Université Fédérale

Toulouse Midi-Pyrénées

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

Délivré par :

Université Toulouse 3 Paul Sabatier (UT3 Paul Sabatier)

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le 28/06/2016

Titre :

Toxicity of three biological derivatives of deoxynivalenol: deepoxy-deoxynivalenol, 3-epi-deoxynivalenol and deoxynivalenol-3-glucoside on pigs

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École doctorale et discipline ou spécialité :
ED SEVAB : Pathologie, Toxicologie, Génétique et Nutrition

Unité de recherche :
UMR 1331 INRA - ToxAlim

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“La plus grande gloire n'est pas de ne jamais tomber, mais de se relever à chaque chute.”
Confucius

The present work was possible by a fellowship from CIFRE (2012/0572, jointly financed by the BIOMIN Holding GmbH, Association Nationale de la Recherche Technique (ANRT) and the laboratory of INRA-ToxAlim, UMR 1331.

♣ I would like to thank all researchers who have accepted to be part of my jury:

– Prof.Chiara Dall'Asta, Dr. Sven Dänicke and Prof. Alexis Valentin, who accepted to evaluate my work.

– Dr. Wulf-Dieter Moll, for having accepted to be my co-director during this PhD, for his support and all his advice and for trusting me during these 4 years.

– Dr. Isabelle Oswald :

Isabelle, merci à toi de m'avoir donné ma chance en stage d'ingénieur, puis d'avoir continué à me faire confiance pour une thèse. Merci de m'avoir permis de faire de nombreuses expérimentations, supplémentaires, non prévues… Je te remercie de m'avoir permis de participer à de nombreux congrès et réunions à l'étranger qui m'ont fait énormément progresser et évoluer! Enfin, merci de m'avoir suivie, conseillée, à toute heure, jour et nuit, 7 jours sur 7, pour rendre sans cesse mon travail meilleur. Ta capacité de travail et de gestion de nombreux projets simultanément est impressionnante. En un mot j'ai adoré ma thèse sous ta direction.

♣ BIOMIN :

I would like to thank the company for funding my thesis, allowing me to make many varied experiments in my research during my PhD. I thank BIOMIN France and BIOMIN Austria, as well as Gerd Schatzmayr, Chirstian Tenier, Wulf-Dieter Moll to make possible this collaboration. They gave me the opportunity to visit them several times in Austria, go to several congresses where I met many researchers. All of this was very rewarding. Dieter, Gerd, it was a great pleasure to work with you during this period.

♣ Merci à tous les collaborateurs qui ont participé directement ou indirectement à la réalisation de ces travaux. Merci à Ting Zhou et à Franz Berthiller pour nous avoir procuré les différentes molécules nécessaires à mes analyses.

Un grand merci au Professeur Ana-Paula Loureiro Bracarensede l'école vétérinaire de Londrina au Brésil, pour avoir réalisé toutes les coupes et analyses histologiques des expérimentations *in vivo*. J'ai été enchantée de te rencontrer et de bénéficier de tes conseils.

♣ Je tiens aussi à remercier toutes les personnes qui m'ont accompagnée pendant ces années de travail au sein de ToxAlim, de près ou de loin, et que j'ai eu le plaisir de rencontrer ; qui m'ont aidé, conseillé, soutenu, encouragé et avec qui j'ai eu le plaisir de papotter!

♣ A ma chère équipe, tu vas me manquer! Ces 4 années et 4 mois tout compris, que j'ai passé au sein de cette équipe ont été super, riches en enseignement et en rencontre. Merci à tous les membres qui la composent, qui font fonctionner la machine et qui font en sorte que notre séjour, plus ou moins long, soit toujours agréable ! Ce sera difficile de trouver une équipe aussi chouette, avec des personnes aussi travailleuses, dynamiques, enjouées et compétentes que celles qui la compose.

Joëlle, telle une abeille butineuse tu t'affaires sans relâche pour que toute la ruche fonctionne correctement et ait toujours assez pour se nourrir et fonctionner! Merci Joëlle.

Philippe, tel le sage de la montagne, tu sais tout ! Toujours patient, tu écoutes et promulgue soit un bon conseil, une astuce ou encore des précautions! Je suis contente d'avoir pu travailler avec toi.

Anne, que dire…, peut-être que de passer la meilleure des journées c'est de la passer avec toi et les cochons! Anne je te souhaite tout le meilleur pour la suite et encore plus.

