidney proximal tubule: focus on the interaction with essential metals. Toxicol Lett 198:13–19. https://doi.org/10.1016/j.toxlet.2010.05.004

Vidal A, Claeys L, Mengelers M, et al (2018) Humans significantly metabolize and excrete the mycotoxin deoxynivalenol and its modified form deoxynivalenol-3-glucoside within 24 hours. Sci Rep 8:. https://doi.org/10.1038/s41598-018-23526-9

Videmann B, Tep J, Cavret S, Lecoeur S (2007) Epithelial transport of deoxynivalenol: Involvement of human P-glycoprotein (ABCB1) and multidrug resistance-associated

protein 2 (ABCC2). Food and Chemical Toxicology 45:1938–1947. https://doi.org/10.1016/j.fct.2007.04.011

Vignal C, Djouina M, Pichavant M, et al (2018) Chronic ingestion of deoxynivalenol at human dietary levels impairs intestinal homeostasis and gut microbiota in mice. Arch Toxicol 92:2327–2338. https://doi.org/10.1007/s00204-018-2228-6

Vitali R, Palone F, Pierdomenico M, et al (2015) Dipotassium glycyrrhizate via HMGB1 or AMPK signaling suppresses oxidative stress during intestinal inflammation. Biochem Pharmacol 97:292–299. https://doi.org/10.1016/j.bcp.2015.07.039

Voss KA, Riley RT (2013) Fumonisin Toxicity and Mechanism of Action: Overview and Current Perspectives. Food Safety 1:2013006–2013006. https://doi.org/10.14252/foodsafetyfscj.2013006

Vrieze A, Holleman F, Zoetendal EG, et al (2010) The environment within: how gut microbiota may influence metabolism and body composition. Diabetologia 53:606–613. https://doi.org/10.1007/s00125-010-1662-7

Waché YJ, Valat C, Postollec G, et al (2009) Impact of deoxynivalenol on the intestinal microflora of pigs. Int J Mol Sci 10:1–17. https://doi.org/10.3390/ijms10010001

Wang H, Engstrom AK, Xia Z (2017) Cadmium impairs the survival and proliferation of cultured adult subventricular neural stem cells through activation of the JNK and p38 MAP kinases. Toxicology 380:30–37. https://doi.org/10.1016/j.tox.2017.01.013

Wang H, Zhang W, Zuo L, et al (2014a) Intestinal dysbacteriosis contributes to decreased intestinal mucosal barrier function and increased bacterial translocation. Lett Appl Microbiol 58:384–392. https://doi.org/10.1111/lam.12201

Wang J, Zhu H, Liu X, Liu Z (2014b) Oxidative stress and Ca(2+) signals involved on cadmium-induced apoptosis in rat hepatocyte. Biol Trace Elem Res 161:180–189. https://doi.org/10.1007/s12011-014-0105-6

Wang L, Wang H, Hu M, et al (2009) Oxidative stress and apoptotic changes in primary cultures of rat proximal tubular cells exposed to lead. Arch Toxicol 83:417–427. https://doi.org/10.1007/s00204-009-0425-z

Wang S, Shi X (2001) Molecular mechanisms of metal toxicity and carcinogenesis. Mol Cell Biochem 222:3–9

Wang X, Xu W, Fan M, et al (2016) Deoxynivalenol induces apoptosis in PC12 cells via the mitochondrial pathway. Environmental Toxicology and Pharmacology 43:193–202. https://doi.org/10.1016/j.etap.2016.03.016

Waśkiewicz A, Beszterda M, Kostecki M, et al (2014) Deoxynivalenol in the gastrointestinal tract of immature gilts under per os toxin application. Toxins (Basel) 6:973–987. https://doi.org/10.3390/toxins6030973

Wek RC, Jiang H-Y, Anthony TG (2006) Coping with stress: eIF2 kinases and translational control. Biochem Soc Trans 34:7–11. https://doi.org/10.1042/BST20060007

Wells JM, Brummer RJ, Derrien M, et al (2017) Homeostasis of the gut barrier and potential biomarkers. Am J Physiol Gastrointest Liver Physiol 312:G171–G193. https://doi.org/10.1152/ajpgi.00048.2015

White SS, Birnbaum LS (2009) An Overview of the Effects of Dioxins and Dioxin-like Compounds on Vertebrates, as Documented in Human and Ecological Epidemiology. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 27:197–211. https://doi.org/10.1080/10590500903310047

Wu Q-H, Wang X, Yang W, et al (2014) Oxidative stress-mediated cytotoxicity and metabolism of T-2 toxin and deoxynivalenol in animals and humans: an update. Arch Toxicol 88:1309–1326. https://doi.org/10.1007/s00204-014-1280-0

Wu S, Liu Y, Duan Y, et al (2018) Intestinal toxicity of deoxynivalenol is limited by supplementation with Lactobacillus plantarum JM113 and consequentially altered gut microbiota in broiler chickens. Journal of Animal Science and Biotechnology 9:74. https://doi.org/10.1186/s40104-018-0286-5

Xu M-Y, Wang P, Sun Y-J, et al (2017) Joint toxicity of chlorpyrifos and cadmium on the oxidative stress and mitochondrial damage in neuronal cells. Food Chem Toxicol 103:246–252. https://doi.org/10.1016/j.fct.2017.03.013

Yang R, Harada T, Li J, et al (2005) Bile modulates intestinal epithelial barrier function via an extracellular signal related kinase 1/2 dependent mechanism. Intensive Care Med 31:709–717. https://doi.org/10.1007/s00134-005-2601-9

Yang W, Yu M, Fu J, et al (2014) Deoxynivalenol induced oxidative stress and genotoxicity in human peripheral blood lymphocytes. Food and Chemical Toxicology 64:383–396. https://doi.org/10.1016/j.fct.2013.12.012

Ye D, Ma I, Ma TY (2006) Molecular mechanism of tumor necrosis factor-alpha modulation of intestinal epithelial tight junction barrier. Am J Physiol Gastrointest Liver Physiol 290:G496-504. https://doi.org/10.1152/ajpgi.00318.2005

Yoshinari T, Takeuchi H, Aoyama K, et al (2014) Occurrence of four Fusarium mycotoxins, deoxynivalenol, zearalenone, T-2 toxin, and HT-2 toxin, in wheat, barley, and Japanese retail food. J Food Prot 77:1940–1946. https://doi.org/10.4315/0362-028X.JFP-14-185

Yu M, Chen L, Peng Z, et al (2017) Mechanism of deoxynivalenol effects on the reproductive system and fetus malformation: Current status and future challenges. Toxicology in Vitro 41:150–158. https://doi.org/10.1016/j.tiv.2017.02.011

Yu X, Sidhu JS, Hong S, et al (2011) Cadmium induced p53-dependent activation of stress signaling, accumulation of ubiquitinated proteins, and apoptosis in mouse embryonic fibroblast cells. Toxicol Sci 120:403–412. https://doi.org/10.1093/toxsci/kfr010

Yuan Y, Jiang C, Hu F, et al (2015) The role of mitogen-activated protein kinase in cadmium-induced primary rat cerebral cortical neurons apoptosis via a mitochondrial apoptotic pathway. J Trace Elem Med Biol 29:275–283. https://doi.org/10.1016/j.jtemb.2014.06.006

Zang Y, Odwin-DaCosta S, Yager JD (2009) Effects of cadmium on estrogen receptor mediated signaling and estrogen induced DNA synthesis in T47D human breast cancer cells. Toxicol Lett 184:134–138. https://doi.org/10.1016/j.toxlet.2008.10.032

Zeng X, Wang Z, Wang J, et al (2015) Health risk assessment of heavy metals via dietary intake of wheat grown in Tianjin sewage irrigation area. Ecotoxicology 24:2115–2124. https://doi.org/10.1007/s10646-015-1547-0

Zhai Q, Tian F, Zhao J, et al (2016) Oral Administration of Probiotics Inhibits Absorption of the Heavy Metal Cadmium by Protecting the Intestinal Barrier. Appl Environ Microbiol 82:4429–4440. https://doi.org/10.1128/AEM.00695-16

Zhang D, Liu J, Gao J, et al (2014) Zinc Supplementation Protects against Cadmium Accumulation and Cytotoxicity in Madin-Darby Bovine Kidney Cells. PLOS ONE 9:e103427. https://doi.org/10.1371/journal.pone.0103427

Zhang P, Xu Y, Sun J, et al (2009a) Protection of pyrroloquinoline quinone against methylmercury-induced neurotoxicity via reducing oxidative stress. Free Radic Res 43:224–233. https://doi.org/10.1080/10715760802677348

Zhang S, Jin Y, Zeng Z, et al (2015) Subchronic Exposure of Mice to Cadmium Perturbs Their Hepatic Energy Metabolism and Gut Microbiome. Chem Res Toxicol 28:2000–2009. https://doi.org/10.1021/acs.chemrestox.5b00237

Zhang X, Jiang L, Geng C, et al (2009b) The role of oxidative stress in deoxynivalenol-induced DNA damage in HepG2 cells. Toxicon 54:513–518. https://doi.org/10.1016/j.toxicon.2009.05.021

Zhang Z-Q, Wang S-B, Wang R-G, et al (2016) Phosphoproteome Analysis Reveals the Molecular Mechanisms Underlying Deoxynivalenol-Induced Intestinal Toxicity in IPEC-J2 Cells. Toxins (Basel) 8:. https://doi.org/10.3390/toxins8100270

Zhao L-L, Ru Y-F, Liu M, et al (2017) Reproductive effects of cadmium on sperm function and early embryonic development in vitro. PLoS ONE 12:e0186727. https://doi.org/10.1371/journal.pone.0186727

Zhao Z, Hyun JS, Satsu H, et al (2006) Oral exposure to cadmium chloride triggers an acute inflammatory response in the intestines of mice, initiated by the over-expression of tissue macrophage inflammatory protein-2 mRNA. Toxicol Lett 164:144–154. https://doi.org/10.1016/j.toxlet.2005.12.004

Zhou H-R, He K, Landgraf J, et al (2014) Direct activation of ribosome-associated double-stranded RNA-dependent protein kinase (PKR) by deoxynivalenol, anisomycin and ricin: a new model for ribotoxic stress response induction. Toxins (Basel) 6:3406–3425. https://doi.org/10.3390/toxins6123406

Zhou H-R, Islam Z, Pestka JJ (2005) Induction of competing apoptotic and survival signaling pathways in the macrophage by the ribotoxic trichothecene deoxynivalenol. Toxicol Sci 87:113–122. https://doi.org/10.1093/toxsci/kfi234

Zhou H-R, Islam Z, Pestka JJ (2003a) Rapid, sequential activation of mitogen-activated protein kinases and transcription factors precedes proinflammatory cytokine mRNA expression in spleens of mice exposed to the trichothecene vomitoxin. Toxicol Sci 72:130–142

Zhou H-R, Lau AS, Pestka JJ (2003b) Role of double-stranded RNA-activated protein kinase R (PKR) in deoxynivalenol-induced ribotoxic stress response. Toxicol Sci 74:335–344. https://doi.org/10.1093/toxsci/kfg148

Zhou X, Hao W, Shi H, et al (2015) Calcium homeostasis disruption - a bridge connecting cadmium-induced apoptosis, autophagy and tumorigenesis. Oncology research and treatment 38:311–5. https://doi.org/10.1159/000431032

Zhou Z, Wang C, Liu H, et al (2013) Cadmium induced cell apoptosis, DNA damage, decreased DNA repair capacity, and genomic instability during malignant transformation of human bronchial epithelial cells. Int J Med Sci 10:1485–1496. https://doi.org/10.7150/ijms.6308

Annex

List of communication and publication

Communications:

Su Luo, Sylvie Puel, Isabelle P. Oswald and Philippe Pinton. Specific intestinal toxicity of deoxynivalenol and cadmium: analysis on pig jejunal explants. 14th International Symposium of Animal Biology and Nutrition, Bucharest, Romania. Sept. 28th, 2017 (Oral).

Su Luo, Ana-Paula Bracarense, Chloe Terciolo, Delphine Payros, Philippe Pinton, Isabelle P. Oswald. In vitro and in vivo effects of a mycotoxin, the deoxynivalenol, and a trace metal, the cadmium, alone or in mixture on the intestinal barrier. 37th CECED, Toulouse, France. 28, Mar. 29th, 2019 (Oral).

Su Luo, Ana-Paula Bracarense, Chloe Terciolo, Delphine Payros, Philippe Pinton, Isabelle P. Oswald. Individual and combined effect of a mycotoxin, the deoxynivalenol, and a metal trace element, the cadmium on the intestinal barrier: in vitro and in vivo analysis. 41st Mycotoxin Workshop, Lisbon, Portugal. May 6- 8th, 2019 (Oral).

Su Luo, Ana-Paula Bracarense, Chloe Terciolo, Delphine Payros, Philippe Pinton, Isabelle P. Oswald. The intestinal toxicity of a mycotoxin, deoxynivalenol and a trace metal, the cadmium alone or in combination: in vitro and in vivo analysis**.** Mytotoxins & Phycotoxins, Gordon Research Conference, Boston, USA. Jun. 16-21st, 2019 (Poster).

Su Luo, Ana-Paula Bracarense, Chloe Terciolo, Delphine Payros, Philippe Pinton, Isabelle P. Oswald. Deoxynivalenol and cadmium alone or in combination disrupt intestinal barrier. ½ Journée Graines de Toxalim 2019, Toulouse, France. Jun. 28th, 2019.

Publications:

Su Luo, Sylvie Puel, Isabelle P. Oswald and Philippe Pinton. Specific intestinal toxicity of deoxynivalenol and cadmium: analysis on pig jejunal explant. Archiva Zootechnica, 20 :2, 39-50, 2017.

Su Luo, Chloe Terciolo, Ana Paula Bracarense, Delphine Payros, Philippe Pinton, Isabelle Oswald. In vitro and in vivo effects of a mycotoxin, deoxynivalenol, and a trace metal, cadmium, alone or in mixture on the intestinal barrier. Environment International, 132:105082, 2019. https://www.ncbi.nlm.nih.gov/pubmed/31400600

Environment International 132 (2019) 105082

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

In vitro and *in vivo* effects of a mycotoxin, deoxynivalenol, and a trace metal, cadmium, alone or in a mixture on the intestinal barrier

Su Luo[a], Chloe Terciolo[a], Ana Paula F.L. Bracarense[b], Delphine Payros[a], Philippe Pinton[a,*], Isabelle P. Oswald[a]

[a] Toxalim (Research Center in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, UPS, Toulouse, France
[b] Laboratory of Animal Pathology, Universidade Estadual de Londrina, Londrina, Brazil

ARTICLE INFO

Handling Editor: Daniel Doerge

Keywords:
Deoxynivalenol
Cadmium
Intestinal barrier function
Mixture exposure
Junctional proteins
Mixture risk assessment

ABSTRACT

Deoxynivalenol (DON), one of the most widespread mycotoxins in Europe, and cadmium (Cd), a widespread environmental pollutant, are common food contaminants. They exert adverse effects on different organs including kidney, liver, and intestine. The intestine is a common target of DON and Cd when they are ingested. Most studies have focused on their individual effects whereas their combined toxicity has rarely been studied. The aim of this study was thus to evaluate their individual and combined effects on the intestinal barrier function *in vitro* and *in vivo*. *In vitro*, Caco-2 cells were treated with increasing concentrations of DON and Cd (1–30 μM). *In vivo*, Wistar rats were used as controls or exposed to DON contaminated feed (8.2 mg/kg feed), Cd-contaminated water (5 mg/l) or both for four weeks. In Caco-2 cells, DON, Cd and the DON + Cd mixture reduced transepithelial electrical resistance (TEER) and increased paracellular permeability in a dose-dependent manner. Impairment of the barrier function was associated with a decrease in the amount of E-cadherin and occludin after exposure to the two contaminants alone or combined. A decrease in E-cadherin expression was observed in rats exposed to the two contaminants alone or combined, whereas occludin expression only decreased in animals exposed to DON and DON + Cd. Jejunal crypt depth was reduced in rats exposed to DON or Cd, whereas villi height was not affected. *In vitro* and *in vivo* results showed that the effects of exposure to combined DON and Cd on the intestinal barrier function in the jejunum of Wistar rats and in the colorectal cancer cell line (Caco-2) was similar to the effects of each individual contaminant. This suggests that regulations for each individual contaminant are sufficiently protective for consumers.

1. Introduction

Food safety is a major concern worldwide. Food and feedstuffs are frequently contaminated by multiple contaminants (Rather et al., 2017; Silins and Högberg, 2011). The toxic effects of these mixtures cannot be predicted based on the toxicity of individual contaminants alone, as simultaneous exposure to several contaminants can have synergistic, antagonistic or additive effects (Alassane-Kpembi et al., 2017b; More et al., 2019). In recent years, combined toxicity studies have assessed the effects of mixtures of food contaminants such as mycotoxins (Alassane-Kpembi et al., 2017a), trace metal elements (Claus Henn et al., 2014), pesticides (Lukowicz et al., 2018) and carcinogens (Miller et al., 2017). However, the toxicity of mixtures of contaminants from different families remains poorly documented (Le et al., 2018; More et al., 2019; Payros et al., 2017).

Mycotoxins are the most frequently occurring natural food contaminants in human and animal diets. Among mycotoxins, Deoxynivalenol (DON), mainly produced by *Fusarium graminearum* and *F. culmorum*, frequently contaminates cereals and cereal products. Almost half of 26,613 cereal samples collected from 21 European countries were found to be contaminated by DON, with the highest levels observed in wheat, maize and oat grains (EFSA, 2017). Tolerable daily intake (TDI) for DON and its derivative was established at 1 μg/kg b.w./day (EFSA, 2017). Analyses of adult urine samples in the United Kingdom revealed that 98% of them had been exposed to DON (Turner et al., 2008), while national and European surveys indicate that the health based guidance values are exceeded in children (EFSA, 2017; Sirot et al., 2013). DON interacts with the peptidyl transferase region of the 60S ribosomal subunit, inducing “ribotoxic stress,” resulting in the activation of mitogen-activated protein kinases and their downstream pathways (Lucioli et al., 2013; Pestka, 2010). Symptoms of intoxication in animals exposed to DON may include reduced food consumption and

* Corresponding author at: Toxalim, INRA, 180 chemin de tournefeuille, 31300 Toulouse, France.
E-mail address: Philippe.Pinton@inra.fr (P. Pinton).

https://doi.org/10.1016/j.envint.2019.105082
Received 27 March 2019; Received in revised form 31 July 2019; Accepted 1 August 2019
0160-4120/ © 2019 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).

weight gain, neuro-endocrine changes as well as alteration of intestinal and immune functions. Following acute exposure, vomiting and bloody diarrhea have been reported (Payros et al., 2016; Pestka, 2010; Pinton and Oswald, 2014).

Cadmium (Cd) is a heavy metal element naturally present in the Earth's crust. It is a common environmental pollutant that occurs both naturally and as a result of industrial and agricultural activities (ATSDR, 2012). Natural sources of Cd include volcanic activity, weathering of rocks, sea aerosols and forest fires. Anthropic origins of Cd include batteries, pigments, plastic stabilizers, pesticides and fertilizers, and photovoltaic devices, as well as rubber processing, galvanization, fossil combustion and waste incineration. Cadmium compounds are soluble in water and can be taken up by plant roots and translocated to edible parts where they accumulate. For example, large quantities of Cd accumulate in tobacco leaves and tobacco smokers are thus exposed by inhalation (Ganguly et al., 2018). Among nonsmokers, ingestion of contaminated food is a major source of Cd exposure. Sixty-six percent of 137,200 food samples analyzed in 20 European countries contained 5% more than the maximum recommended level of Cd (EFSA, 2009). Tolerable weekly intake (TWI) for Cd is set at 2.5 μg/kg b.w. (EFSA, 2009). European adults' mean dietary exposure to Cd was shown to be close to this TWI, and subgroups, including vegetarians, children, and smokers, exceeded the TWI about 2-fold (EFSA, 2009). Grain and cereal products, as well as fish and offal were the major contributors to human Cd exposure (ATSDR, 2012; Filipič et al., 2006). Cd causes inflammation, apoptosis and oxidative stress in liver and kidney (Kim et al., 2015; Liu et al., 2015). Cadmium absorbed by inhalation and ingestion mainly accumulates in the liver and kidney, but the small intestine and lung are other targets (Tinkov et al., 2018; Zhang et al., 2015). Cd increases the risk of cancer through oxidative stress, which damages DNA and inhibits DNA repair systems, and cadmium is classified as a (group 1) human carcinogen (Bishak et al., 2015).

The intestinal epithelium is the first barrier against ingested chemicals and food contaminants. The gut barrier is formed to a large extent by intercellular junctions on the apical side of epithelial cells. These junctions seal the cells together and regulate the passage of ions and water across the epithelium (Suzuki, 2013; Terciolo et al., 2019). Following ingestion of contaminated food, intestinal epithelial cells may be chronically exposed to contaminants including DON and Cd. The effects of DON on the intestine are well described (Maresca, 2013; Pinton and Oswald, 2014). DON alters the intestinal structure, reduces the expression of several junctional proteins, reduces the barrier function, affects nutrient absorption, modulates intestinal microbiota and the local immune responses (Payros et al., 2016; Pestka, 2010; Pinton and Oswald, 2014). The effects of Cd on the intestine are less well documented, but recent studies have shown that it alters the gut microbiota, triggers a local inflammatory response and disrupts tight junctions (Breton et al., 2016; Tinkov et al., 2018).

Human and animals are exposed to both compounds through ingestion of contaminated food and feed, especially cereals and cereal by-products (Arnich et al., 2012; Sirot et al., 2013). To the best of our knowledge, with the exception of one study (Le et al., 2018), the combined effects of these two contaminants have not been documented to date. The aim of the present study was thus to assess the individual and combined effects of DON and Cd on the intestinal barrier using *in vitro* and *in vivo* models. *In vitro*, the individual and combined effects of DON and Cd (0–30 μM) on Caco-2 cells were analyzed by measuring transepithelial electrical resistance (TEER), paracellular permeability and the abundance of junctional proteins. *In vivo* experiments were performed to investigate the effects on intestinal histomorphology, the abundance and localization of junctional proteins in rats exposed to DON (8.2 mg/kg feed) and Cd (5 ml/l of drinking water). Our *in vitro* and *in vivo* data on the parameters cited above demonstrated that the effects of different combinations of DON and Cd tested were comparable to those of the highest dose of each individual contaminant.

2. Materials and methods

2.1. Reagents

DON and $CdCl_2$ were purchased from Sigma (St Quentin Fallavier, France). For the *in vitro* experiments, DON and $CdCl_2$ were dissolved in water and stock solutions (5 mM) were stored at −20 °C before dilution in complete cell culture medium. For the *in vivo* experiments, DON was included in the rats' diet as previously described (Bracarense et al., 2017; Payros et al., 2016) while $CdCl_2$ was added to drinking water at a concentration of 5 mg/l.

2.2. Cells

The Caco-2 cell line (Sigma, 86010202) was originally isolated from a primary colonic tumor in a 72-year-old Caucasian male. The cells were maintained in DMEM-Glutamax (Gibco, Life Technologies, Courtaboeuf, France) supplemented with 10% fetal calf serum (FCS) (Eurobio, Courtaboeuf, France), 1% non-essential amino acid (Sigma) and 0.5% gentamycin (Eurobio). Cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2 and passaged by trypsinization (0.5% trypsin in 0.5 mM EDTA) when they reached 80% confluency. Caco-2 cells were differentiated on 0.3 cm^2 polyethylene terephthalate membrane inserts with 0.4 μm pores (Corning Inc., Corning, NY, USA) for the assessment of transepithelial electrical resistance (TEER) and paracellular permeability, as previously described (Pierron et al., 2016b; Pinton et al., 2009).

2.3. Animals

The experimental protocol was carried out in accordance with European Guidelines for the Care and Use of Animals for Research Purposes and was validated by the Toxcomethique Ethics Committee and the French Ministry of National Education Higher Education and Research (TOXCOM/0142/PP). Three of the authors (DP, PP and IPO) have official authorization from the French Veterinary Services for animal experimentation.

Four-week-old male Wistar rats (140–170 g body weight), (Janvier Labs, Le Genest Saint Isle, France) were kept under the normal conditions with a 12-hour day/night cycle, 19–25 °C temperature, 50–70% humidity, at the Toxalim animal facility (INRA, UMR 1331, Toulouse) with *ad libitum* access to food and water throughout the study. After five days of acclimatization, the rats were divided into four groups of eight animals: a control group, rats exposed to DON contaminated-feed (8.2 mg DON/kg feed), rats exposed to Cd-contaminated water (5 mg $CdCl_2$/l) and rats exposed to both DON and Cd. No other mycotoxin was detected in the feed (Supplementary Table 1). The rats were weighed weekly. After four weeks, the animals were euthanized, and the intestinal tissue was collected and fixed in 10% buffered formalin for histological assessment and immunohistochemical staining.