Je remercie aussi tous les autres membres de l'équipe pour leur disponibilité, conseils et bonne humeur : Olivier, Sophie, Soraya, Sylviane, Jean-Denis, Arlette ; cela a été un plaisir de travailler à vos côtés.

Une pensée à tous les stagiaires, thésards, post-docs que j'ai eu le plaisir de rencontrer et de côtoyer de plus ou moins près pendant ma thèse, et pour tous les bons moments passés en vôtre compagnie : Pascal, Sabria, Julien, Julie, Joanna, Rhoda, Patricia, Yann, Stella, Anwar, HK, Stéphanie, Florian, Inès, Pauline, Duncan, Joanne, Leticia, Viviane, Sophal, Joya… j'espère que je n'oublie personne. Aux post-docs, Delphine, Selma et Imourane courage pour la suite et les concours !

♣ Pendant cette thèse j'ai eu l'occasion de rencontrer des étudiantes supers (eh oui que des filles), dynamiques, intelligentes et courageuses, pour certaines prêtes à faire leurs études loin de leur famille. Les filles tout le meilleur pour la suite avec une super thèse, un super post-doc et un super échantillon à la clé… ! ;)

Elisabeth, Marion, Yann et Sarah, merci pour tous les moments de rigolades partagés, les longues discussions…, les aprems détentes/jeux, tout le meilleur pour la suite ! Courage Marion et Sarah bientôt la fin pour vous aussi !;)

Une pensée spéciale aux filles sublimes et exotiques! ;), qui m'ont soutenu, aidé avec plein d'entrain et de bonnes humeur!
Joanna, Rhoda, Joanne, Joya et Christelle, merci de m'avoir fait découvrir la culture libanaise à travers vous. Christelle merci énormément pour tes corrections d'anglais de dernière minute !
Isaura, Amaranta, associées aux brésiliennes, merci de m'avoir fait découvrir un brin de votre culture et la joie de vivre des filles d'Amérique du Sud !;)
Bianca, j'ai été ravie de découvrir ton beau pays !

Je tiens aussi à remercier et à citer Beyoncé pour son soutient (même si elle n'est pas au courant) pendant toutes ces soirées de travail et de rédaction… : « I'm a survivor, I'm not gonna give up, I'm not gon stop, I'm gon work harder… ».

Une pensée pour tous mes cochons, décédés mais non moins aimés. A mon Marshmallow trop vite parti…, ainsi que Baloo et tous les autres.

♣ Un grand merci à mon club de Kung Fu, mon équipe de volley et à mes compagnons de sorties roller et danse…, qui m'ont permis de bien me défouler et de me vider la tête au besoin.

♣ Merci à tout mon entourage, de plus ou moins loin, qui m'ont soutenu, réconforté et aussi permis de me changer les idées, merci d'être là ! Je citerai en particulier Sarah (ma BF), Florent (mon BF), Christelle pour votre soutien actif !

♣ Un mot à ma famille (plus ou moins éloignée), inébranlable, parfaite, qui m'a toujours soutenue, encouragée, portée, cette thèse est aussi pour vous. **Je vous aime** : Jacky, Jojo, Maman.

Original papers

Pierron A., Mimoun S., Murate L. S., Lippi Y., Loiseau N., Bracarense A-P., Liaubet L., Schatmayr G., Berthiller F., Moll W.D. and Oswald I. P. Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-glucoside (D3G). 2016. Archive of Toxicology

Pierron A., Mimoun S., Murate L. S., Lippi Y., Loiseau N., Bracarense A-P., Zhou T., Schatmayr G., Moll D. and Oswald I. P. 2016. Microbial biotransformation of DON reduces its intestinal toxicity. Scientific Report **(***In press***).**

Pierron A., Cossalter A-M., Bracarense A-P., Schwartz-Zimmermann H., Pinton P., Laffitte J., Schatzmayr G., Moll D. and Oswald I. P. Evaluation of metabolisation and toxicity of purified deepoxy-deoxynivalenol (DOM-1) in piglets (*Under writing*).

Review

Pierron A., Alassane-Kpembi I., Payros D., Pinton P. and Oswald I. P. Masked mycotoxins : the risk on porcine production ? 2016. 48th days of Journée de la recherche Porcine (JRP).

Pierron A., Alassane-Kpembi I. and Oswald I. P. 2016. Impact of mycotoxins on immune response and consequences for pig health. Animal Nutrition.

Pierron A., Alassane-Kpembi I. and Oswald I. P. 2016. Feed mycotoxins: impact on pig intestinal health. PHM (*Under review*).