2.4. Transepithelial electrical resistance assay

Caco-2 cells grown on inserts differentiated and acquired an epithelial phenotype with polarity properties (apical and basolateral sides). They were treated apically with increasing concentrations of DON and Cd (0, 3, 10, 30 μM) alone or combined. Transepithelial electrical resistance (TEER) was measured at 4 hourly intervals for 48 h using a cellZscope device (nanoAnalytics, Münster, Germany). Measurements were made on four replicates of four independent experiments.

2.5. Paracellular tracer flux assay

To assess paracellular flux, the 4-kDa fluorescein isothiocyanate-dextran (FITC-dextran) (Sigma), dissolved in cell culture medium, was

added in the apical compartment (transwell, final concentration 2.2 mg/ml) at 48 h post DON and/or Cd exposure. After 1 h of incubation, fluorescence was measured in the basal compartment (well of plate) with a microplate fluorimeter reader (Tecan, Lyon, France). The excitation and emission wavelengths were 490 and 520 nm, respectively. The background signal resulted from reagent-treated medium without cells. Measurements were made on four replicates of four independent experiments.

2.6. Western blot analysis of junctional proteins

Differentiated Caco-2 cells, cultured in 6-well plates, were exposed for 24 h to DON and Cd individually or combined to analyze junctional proteins. Three independent experiments were performed for each cell culture condition. After cell treatment, proteins were extracted as previously described (Pinton et al., 2009) and separated on SDS-PAGE membranes probed with rabbit polyclonal Abs anti-occludin (#71-1500), -ZO-1 (#61-7300) (Thermo Fisher Scientific, Illkirch, France), -claudin-3 (#34-1700), -claudin-7 (#34-9100) (Invitrogen), rabbit monoclonal Ab anti-E-cadherin (Cell Signaling Technology, Leiden, The Netherlands), or mouse monoclonal Ab anti-claudin-4 (Invitrogen), diluted 1:250. Mouse monoclonal Ab or rabbit polyclonal anti-β-actin (Cell Signaling Technology, #3700 or #4967) was used as control (diluted 1:1000). Membranes were then washed and incubated with secondary antibodies CF680 goat anti rabbit IgG (#20067) or CF680 goat anti mouse IgG (#20065, diluted 1:10000) obtained from Biotium (Hayward, CA). Infrared fluorescence intensity of the specific bands was obtained with Odyssey Infrared Imaging System (LI-COR ScienceTec, Les Ulis, France). Protein expression was estimated after normalization calculated by the ratio of the intensity of the band of interest to that of the β-actin band.

2.7. Immunofluorescence analysis

Differentiated Caco-2 cells cultured on glass cover slips were exposed for 24 h to DON or Cd. The cells were fixed with 4% paraformaldehyde at 4 °C for 30 min and permeabilized with PBS containing 0.1% Triton X-100 for 10 min, then blocked with 10% goat serum at room temperature for 1 h. Occludin and E-cadherin were detected by incubation with rabbit polyclonal Abs anti-occludin (Thermo Fisher Scientific, #71-1500) and monoclonal anti-E-cadherin (Cell Signaling Technology, #3195) respectively. After three washes, the cells were incubated for 1 h with Alexa Fluor 546-conjugated goat immunoglobulin (1:500; Life Technologies, #A11035) raised against rabbit IgGs. Nuclei were stained with DAPI (Vector Laboratories) for 10 min. Cells were washed and mounted in Prolong® Gold antifade reagent (Invitrogen, Oregon, USA). Images were captured using an SP8 Leica confocal microscope and analyzed using FIJI software.

2.8. Histological and morphometric assessment of rat jejunum

The pieces of rat jejunum were fixed in 10% buffered formalin, embedded in paraffin and cut into 5 μm sections. The sections were stained with hematoxylin & eosin for histopathological evaluation. A lesional score, including the morphology of villi and enterocytes, interstitial edema, and lymph vessel dilation was used to compare histological changes in different conditions as previously described (Pierron et al., 2018). Villus height and crypt depth were measured randomly using a MOTIC Image Plus 2.0 ML software (Motic Instruments, Richmond, Canada).

2.9. Immunohistochemical assessment of junctional proteins in the jejunum

After dewaxing and heat-induced antigen retrieval as described previously (Bracarense et al., 2012), paraffin-embedded 5 μm sections of rat jejunum were incubated overnight at 4 °C with the primary antibody anti-E-cadherin (Zymed, San Francisco, CA, USA) and anti-occludin (*Santa Cruz*. Biotechnology Inc., USA). The secondary antibody (SuperPicTure™ Polymer, Zymed) was applied followed by the addition of a chromogen (3,30-diaminobenzidine). Finally, tissue sections were counterstained with hematoxylin. The sections were then examined and the proportion of intestinal section expressing E-cadherin was estimated. Each sample was classified as having either normal or reduced staining.

2.10. Statistical analysis

The results are presented as the means ± standard error of the mean (SEM) of independent experiments. Statistical analysis was performed using GraphPad Software (La Jolla, CA, USA). Significant differences between groups were analyzed by one-way ANOVA (non-parametric) with Bonferroni's multiple comparison test. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Individual effects of DON and Cd on intestinal barrier function

The individual effects of DON and Cd were first assessed on two parameters that reveal the integrity of the intestinal epithelium: the TEER and the paracellular passage of dextran.

As shown in Fig. 1A, both DON and Cd reduced the TEER of Caco-2 monolayers in a dose- and time-dependent manner. The TEER was not affected by 1 and 3 μM of either contaminant but was significantly reduced by 10 μM DON as early as 8 h after exposure (44% decrease, $p < 0.001$) and by 10 μM Cd after 12 h of exposure (40% decrease, $p < 0.001$). Exposure to 30 μM of DON or Cd reduced the TEER as early as 4 h (27%, $p < 0.05$), and the TEER decreased over time: respectively 82% and 87% decrease ($p < 0.001$) at 12 h.

The permeability of Caco-2 monolayers to 4-kDa FITC-dextran was measured at the end of the experiment. As shown in Fig. 1B, this parameter was not affected in cells exposed to 1, 3 μM DON or 10 μM Cd. Higher doses of DON and/or Cd significantly increased the passage of FITC-dextran (18, 67 and 53-fold upon exposure to 10 μM DON, 30 μM DON and 30 μM Cd, respectively).

These results show that both DON and Cd alter the intestinal barrier function as measured by decreased TEER and increased passage of dextran.

3.2. Individual effects of DON and Cd on the abundance of junctional proteins

Adherent and tight junctions belong to apical multiprotein complexes that link adjacent epithelial cells together and play an important role in the formation and the maintenance of the intestinal barrier (Suzuki, 2013). Consequently, the effect of DON and Cd on the abundance of several junctional proteins was examined.

As shown in Fig. 2, DON induced a dose-dependent decrease in the amount of occludin, E-cadherin and claudin-7. In the case of occludin, upon exposure to 10 and 30 μM DON, the decrease reached 54% ($p < 0.05$) and 66% ($p < 0.01$); the reduction in E-cadherin was 50% ($p < 0.05$) and 80% ($p < 0.001$) respectively. For claudin-7, a significant decrease was only observed at 30 μM DON (80%, $p < 0.05$). By contrast, exposure to DON at rates of up to 30 μM, did not affect the amount of claudin-3, -4 and ZO-1.

Cd only caused a significant reduction in the abundance of occludin and E-cadherin upon exposure to 30 μM (58% ($p < 0.05$) and 70% ($p < 0.01$) decrease respectively), whereas claudin-3, -4, -7 and ZO-1 were not affected by the trace metal element.

These results suggest that DON and Cd impair the intestinal barrier function *via* a specific decrease in the abundance of E-cadherin and occludin.

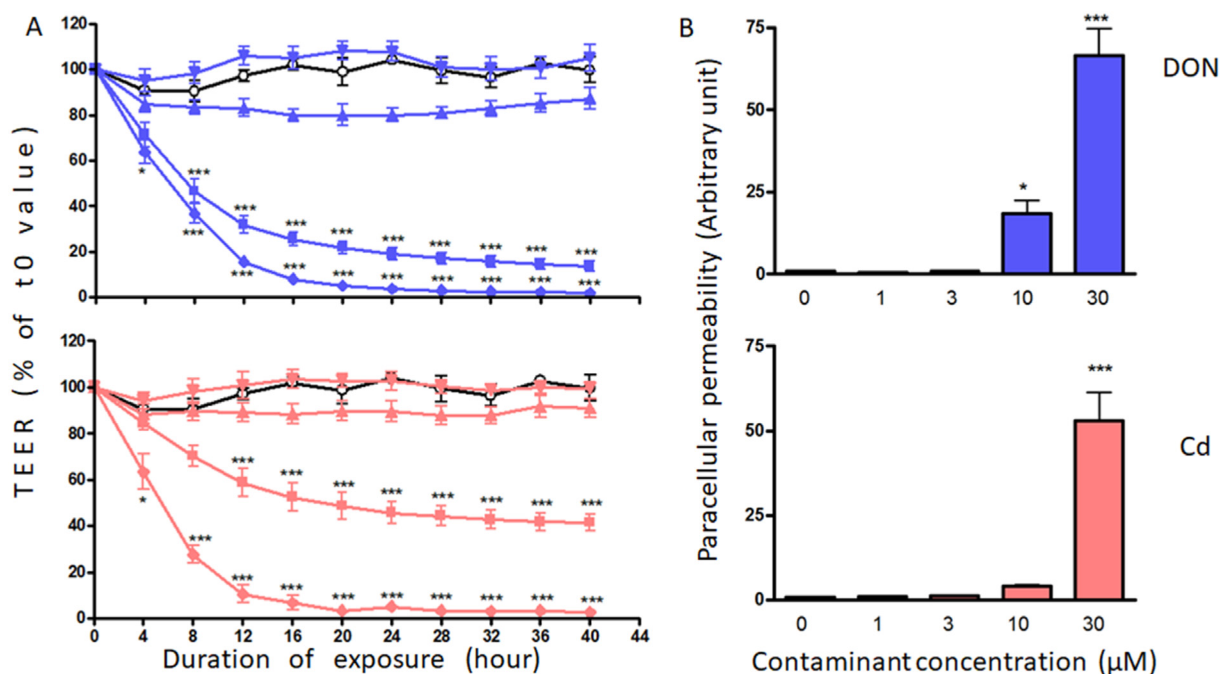


Fig. 1. Effects of DON and Cd on TEER and on the paracellular permeability of differentiated Caco-2 cells.
(A) Effect on the TEER. Caco-2 cells were grown and differentiated on inserts and treated apically with DON (blue upper panel) or Cd (red lower panel) at 1 µM (inverted triangles), 3 µM (triangles), 10 µM (squares) or 30 µM (diamonds); control cells (circles, black line) were untreated. The TEER was recorded over a period of 40 h.
(B) Effect on paracellular permeability. At 48 h, 4-kDa FITC-dextran was added in the apical compartment and fluorescence was assessed in the basal compartment 1 h later. Fluorescent intensity was measured, and background levels subtracted.
Results, normalized to the controls, are expressed as the mean of 4 independent experiments ± SEM (*, $p < 0.05$; ***, $p < 0.001$). Data were analyzed by non-parametric one-way ANOVA.

3.3. Individual effects of DON and Cd on the distribution of junctional proteins

We next investigated the effect of DON and Cd on the distribution of junctional proteins in Caco-2 cells. In control cultures, a well-defined band of membrane-associated immunofluorescence staining was observed for E-cadherin and occludin (Fig. 3A and B). Exposure to DON or Cd for 24 h caused significant changes in the E-cadherin pattern analyzed by fluorescence (white arrow) (Fig. 3A). The main modification caused by DON was reduced membranous expression of E-cadherin. At the same time, increased granular fluorescence was observed in the cytoplasm. After exposure to Cd, the redistribution of E-cadherin into the cytoplasm was more visible. The loss of membranous expression with partial redistribution into the cytoplasm was also observed for occludin (white arrow) (Fig. 3B). The effects of DON and Cd on E-cadherin and occludin distribution were dose dependent.

Taken together, these results show that exposure to DON or Cd caused alterations in the cellular localization of E-cadherin and occludin.

3.4. Combined effects of DON and Cd on intestinal barrier function

The data presented above show that individual exposure to DON or Cd has harmful effects on the intestinal barrier; however, in a mixture, their combined effects remain unknown.

The combined effects of DON and Cd on the TEER are summarized in Fig. 4. In this heat-map, the first line and column show the individual effects of DON and Cd while the other cells show the effects of different combinations, with a color code indicating the strength of the effect. A time and dose dependent decrease in the TEER was observed both when the toxins were present alone or in a mixture. Comparison of the combined effect with that of each individual compound revealed that after 8 h of exposure, the impact of Caco-2 cells exposed to 3 µM Cd combined with 10 µM DON was always stronger than that of 3 µM Cd alone ($p < 0.001$) but was similar to that of DON alone. The same effects were observed for 10 µM Cd combined with DON (10 or 30 µM) ($p < 0.001$). Moreover, at 12 h of exposure, 10 µM Cd combined with DON (10 or 30 µM) also had a stronger impact than DON alone ($p > 0.05$). Conversely, 10 µM of Cd combined with 3 µM of DON led to a greater decrease in the TEER than DON alone ($p < 0.001$). These results were also observed when 30 µM Cd was combined with 10 µM DON ($p < 0.001$ from 8 h to 12 h and $p < 0.05$ from 24 h), except for 36 h. At 4 h, 24 h and 36 h, the effect of the combination of DON and Cd 30 µM was stronger than the effect observed with DON or 30 µM Cd alone ($p < 0.01$ at 4 h, 24 h and 36 h). These data show that, as far as the TEER is concerned, in most cases, the combination of DON and Cd had a similar effect to that of the individual contaminants.

Paracellular permeability to 4-kDa FITC-dextran was assessed on cell monolayers exposed to mixtures of DON and Cd for 48 h (Fig. 5). The results showed that, except for the combination 3 µM DON and 10 µM Cd, combinations of low doses of DON and Cd (1 to 3 µM) did not cause any significant changes in paracellular permeability. In this case, the mixture had a stronger effect than the contaminant alone (DON, $p < 0.001$; Cd, $p < 0.05$). Moderate to high doses (10 to 30 µM) resulted in a marked increase in permeability to 4-kDa FITC-dextran. For example, the combination of DON and Cd at 10/10, 30/30, 10/30 and 30/10 µM increased the passage of dextran 46-, 82-, 76- and 81-fold, respectively, compared to untreated cells. Notably, 10 µM DON combined with 10 µM Cd resulted in a greater increase in paracellular permeability than 10 µM of Cd alone ($p < 0.001$). DON (10 µM) combined with 30 µM Cd had a stronger effect than 10 µM DON alone ($p < 0.001$). Similarly, 30 µM DON combined with 10 µM Cd led to a greater increase in paracellular permeability than 10 µM Cd alone ($p < 0.001$). These data show that the combination of DON and Cd has

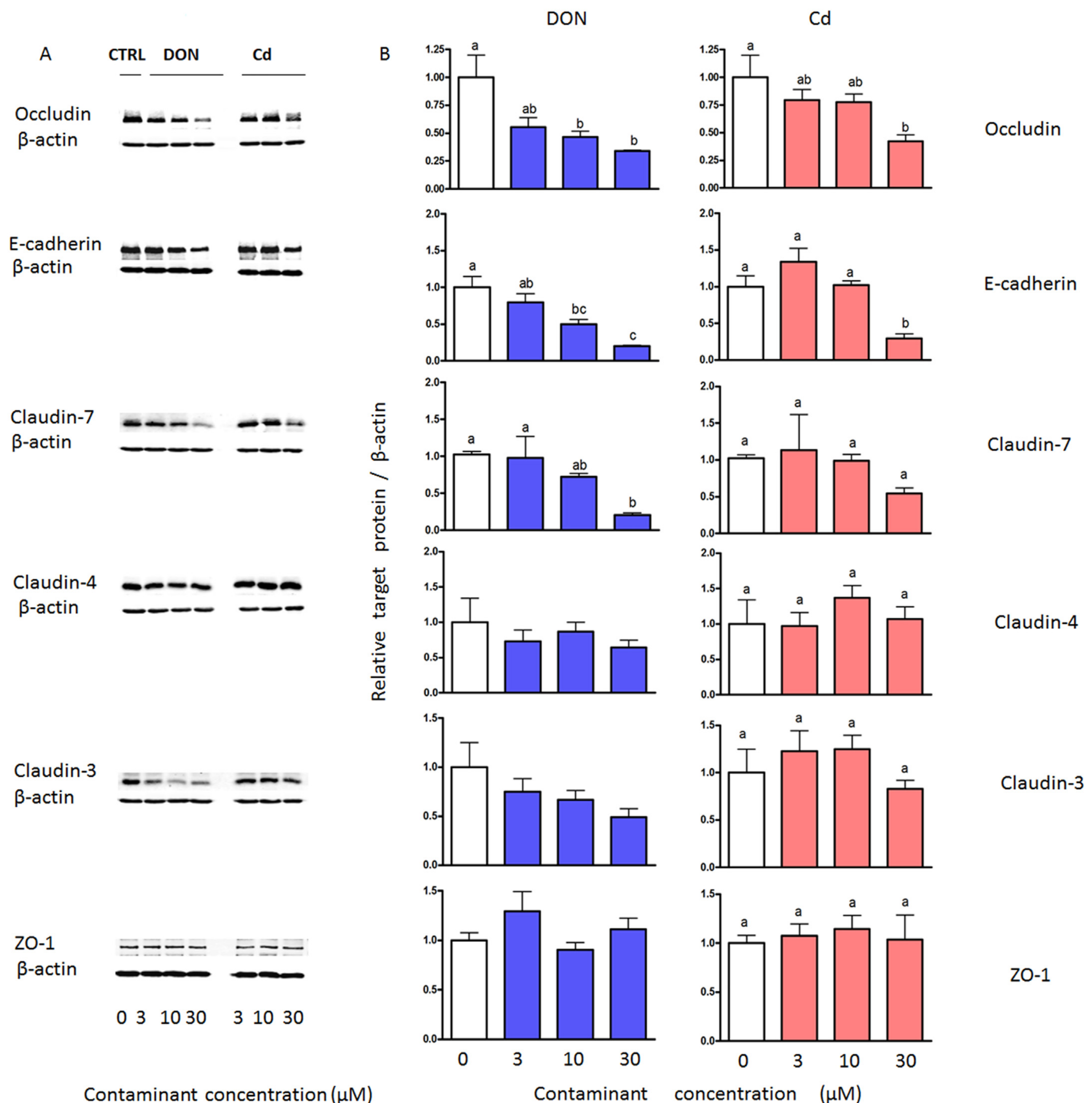


Fig. 2. Effects of DON and Cd on the expression of junctional proteins in Caco-2 cells. Differentiated Caco-2 cells were exposed to different concentrations of DON and Cd for 24 h. (A) After extraction, proteins were analyzed by immunoblotting for claudin-3, 4, 7, occludin, E-cadherin and ZO-1.
(B) Protein expression was analyzed by densitometry and normalized to that of β-actin. Data are presented as means ± SEM of 3 independent experiments. Means with different letters differ ($p < 0.05$). Results were analyzed by one-way ANOVA with Bonferroni's multiple comparison test.

a similar effect on the intestinal permeability to that of the individual contaminant.

3.5. Combined effects of DON and Cd on junctional protein abundance

To investigate the combined effect of DON and Cd on the intestinal barrier function in more detail, the abundance of two junctional proteins, occludin and E-cadherin, was analyzed. Fig. 6 is a heatmap of the individual and combined effects of DON and Cd. The levels of the two proteins decreased continuously with increasing concentrations of combined compounds. The combination of DON and Cd at a rate of 3 μM led to a similar decrease in occludin and E-cadherin as 3 μM of DON or 3 μM of Cd alone. Only 10 μM Cd combined with 10 μM DON led to a bigger decrease in occludin and E-cadherin abundance than 10 μM of Cd alone ($p < 0.001$). When the cells were exposed to a combination of 30 μM DON and 30 μM Cd, the levels of occludin and E-cadherin were lower than the level observed in cells only exposed to DON or Cd alone (DON, $p < 0.001$; Cd, $p < 0.05$). The data show that except for very high concentrations, the effect of a combination of DON and Cd on the abundance of occludin and E-cadherin was similar to the

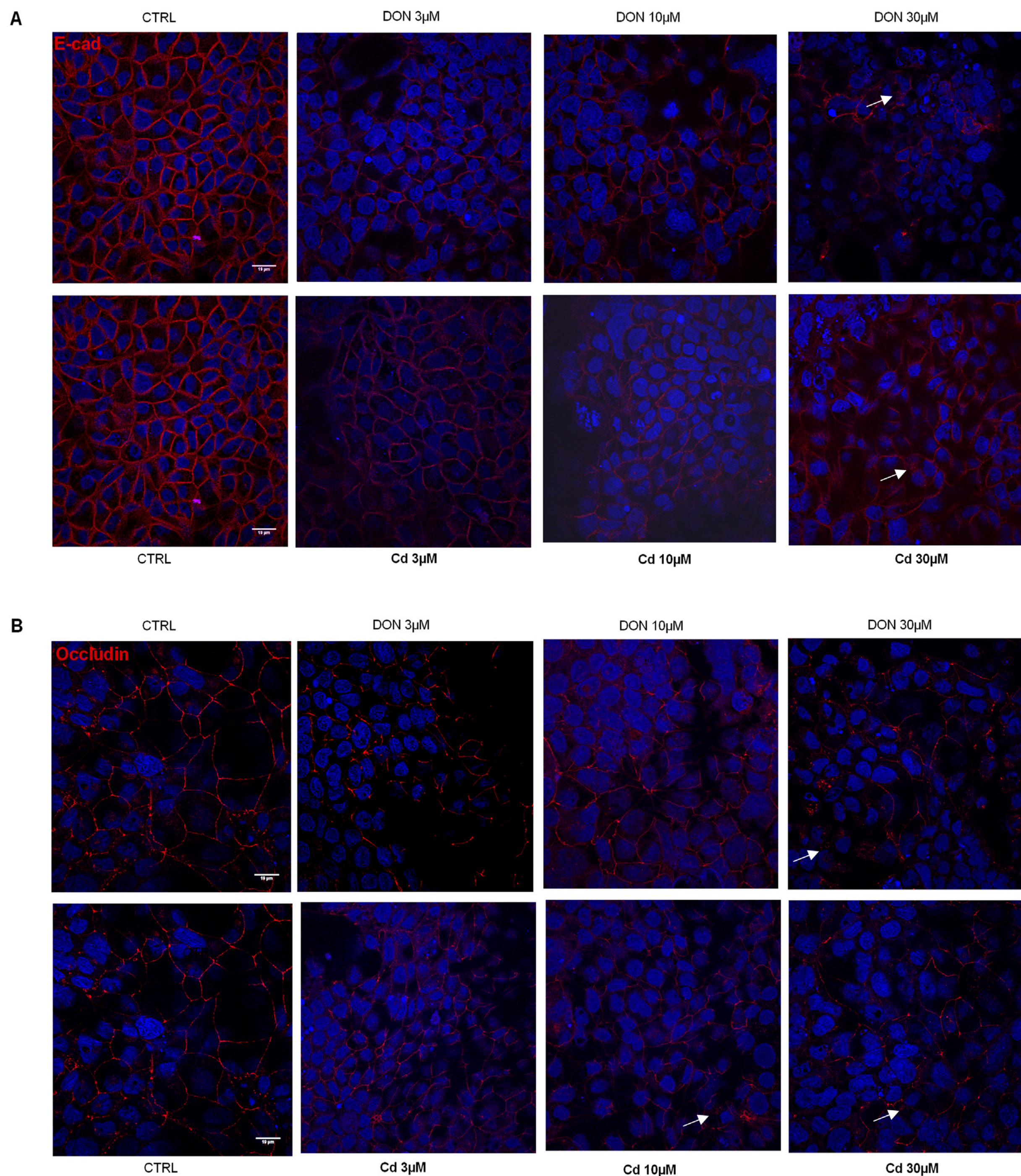


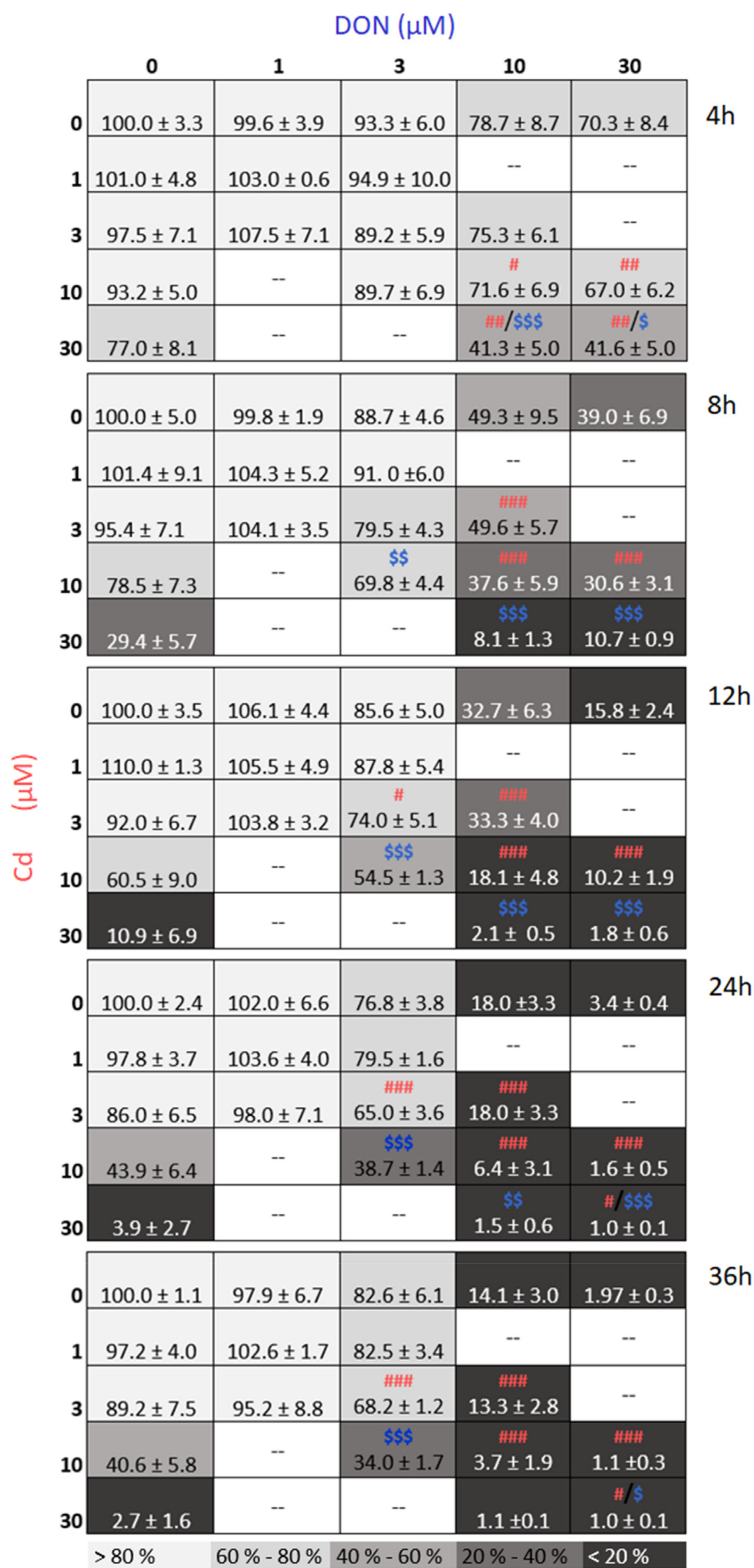
Fig. 3. Effect of DON and Cd on E-cadherin and occludin distribution in differentiated Caco-2 cells.
Differentiated Caco-2 cells were exposed to different concentrations of DON or Cd for 24 h. E-cadherin (A) and occludin (B) distribution (white arrow) was analyzed after immunostaining with specific antibody.

effect of the individual compounds.

3.6. *In vivo effects of DON and Cd alone or in combination on the histomorphometry of the jejunum*

Given the impact of DON and Cd on intestinal barrier function on Caco-2 cells, experiments were also performed on animals. Rats were exposed to DON-contaminated feed (8.2 mg/kg), Cd-contaminated water (5 mg/l) or both. The individual and combined effects of these contaminants were assessed in the intestine after four weeks of exposure.

The animals' body weight gain was not affected in any of the conditions (Fig. 7). Histomorphometrical changes were analyzed in the jejunum of rats fed DON and Cd alone or in combination. These results showed moderate but significant lesions compared to the intestine of control rats. The main histological changes were atrophy and fusion of the villi. Interstitial edema and enterocyte apical flattening were also observed (Fig. 8A, B, C, D). Lesional scores were about 4-fold higher in rats exposed to DON, Cd or both than that in control rats (Fig. 8E). These results show that intestinal damage caused by the combination of DON and Cd were as severe as the damage caused by each contaminant individually.



DON (μM) / Cd (μM)

4h

Cd \ DON	0	1	3	10	30
0	100.0 ± 3.3	99.6 ± 3.9	93.3 ± 6.0	78.7 ± 8.7	70.3 ± 8.4
1	101.0 ± 4.8	103.0 ± 0.6	94.9 ± 10.0	--	--
3	97.5 ± 7.1	107.5 ± 7.1	89.2 ± 5.9	75.3 ± 6.1	--
10	93.2 ± 5.0	--	89.7 ± 6.9	# 71.6 ± 6.9	## 67.0 ± 6.2
30	77.0 ± 8.1	--	--	##/$$$ 41.3 ± 5.0	##/$ 41.6 ± 5.0

8h

Cd \ DON	0	1	3	10	30
0	100.0 ± 5.0	99.8 ± 1.9	88.7 ± 4.6	49.3 ± 9.5	39.0 ± 6.9
1	101.4 ± 9.1	104.3 ± 5.2	91. 0 ±6.0	--	--
3	95.4 ± 7.1	104.1 ± 3.5	79.5 ± 4.3	### 49.6 ± 5.7	--
10	78.5 ± 7.3	--	$$ 69.8 ± 4.4	### 37.6 ± 5.9	### 30.6 ± 3.1
30	29.4 ± 5.7	--	--	$$$ 8.1 ± 1.3	$$$ 10.7 ± 0.9

12h

Cd \ DON	0	1	3	10	30
0	100.0 ± 3.5	106.1 ± 4.4	85.6 ± 5.0	32.7 ± 6.3	15.8 ± 2.4
1	110.0 ± 1.3	105.5 ± 4.9	87.8 ± 5.4	--	--
3	92.0 ± 6.7	103.8 ± 3.2	# 74.0 ± 5.1	### 33.3 ± 4.0	--
10	60.5 ± 9.0	--	$$$ 54.5 ± 1.3	### 18.1 ± 4.8	### 10.2 ± 1.9
30	10.9 ± 6.9	--	--	$$$ 2.1 ± 0.5	$$$ 1.8 ± 0.6

24h

Cd \ DON	0	1	3	10	30
0	100.0 ± 2.4	102.0 ± 6.6	76.8 ± 3.8	18.0 ±3.3	3.4 ± 0.4
1	97.8 ± 3.7	103.6 ± 4.0	79.5 ± 1.6	--	--
3	86.0 ± 6.5	98.0 ± 7.1	### 65.0 ± 3.6	### 18.0 ± 3.3	--
10	43.9 ± 6.4	--	$$$ 38.7 ± 1.4	### 6.4 ± 3.1	### 1.6 ± 0.5
30	3.9 ± 2.7	--	--	$$ 1.5 ± 0.6	#/$$$ 1.0 ± 0.1

36h

Cd \ DON	0	1	3	10	30
0	100.0 ± 1.1	97.9 ± 6.7	82.6 ± 6.1	14.1 ± 3.0	1.97 ± 0.3
1	97.2 ± 4.0	102.6 ± 1.7	82.5 ± 3.4	--	--
3	89.2 ± 7.5	95.2 ± 8.8	### 68.2 ± 1.2	### 13.3 ± 2.8	--
10	40.6 ± 5.8	--	$$$ 34.0 ± 1.7	### 3.7 ± 1.9	### 1.1 ±0.3
30	2.7 ± 1.6	--	--	1.1 ±0.1	#/$ 1.0 ± 0.1

> 80 %	60 % - 80 %	40 % - 60 %	20 % - 40 %	< 20 %

Fig. 4. Heatmap showing the effects of single and binary combinations of DON and Cd on the TEER of Caco-2 monolayers after 4 h, 8 h, 12 h, 24 h and 36 h of exposure. At each time-point, the TEER of control untreated cells was considered to be 100%. Results are expressed as the mean of four independent experiments ± SEM (#, combined effects *vs* effects of Cd alone, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$. $, combined effects *vs* effects of DON alone, $p < 0.05$; $$, $p < 0.01$; $$$, $p < 0.001$). Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test. The intensity of the color reflects the strength of the effect on the TEER.

Cd (μM) \ DON (μM)	0	1	3	10	30
0	1.0 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	18.4 ± 6.1	66.7 ± 10.7
1	1.1 ± 0.2	0.6 ± 0.2	1.2 ± 0.2	--	--
3	1.3 ± 0.2	1.0 ± 0.1	$ 1.2 ± 0.1	18.8 ± 5.4	--
10	4.2 ± 0.8	--	##/$$$ 8.1 ± 0.7	### 46.2 ± 5.3	### 81.5 ± 18.5
30	53.0 ± 10.2	--	--	$$$ 75.8 ± 20.1	81.7 ± 17.9

< 20	20 - 40	40 - 60	60 - 80	> 80

Fig. 5. Heatmap showing the effects of single compound and binary combinations of DON and Cd on the paracellular permeability of Caco-2 monolayers after 48 h of exposure. After 1 h of incubation with FITC-Dextran, the intensity of fluorescent was measured in the basal compartment. Results are expressed as fold increase in fluorescent intensity relative to controls. Results are expressed as the mean of four independent experiments ± SEM. (#, combined effects *versus* effects of Cd alone, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$. $, combined effects *VS* effects of DON alone, $p < 0.05$; $$, $p < 0.01$; $$$, $p < 0.001$). The depth of color reflects the strength of the effect on permeability. Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

The *in vivo* effects of the mycotoxin and the heavy metal on intestinal cell proliferation were also assessed by measuring villus height and crypt depth. A significant reduction in crypt depth was observed in the jejunum of rats exposed to the contaminants compared with control animals (Fig. 8F). The reduction in crypt depth in animals exposed to both DON and Cd was similar to the reduction observed in animals exposed to Cd alone but slightly greater than the reduction observed in the animals only exposed to DON (−16%, $p < 0.01$). Villus heights did not differ significantly whatever the group of animals considered (Fig. 8G).

Taken together, these results indicate that ingestion of a combination of DON and Cd induces similar or slightly bigger lesions and has greater histomorphological effects on the intestine than ingestion of the individual contaminants.

3.7. In vivo effects of DON and Cd alone or in combination on the abundance of E-cadherin and occludin in the jejunum

As DON and Cd significantly reduced the amount of E-cadherin and occludin in Caco-2 cells, their abundance was also assessed in the jejunum of rats exposed to the same contaminants. Immunohistochemical staining revealed a significant reduction in E-cadherin in all exposed animals (Fig. 9). In control rats, strong homogeneous immunostaining was observed at the intercellular borders of epithelial cells (Fig. 9A). The intensity of E-cadherin staining was significantly reduced in rats exposed to either DON or Cd (Fig. 9B, C). Weak heterogeneous staining was also observed in rats exposed to the mixture (Fig. 9D). The

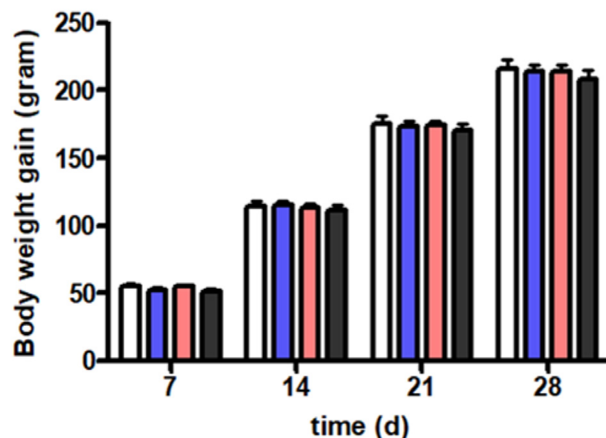


Fig. 7. Individual and combined effects of DON and Cd on body weight gain. Male Wistar rats were divided into four groups: control (white column), exposed to 10 mg/kg DON-contaminated feed (blue column), exposed to 5 mg/l Cd-contaminated feed (red column) or exposed to both DON and Cd (black column). Values are mean ± SEM ($n = 8$ animals). Data were analyzed by non-parametric one-way ANOVA.

Occludin

Cd (μM) \ DON (μM)	0	3	10	30
0	100.0 ± 26.3	55.6 ± 10.3	46.5 ± 7.5	33.9 ± 1.4
3	79.3 ± 11.9	56.7 ± 9.6	--	--
10	77.6 ± 10.5	--	### 31.9±6.1	--
30	42.1 ± 9.1	--	--	#/$$ 22.7 ± 2.8

E-cadherin

Cd (μM) \ DON (μM)	0	3	10	30
0	100.0 ± 18.3	79.7 ± 15.7	50.0 ± 9.6	19.9 ± 2.0
3	134.1 ± 23.8	98.0 ± 17.5	--	--
10	98.8 ± 8.9	--	### 11.0 ± 3.6	--
30	29.7 ± 9.6	--	--	#/$$$ 3.3 ± 0.4

> 80 %	60 % - 80 %	40 % - 60 %	20 % - 40 %	< 20 %

Fig. 6. Heatmap showing the effects of individual compounds and binary combinations of DON and Cd on the expression of junctional proteins after 24 h of exposure. Results are expressed as means of three independent experiments ± SEM. (# combined effects *versus* effects of Cd alone, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$. $, combined effects *versus* effects of DON alone, $p < 0.05$; $$, $p < 0.01$; $$$, $p < 0.001$). The intensity of the color reflects the extent of the decrease in the junctional proteins. Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

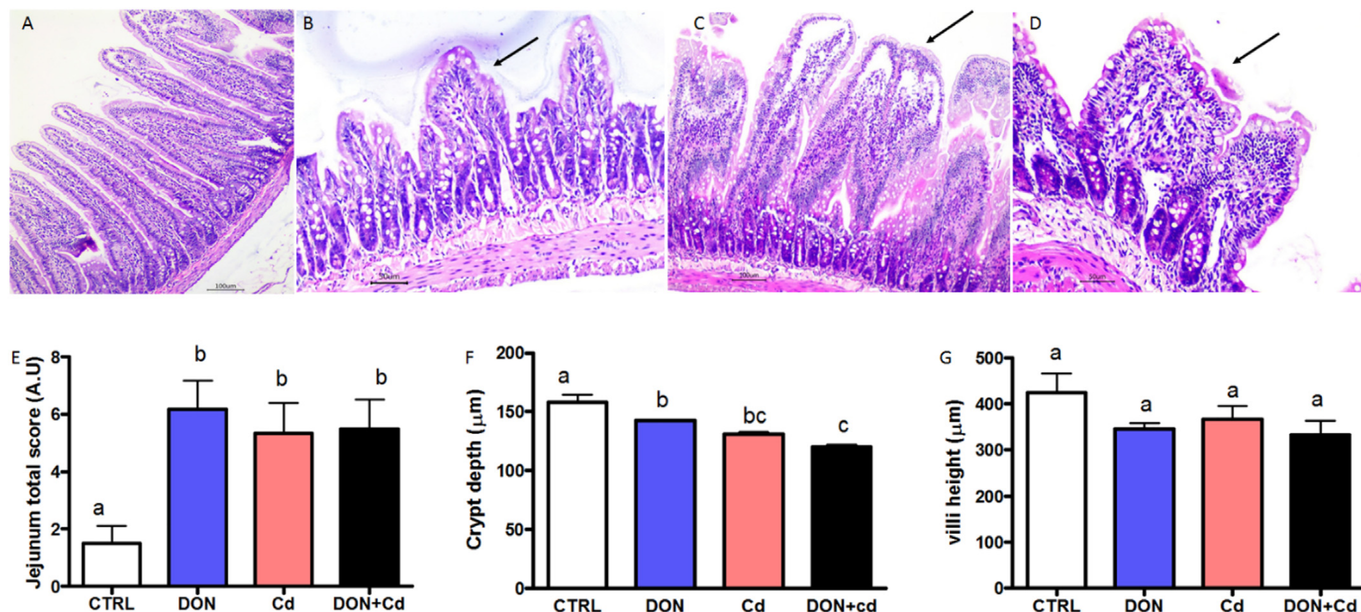


Fig. 8. Individual and combined effects of DON and Cd on jejunum histology. Histology of the jejunum after hematoxylin–eosin staining: control rat (A), DON treated rat (B, villus atrophy and fusion, arrow; 20×), Cd treated rat (C, moderate interstitial edema and enterocyte flattening, arrow; 10×) and DON + Cd treated rat (D, villus atrophy and fusion, arrow; 20×). Lesional score (E); crypt depth (F) and villus height (G). Values are means ± SEM (n = 8). Mean values with different letters differ ($p < 0.05$). Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

abundance of E-cadherin decreased by 57%, 49% and 71% in animals treated with DON, Cd, and DON + Cd respectively (Fig. 9E).

For occludin, strong homogeneous immunostaining at the cell membrane was observed in control rats as well as in rats exposed to Cd (Fig. 10A, C). By contrast, weak heterogeneous staining was observed in the jejunum of animals exposed to DON and to the combination of DON and Cd, (Fig. 10B, D) compared to that in control rats, the abundance of occludin decreased by 84% and 62% ($p < 0.001$), respectively (Fig. 10E).

These results show that exposure to either DON or Cd reduces the expression of the E-cadherin and occludin at the cell membrane. Exposure to the mixture led to the same reduction in junctional protein as ingestion of DON alone.

4. Discussion

The combined toxicity of pollutants is hard to predict based on the toxic effect of a single compound (Alassane-Kpembi et al., 2017b; More et al., 2019). In the last few years, the number of studies of the combined toxicity of pollutants has increased but knowledge of the impact on animal and human health of exposure to mixtures of pollutants is nevertheless still very poor (Silins and Högberg, 2011). The main objective of the present study was to investigate the effects of a mycotoxin, DON, and a heavy metal, Cadmium, alone and in combination, on the intestinal epithelial barrier function. Two experimental approaches were used: *in vitro* exposure of human intestinal epithelial cells (Caco-2) to DON and Cd alone or in combination, and the *in vivo* dietary exposure of rats to these contaminants. The range of concentrations tested *in vitro* (1 to 30 μM) was chosen to screen a large panel of plausible exposure and adverse effects. In human, assuming that DON consumption in one meal is diluted in 1 l of gastrointestinal fluid and is totally bioaccessible (Sergent et al., 2006). The doses used in this study correspond to 0.296, 0.89, 2.96 and 8.89 μg/ml, which lay in the range of the plausible intestinal concentrations of DON (0.25–10 μg/ml) (De

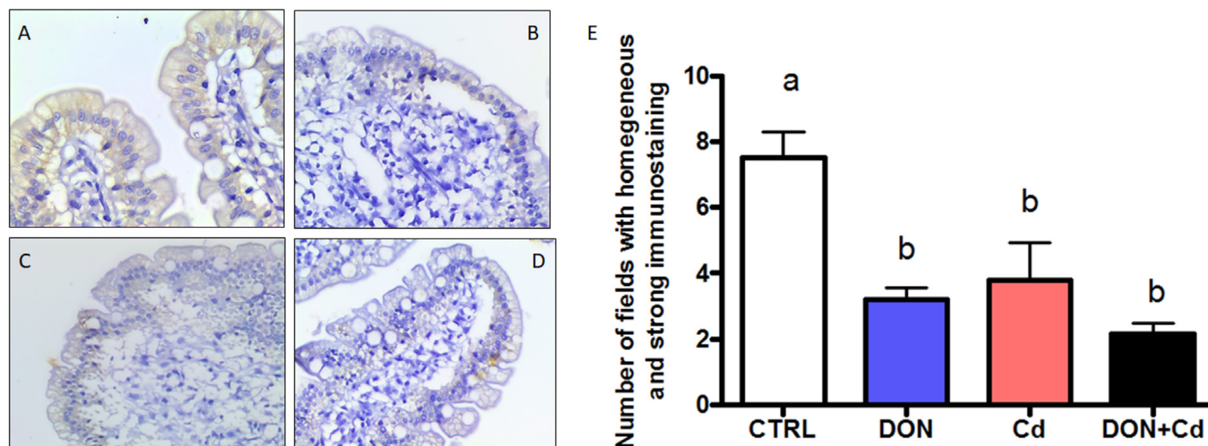


Fig. 9. Individual and combined effects of DON and Cd on E-cadherin expression in jejunum. (A, 60×) Jejunum of rats in the control group showed strong homogeneous staining on E-cadherin; less intense immunostaining on E-cadherin occurred in the jejunum of rats in the groups treated with DON (B, 40×), Cd (C, 40×) and DON + Cd (D, 40×). (E) The number of fields with strong homogeneous immunostaining. Values are mean ± SEM (n = 8). Means with a different letter differ ($p < 0.05$). Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

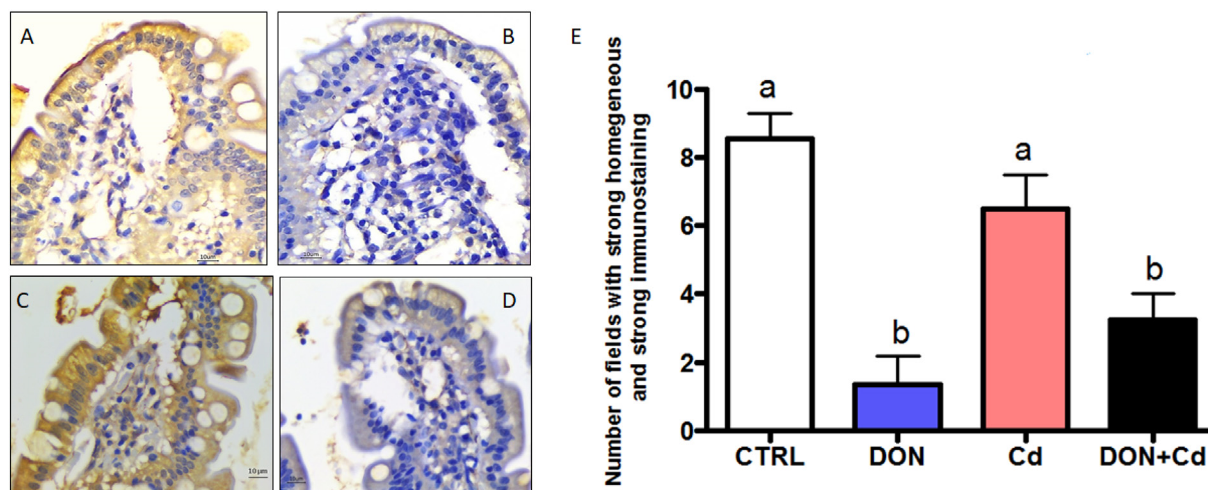


Fig. 10. Individual and combined effect of DON and Cd on occludin expression in jejunum. (A, 60×) The jejunum of rats in the control group showed strong homogeneous staining on occludin; less intense immunostaining on occludin occurred in the jejunum of rats in the groups treated with DON (B, 40×), Cd (C, 40×) and the combination of DON and Cd (D, 40×). (E) The number of fields with strong homogeneous immunostaining. Values are mean ± SEM (n = 8). Means with a different letter differ ($p < 0.05$). Data were analyzed by non-parametric one-way ANOVA with Bonferroni's multiple comparison test.

Walle et al., 2008). Furthermore, as previous report indicates that the molar ratio of mean exposure to DON and Cd in food in Europe is 1:1 (Le et al., 2018), the doses chosen for Cd were in the same range of doses as DON. The recommendation for DON in feed intended for farm animals is 5 mg/kg feed (European Union, 2006). In our experiment, the concentration of DON in feed was 8.2 mg/kg. Indeed, the concentrations of 2–10 mg/kg feed of DON are the most frequently used in toxicological studies of farm and experimental animal models and demonstrate adverse effects in specific target organs (intestine, liver, kidney) (Bracarense et al., 2012; Pinton et al., 2009, 2008). The concentration of 5 mg/l CdCl2 in drinking water is an environmentally realistic low dose (Benoff et al., 2008; Brzóska et al., 2003). It led only to modification of the phenotype of innate immune cells in the mesenteric lymph nodes in a rat model (Ninkov et al., 2015). Our proposal was to investigate the potential increase of the adverse effects when cadmium was in combination with another toxic compound. We took into account that the molar ratio of mean exposure of European population to DON and Cd in food is estimated to be 1:1 (Le et al., 2018). Considering the daily ingestion of food and water by the rat and the content of DON in food, the concentration of 5 mg of Cd/l of drinking was in accordance to reach this realistic ratio.

To evaluate the effects of DON and Cd alone or in combination on the intestine *in vivo*, rats were exposed to the contaminants for four weeks. The body weight gain of animals was not affected in any of the conditions tested. However, alterations in the histology and the morphology of the intestine were observed after exposure to DON and Cd alone or in combination. A significant decrease was observed in crypt depth in the jejunum of rats exposed to DON, Cd and DON + Cd. The effect of DON on crypt depth has already been reported in pigs (Gerez et al., 2015) and might be due to the DON-induced ribotoxic stress that compromised protein synthesis and triggered apoptosis (Pestka, 2010; Pierron et al., 2016a; Pinton et al., 2010). In piglets exposed to DON, a decrease in the number of proliferating cells in the intestinal crypt in response to DON has been already observed (Bracarense et al., 2012; Gerez et al., 2015). A study on broiler chickens showed that Cd ingestion caused histological alterations such as a decrease in leaf-like villi and a reduction in crypt depth (Teshfam et al., 2006). Moreover, inhibition of proliferation and induction of apoptosis has been observed in human renal mesangial cells and subventricular neural stem cells exposed to Cd. These effects were mediated by activation of the JNK or p-38 pathway (Wang et al., 2017). It is thus tempting to hypothesize that Cd inhibits the proliferation of intestinal epithelial cells *via* the same MAPK-dependent mechanism. By contrast, villus height was not affected irrespective of the contaminant to which the rats were exposed. This suggests proliferating cells are more sensitive to DON and/or Cd than differentiated ones, as already described *in vitro* for DON (Bony et al., 2006; Pierron et al., 2016a).

The histological and morphological alterations induced by the two contaminants tested here suggest that the intestinal barrier function may also be impaired. A dose-dependent reduction in the TEER and an increase in paracellular permeability were observed in DON and in Cd treated cells. The reduction in the barrier function caused by DON is mediated by MAPKs/ERK activation, as inhibition of this signaling pathway restored TEER in porcine and human intestinal epithelial cells (Du et al., 2018; Pinton et al., 2010; Springler et al., 2016). A MAPKs/ERK activation in the differentiated Caco-2 cells treated with Cd has also been reported (Mantha and Jumarie, 2010), and may be involved in the disruption of the intestinal barrier by Cd.

The intestinal barrier function is closely linked to the junctional protein network and several studies have shown that DON or Cd alter some junctional proteins including occludin or E-cadherin (De Walle et al., 2010; Pinton et al., 2009; Templeton and Liu, 2013). However, the effects of combinations of DON and Cd on the junctional proteins have not previously been investigated. Here, we observed a decrease in E-cadherin expression in the three groups of exposed rats (DON, CD and DON + Cd) while the decrease in occludin was only observed in animals exposed to DON (with or without Cd). By contrast, in Caco-2 cells, the amount of E-cadherin and occludin decreased after exposure to the two contaminants (DON and Cd) alone or in combination. This was associated with redistribution of the junctional proteins from the membrane to the cytoplasm. A similar decrease in the expression of E-cadherin has already been observed in pigs fed a DON-contaminated diet (Bracarense et al., 2012; Pierron et al., 2018). As far as Cd is concerned, several studies have shown that, in intestinal cells, intercellular junctions, mainly E-cadherin and actin cytoskeleton, are sensitive to this contaminant (Duizer et al., 1999; Rusanov et al., 2015). In MCF-7 cells, Cd has been shown to disturb calcium homeostasis and cause a depletion of Ca^{2+} (Zhou et al., 2015); this induces the activation of Cdc42, a member of Rho family GTPases, and leads to the ubiquitination and degradation of E-cadherin *via* Src-mediated pathway (Shen et al., 2008). In a human airway tissue model, Cd induced redistribution of occludin by tyrosine phosphorylation through c-Src and PKC activation, resulting in TJ disruption (Cao et al., 2015). Whether these modes of action of Cd also occur in the intestine remains to be determined. Disruption of junctional adhesion associated with increased paracellular permeability can facilitate the passage of pathogens and/or

harmful substances into the body. Therefore, animals or humans exposed to these toxins are likely to be more sensitive to intestinal pathogens. In addition, increased intestinal permeability may also favor permeability to other contaminants (Payros et al., 2017).

Humans and animals are exposed to multi-component mixtures in the environment and food, whereas chemical risk assessment generally concerns the toxicity of single compounds (More et al., 2019). The effects of combinations of several food contaminants are very poorly documented. In the case of DON and Cd, apart from a previous study by our team (Le et al., 2018), no study has investigated their combined effect. The *in vitro* and *in vivo* data presented in this paper indicate that, whatever the parameter analyzed (TEER, paracellular permeability or the abundance of junction protein), the toxicity resulting from exposure to a combination of DON and Cd is similar to that caused by each individual contaminant. It is well known that chemical compounds have multiple modes of action, a range of targets and different degrees of affinity for these targets, and, conversely, that each mode of action can be used by a variety of chemical compounds (Thrupp et al., 2018). As described above, the mechanisms of action of DON and Cd share some pathways, one of which is the MAPkinase pathway. Indeed, DON and Cd induce the activation of ERK1/2 and p38 respectively, leading to disruption in tight junctions (Pinton et al., 2010; Rather et al., 2017). We hypothesize that, when combined, DON and Cd play competitive roles in the pathway, which would explain why the effect of a mixture of DON and Cd is no greater than the effect of each individual contaminant.

From the point of view of human health, our results suggest that existing regulation for DON and Cd are sufficient to protect consumers exposed to a mixture of these contaminants. Indeed, their combined effects on the intestine were identical to the effect of the most toxic compound alone, whatever the parameter analyzed. These results point to a less than additive effect. More data are needed to determine if the results concerning the effect of DON and Cd on the intestine can be extended to other organs and/or to other mixtures.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105082.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgments

We are grateful to the staff of the team EZOP for animal care. We thank the UPAE (*Unité de Preparation des Aliments Expérimentaux*, UE03000, Jouy-en-Josas, France) for the preparation of the diets.

Funding

The work was supported, in part, by the French National Research Agency (project CaDON 15-CE21-0001-02) and by the CAPES Cofecub SV 947 19.

Su Luo was supported by the China Scholarship Council.

References

Alassane-Kpembi, I., Puel, O., Pinton, P., Cossalter, A.-M., Chou, T.-C., Oswald, I.P., 2017a. Co-exposure to low doses of the food contaminants deoxynivalenol and nivalenol has a synergistic inflammatory effect on intestinal explants. Arch. Toxicol. 91, 2677–2687. https://doi.org/10.1007/s00204-016-1902-9.

Alassane-Kpembi, I., Schatzmayr, G., Taranu, I., Marin, D., Puel, O., Oswald, I.P., 2017b. Mycotoxins co-contamination: methodological aspects and biological relevance of combined toxicity studies. Crit. Rev. Food Sci. Nutr. 57, 3489–3507. https://doi.org/10.1080/10408398.2016.1140632.

Arnich, N., Sirot, V., Rivière, G., Jean, J., Noël, L., Guérin, T., Leblanc, J.-C., 2012. Dietary exposure to trace elements and health risk assessment in the 2nd French Total Diet Study. Food Chem. Toxicol. Int. J. Publ. Br. Ind. Biol. Res. Assoc. 50, 2432–2449. https://doi.org/10.1016/j.fct.2012.04.016.

ATSDR, 2012. Toxicological Profile for Cadmium. Available on line.. https://www.atsdr.cdc.gov/toxprofiles/tp5.pdf, Accessed date: 7 December 2018.

Benoff, S., Auborn, K., Marmar, J.L., Hurley, I.R., 2008. Link between low-dose environmentally relevant cadmium exposures and asthenozoospermia in a rat model. Fertil. Steril. 89, e73–e79. https://doi.org/10.1016/j.fertnstert.2007.12.035.

Bishak, Y.K., Payahoo, L., Osatdrahimi, A., Nourazarian, A., 2015. Mechanisms of cadmium carcinogenicity in the gastrointestinal tract. Asian Pac. J. Cancer Prev. 16, 9–21.

Bony, S., Carcelen, M., Olivier, L., Devaux, A., 2006. Genotoxicity assessment of deoxynivalenol in the Caco-2 cell line model using the Comet assay. Toxicol. Lett. 166, 67–76. https://doi.org/10.1016/j.toxlet.2006.04.010.

Bracarense, A.-P.F.L., Lucioli, J., Grenier, B., Drociunas Pacheco, G., Moll, W.-D., Schatzmayr, G., Oswald, I.P., 2012. Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets. Br. J. Nutr. 107, 1776–1786. https://doi.org/10.1017/S0007114511004946.

Bracarense, A.P.F.L., Basso, K.M., Da Silva, E.O., Payros, D., Oswald, I.P., 2017. Deoxynivalenol in the liver and lymphoid organs of rats: effects of dose and duration on immunohistological changes. World Mycotoxin J. 10, 89–96. https://doi.org/10.3920/WMJ2016.2094.

Breton, J., Daniel, C., Vignal, C., Body-Malapel, M., Garat, A., Plé, C., Foligné, B., 2016. Does oral exposure to cadmium and lead mediate susceptibility to colitis? The dark-and-bright sides of heavy metals in gut ecology. Sci. Rep. 6, 19200. https://doi.org/10.1038/srep19200.

Brzóska, M.M., Kamiński, M., Supernak-Bobko, D., Zwierz, K., Moniuszko-Jakoniuk, J., 2003. Changes in the structure and function of the kidney of rats chronically exposed to cadmium. I. Biochemical and histopathological studies. Arch. Toxicol. 77, 344–352. https://doi.org/10.1007/s00204-003-0451-1.

Cao, X., Lin, H., Muskhelishvili, L., Latendresse, J., Richter, P., Heflich, R.H., 2015. Tight junction disruption by cadmium in an in vitro human airway tissue model. Respir. Res. 16, 30. https://doi.org/10.1186/s12931-015-0191-9.

Claus Henn, B., Coull, B.A., Wright, R.O., 2014. Chemical mixtures and children's health. Curr. Opin. Pediatr. 26, 223–229. https://doi.org/10.1097/MOP.0000000000000067.

De Walle, J.V., Romier, B., Larondelle, Y., Schneider, Y.-J., 2008. Influence of deoxynivalenol on NF-kappa B activation and IL-8 secretion in human intestinal Caco-2 cells. Toxicol. Lett. 177, 205–214. https://doi.org/10.1016/j.toxlet.2008.01.018.

De Walle, J. Van, Sergent, T., Piront, N., Toussaint, O., Schneider, Y.-J., Larondelle, Y., 2010. Deoxynivalenol affects in vitro intestinal epithelial cell barrier integrity through inhibition of protein synthesis. Toxicol. Appl. Pharmacol. 245, 291–298. https://doi.org/10.1016/j.taap.2010.03.012.

Du, Z., Kim, K.H., Kim, J., Moon, Y., 2018. Fungal deoxynivalenol-induced enterocyte distress is attenuated by adulterated adlay: in vitro evidences for mucoactive counteraction. Front. Immunol. 9, 186. https://doi.org/10.3389/fimmu.2018.00186.

Duizer, E., Gilde, A.J., Versantvoort, C.H., Groten, J.P., 1999. Effects of cadmium chloride on the paracellular barrier function of intestinal epithelial cell lines. Toxicol. Appl. Pharmacol. 155, 117–126. https://doi.org/10.1006/taap.1998.8589.

EFSA, 2009. Cadmium in food - scientific opinion of the panel on contaminants in the food chain. EFSA J. 7. https://doi.org/10.2903/j.efsa.2009.980.

EFSA, 2017. Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. EFSA J. 15. https://doi.org/10.2903/j.efsa.2017.4718.

European Union, 2006. Commission Recommendation of 17 August 2006 on the Presence of Deoxynivalenol, Zearalenone, Ochratoxin A, T-2 and HT-2 and Fumonisins in Products Intended for Animal Feeding [WWW Document]. URL. https://eur-lex.europa.eu/eli/reco/2006/576/oj, Accessed date: 7 March 2019.

Filipič, M., Fatur, T., Vudrag, M., 2006. Molecular mechanisms of cadmium induced mutagenicity. Hum. Exp. Toxicol. 25, 67–77. https://doi.org/10.1191/0960327106ht590oa.

Ganguly, K., Levänen, B., Palmberg, L., Åkesson, A., Lindén, A., 2018. Cadmium in tobacco smokers: a neglected link to lung disease? Eur. Respir. Rev. 27, 170122. https://doi.org/10.1183/16000617.0122-2017.

Gerez, J.R., Pinton, P., Callu, P., Grosjean, F., Oswald, I.P., Bracarense, A.P.F.L., 2015. Deoxynivalenol alone or in combination with nivalenol and zearalenone induce systemic histological changes in pigs. Exp. Toxicol. Pathol. 67, 89–98. https://doi.org/10.1016/j.etp.2014.10.001.

Kim, H.S., Kim, Y.J., Seo, Y.R., 2015. An overview of carcinogenic heavy metal: molecular toxicity mechanism and prevention. J. Cancer Prev. 20, 232–240. https://doi.org/10.15430/JCP.2015.20.4.232.

Le, T.-H., Alassane-Kpembi, I., Oswald, I.P., Pinton, P., 2018. Analysis of the interactions between environmental and food contaminants, cadmium and deoxynivalenol, in different target organs. Sci. Total Environ. 622–623, 841–848. https://doi.org/10.1016/j.scitotenv.2017.12.014.

Liu, L., Tao, R., Huang, J., He, X., Qu, L., Jin, Y., Zhang, S., Fu, Z., 2015. Hepatic oxidative stress and inflammatory responses with cadmium exposure in male mice. Environ. Toxicol. Pharmacol. 39, 229–236. https://doi.org/10.1016/j.etap.2014.11.029.

Lucioli, J., Pinton, P., Callu, P., Laffitte, J., Grosjean, F., Kolf-Clauw, M., Oswald, I.P., Bracarense, A.P.F.R.L., 2013. The food contaminant deoxynivalenol activates the mitogen activated protein kinases in the intestine: interest of ex vivo models as an alternative to in vivo experiments. Toxicon 66, 31–36. https://doi.org/10.1016/j.toxicon.2013.01.024.

Lukowicz, C., Ellero-Simatos, S., Régnier, M., Polizzi, A., Lasserre, F., Montagner, A., Lippi, Y., Jamin, E.L., Martin, J.-F., Naylies, C., Canlet, C., Debrauwer, L., Bertrand-Michel, J., Al Saati, T., Théodorou, V., Loiseau, N., Mselli-Lakhal, L., Guillou, H., Gamet-Payrastre, L., 2018. Metabolic effects of a chronic dietary exposure to a low-dose pesticide cocktail in mice: sexual dimorphism and role of the constitutive

androstane receptor. Environ. Health Perspect. 126, 067007. https://doi.org/10.1289/EHP2877.
Mantha, M., Jumarie, C., 2010. Cadmium-induced hormetic effect in differentiated Caco-2 cells: ERK and p38 activation without cell proliferation stimulation. J. Cell. Physiol. 224, 250–261. https://doi.org/10.1002/jcp.22128.
Maresca, M., 2013. From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol. Toxins Basel 5, 784–820. https://doi.org/10.3390/toxins5040784.
Miller, M.F., Goodson, W.H., Manjili, M.H., Kleinstreuer, N., Bisson, W.H., Lowe, L., 2017. Low-dose mixture hypothesis of carcinogenesis workshop: scientific underpinnings and research recommendations. Environ. Health Perspect. 125, 163–169. https://doi.org/10.1289/EHP411.
More, S.J., Hardy, A., Bampidis, V., Benford, D., Hougaard Bennekou, S., Bragard, C., Boesten, J., Halldorsson, T.I., Hernández-Jerez, A.F., Jeger, M.J., Knutsen, H.K., Koutsoumanis, K.P., Naegeli, H., Noteborn, H., Ockleford, C., Ricci, A., Rychen, G., Schlatter, J.R., Silano, V., Nielsen, S.S., Schrenk, D., Solecki, R., Turck, D., Younes, M., Benfenati, E., Castle, L., Cedergreen, N., Laskowski, R., Leblanc, J.C., Kortenkamp, A., Ragas, A., Posthuma, L., Svendsen, C., Testai, E., Dujardin, B., Kass, G.E., Manini, P., Zare Jeddi, M., Dorne, J.C., Hogstrand, C., Hogstrand, C., 2019. Guidance on harmonised methodologies for human health, animal health and ecological risk assessment of combined exposure to multiple chemicals. EFSA J. 17. https://doi.org/10.2903/j.efsa.2019.5634.
Ninkov, M., Popov Aleksandrov, A., Demenesku, J., Mirkov, I., Mileusnic, D., Petrovic, A., Grigorov, I., Zolotarevski, L., Tolinacki, M., Kataranovski, D., Brceski, I., Kataranovski, M., 2015. Toxicity of oral cadmium intake: impact on gut immunity. Toxicol. Lett. 237, 89–99. https://doi.org/10.1016/j.toxlet.2015.06.002.
Payros, D., Alassane-Kpembi, I., Pierron, A., Loiseau, N., Pinton, P., Oswald, I.P., 2016. Toxicology of deoxynivalenol and its acetylated and modified forms. Arch. Toxicol. 90, 2931–2957. https://doi.org/10.1007/s00204-016-1826-4.
Payros, D., Dobrindt, U., Martin, P., Secher, T., Bracarense, A.P.F.L., Boury, M., Laffitte, J., Pinton, P., Oswald, E., Oswald, I.P., 2017. The food contaminant deoxynivalenol exacerbates the genotoxicity of gut microbiota. mBio 8. https://doi.org/10.1128/mBio.00007-17.
Pestka, J.J., 2010. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Arch. Toxicol. 84, 663–679. https://doi.org/10.1007/s00204-010-0579-8.
Pierron, A., Mimoun, S., Murate, L.S., Loiseau, N., Lippi, Y., Bracarense, A.P., Liaubet, L., Schatzmayr, G., Berthiller, F., Moll, W.D., Oswald, I.P., 2016a. Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-glucoside. Arch. Toxicol. 90, 2037–2046. https://doi.org/10.1007/s00204-015-1592-8.
Pierron, A., Mimoun, S., Murate, L.S., Loiseau, N., Lippi, Y., Bracarense, A.P., Schatzmayr, G., He, J.W., Zhou, T., Moll, W.D., Oswald, I.P., 2016b. Microbial biotransformation of DON: molecular basis for reduced toxicity. Sci. Rep. 6, 29105. https://doi.org/10.1038/srep29105.
Pierron, A., Bracarense, A.P.F.L., Cossalter, A.-M., Laffitte, J., Schwartz-Zimmermann, H.E., Schatzmayr, G., Pinton, P., Moll, W.-D., Oswald, I.P., 2018. Deepoxy-deoxynivalenol retains some immune-modulatory properties of the parent molecule deoxynivalenol in piglets. Arch. Toxicol. 92, 3381–3389. https://doi.org/10.1007/s00204-018-2293-x.
Pinton, P., Oswald, I.P., 2014. Effect of deoxynivalenol and other type B trichothecenes on the intestine: a review. Toxins Basel 6, 1615–1643. https://doi.org/10.3390/toxins6051615.
Pinton, P., Accensi, F., Beauchamp, E., Cossalter, A.-M., Callu, P., Grosjean, F., Oswald, I.P., 2008. Ingestion of deoxynivalenol (DON) contaminated feed alters the pig vaccinal immune responses. Toxicol. Lett. 177, 215–222. https://doi.org/10.1016/j.toxlet.2008.01.015.
Pinton, P., Nougayrède, J.-P., Del Rio, J.-C., Moreno, C., Marin, D.E., Ferrier, L., Bracarense, A.-P., Kolf-Clauw, M., Oswald, I.P., 2009. The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression. Toxicol. Appl. Pharmacol. 237, 41–48. https://doi.org/10.1016/j.taap.2009.03.003.
Pinton, P., Braicu, C., Nougayrede, J.-P., Laffitte, J., Taranu, I., Oswald, I.P., 2010. Deoxynivalenol impairs porcine intestinal barrier function and decreases the protein expression of Claudin-4 through a mitogen-activated protein kinase-dependent mechanism. J. Nutr. 140, 1956–1962. https://doi.org/10.3945/jn.110.123919.
Rather, I.A., Koh, W.Y., Paek, W.K., Lim, J., 2017. The sources of chemical contaminants in food and their health implications. Front. Pharmacol. 8, 830. https://doi.org/10.3389/fphar.2017.00830.
Rusanov, A.L., Smirnova, A.V., Poromov, A.A., Fomicheva, K.A., Luzgina, N.G., Majouga, A.G., 2015. Effects of cadmium chloride on the functional state of human intestinal cells. Toxicol. Vitro Int. J. Publ. Assoc. BIBRA 29, 1006–1011. https://doi.org/10.1016/j.tiv.2015.03.018.
Sergent, T., Parys, M., Garsou, S., Pussemier, L., Schneider, Y.-J., Larondelle, Y., 2006. Deoxynivalenol transport across human intestinal Caco-2 cells and its effects on cellular metabolism at realistic intestinal concentrations. Toxicol. Lett. 164, 167–176. https://doi.org/10.1016/j.toxlet.2005.12.006.
Shen, Y., Hirsch, D.S., Sasiela, C.A., Wu, W.J., 2008. Cdc42 regulates E-cadherin ubiquitination and degradation through an epidermal growth factor receptor to Src-mediated pathway. J. Biol. Chem. 283, 5127–5137. https://doi.org/10.1074/jbc.M703300200.
Silins, I., Högberg, J., 2011. Combined toxic exposures and human health: biomarkers of exposure and effect. Int. J. Environ. Res. Public Health 8, 629–647. https://doi.org/10.3390/ijerph8030629.
Sirot, V., Fremy, J.-M., Leblanc, J.-C., 2013. Dietary exposure to mycotoxins and health risk assessment in the second French total diet study. Food Chem. Toxicol. 52, 1–11. https://doi.org/10.1016/j.fct.2012.10.036.
Springler, A., Hessenberger, S., Schatzmayr, G., Mayer, E., 2016. Early activation of MAPK p44/42 is partially involved in DON-induced disruption of the intestinal barrier function and tight junction network. Toxins 8, 264. https://doi.org/10.3390/toxins8090264.
Suzuki, T., 2013. Regulation of intestinal epithelial permeability by tight junctions. Cell. Mol. Life Sci. 70, 631–659. https://doi.org/10.1007/s00018-012-1070-x.
Templeton, D.M., Liu, Y., 2013. Effects of cadmium on the actin cytoskeleton in renal mesangial cells. Can. J. Physiol. Pharmacol. 91, 1–7. https://doi.org/10.1139/cjpp-2012-0229.
Terciolo, C., Dapoigny, M., Andre, F., 2019. Beneficial effects of Saccharomyces boulardii CNCM I-745 on clinical disorders associated with intestinal barrier disruption. Clin. Exp. Gastroenterol. 12, 67–82. https://doi.org/10.2147/CEG.S181590.
Teshfam, M., Gharagozlou, M.J., Salaramoli, J., Hassanpour, H., 2006. Morphological alterations of the small intestine mucosa following oral administration of cadmium in broiler chickens. J. Appl. Anim. Res. 29, 65–68. https://doi.org/10.1080/09712119.2006.9706573.
Thrupp, T.J., Runnalls, T.J., Scholze, M., Kugathas, S., Kortenkamp, A., Sumpter, J.P., 2018. The consequences of exposure to mixtures of chemicals: something from 'nothing' and 'a lot from a little' when fish are exposed to steroid hormones. Sci. Total Environ. 619–620, 1482–1492. https://doi.org/10.1016/J.SCITOTENV.2017.11.081.
Tinkov, A.A., Gritsenko, V.A., Skalnaya, M.G., Cherkasov, S.V., Aaseth, J., Skalny, A.V., 2018. Gut as a target for cadmium toxicity. Environ. Pollut. 235, 429–434. https://doi.org/10.1016/j.envpol.2017.12.114.
Turner, P.C., Rothwell, J.A., White, K.L.M., Gong, Y., Cade, J.E., Wild, C.P., 2008. Urinary deoxynivalenol is correlated with cereal intake in individuals from the United Kingdom. Environ. Health Perspect. 116, 21–25. https://doi.org/10.1289/ehp.10663.
Wang, H., Engstrom, A.K., Xia, Z., 2017. Cadmium impairs the survival and proliferation of cultured adult subventricular neural stem cells through activation of the JNK and p38 MAP kinases. Toxicology 380, 30–37. https://doi.org/10.1016/j.tox.2017.01.013.
Zhang, S., Jin, Y., Zeng, Z., Liu, Z., Fu, Z., 2015. Subchronic exposure of mice to cadmium perturbs their hepatic energy metabolism and gut microbiome. Chem. Res. Toxicol. 28, 2000–2009. https://doi.org/10.1021/acs.chemrestox.5b00237.
Zhou, X., Hao, W., Shi, H., Hou, Y., Xu, Q., 2015. Calcium homeostasis disruption - a bridge connecting cadmium-induced apoptosis, autophagy and tumorigenesis. Oncol. Res. Treat. 38, 311–315. https://doi.org/10.1159/000431032.