Communications (Oral and Poster)

A. Pierron, S. Mimoun, L. S. Murate, Y. Lippi, L. Liaubet, F. Berthiller, G. Schatmayr, W.D. Moll and I. P. Oswald. Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-glucoside (D3G). Day of PhD student of SEVAB, Toulouse (France), March 18, 2016. (Oral).

A.Pierron and I. P. Oswald. Masked mycotoxins : the risk on porcine production ? Journée de la recherche Porcine, February, Paris (France), February3-4, 2016. (Oral)

A.Pierron, S. Mimoun, L. S. Murate, Y. Lippi, L. Liaubet, F. Berthiller, G. Schatmayr, W.D. Moll and I. P. Oswald. Microbial de-epoxidation of deoxynivalenol (DON) decreases its immunotoxicity for piglets. EVIW2015, Vienna (Austria), September 2-4, 2015. (Oral)

A.Pierron, S. Mimoun, L. S. Murate, Y. Lippi, L.Liaubet, F. Berthiller, G. Schatmayr, W.D. Moll and I. P. Oswald. Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-glucoside (D3G). Journée des doctorants, Toxalim (Toulouse), June 26, 2015. (Oral)

Mimoun S., **Pierron A.**, L. S. Murate, Y. Lippi, L.Liaubet, F. Berthiller, G. Schatmayr, W.D. Moll and I. P. Oswald. Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-glucoside. 37th Mycotoxin Workshop, Bratislava (Slovakia), June 1-3, 2015.

A.Pierron, S. Mimoun, L. S. Murate, Y. Lippi, L.Liaubet, F. Berthiller, G. Schatmayr, W.D. Moll and I. P. Oswald. Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-glucoside (D3G). EFS13, Martina Franca (Italy), May 10 - 14, 2015. (Oral).

Souto P.C.M.C., Jager A.V., Tonin F.G., Bordin K., **Pierron A.**, Cossalter A.M., Laffitte J., Oswald I.P., Oliveira C.A.F. 2014. Immune response and urinary fumonisin B1 excretion in piglet fed low dietary levels of Fumonisin. The World Mycotoxin 8th Forum Conference, Vienna (Austria), November 10-12, 2014**.**

Pierron A., Mimoun S., Lippi Y., Gourbeyre P., Liaubet L., Zhou T., Berthiller F., Schatmayr G., Moll D. and Oswald I.P. Plant or microbial transformation of DON reduce its intestinal toxicity. The 6th World Nutrition Forum, Munich (Germany), October 15-18, 2014. (Poster)

Pierron A., Mimoun S., Lippi Y., Gourbeyre P., Liaubet L., Zhou T., Berthiller F., Schatmayr G., Moll D. and Oswald I.P. Masked DON and other DON metabolites do not exert intestinal toxicity : Transcriptomic analysis of jejuna explants. The World Mycotoxin 8th Forum Conference, Vienna (Austria), November 10-12, 2014. (Poster)

Pierron A., Mimoun S., Lippi Y., Gourbeyre P., Liaubet L., Zhou T., Berthiller F., Schatmayr G., Moll D. and Oswald I.P. Plant or microbial biotransformation of DON reduce its intestinal toxicity. 36th Mycotoxin Workshop, Göttingen (Germany), June 16 - 18, 2014. (Oral)

F. Accensi, P. Pinton, M. Sibila, **A. Pierron**, A.M. Cossalter, J. Segalés, I.P. Oswald. Effect of deoxynivalenol (DON) intoxication on a PCV2 infection in piglets. 23th International Pig Veterinary Society Congress, Cancun (Mexico), June 8-11, 2014.

Mimoun S., **Pierron A**., Lippi Y., Gourbeyre P., Liaubet L., Zhou T., Berthiller F., Schatmayr G., Moll D. and Oswald I.P. La décontamination biologique du DON réduit sa toxicité intestinale chez le porc. 5ème journée Mycotoxines, France (Montpellier), Juin 5-6 , 2014,

Awards and distinctions

Marasas Oral Prize Winner :

A.Pierron, S. Mimoun, L. S. Murate, Y. Lippi, L.Liaubet, F. Berthiller, G. Schatmayr, W.D. Moll and I. P. Oswald. Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-glucoside (D3G). EFS13, Martina Franca (Italy), May 10 - 14, 2015. (Oral).

List of Abbreviations

15ADON	15-acetyl-deoxynivalenol
3ADON	3-acetyl-deoxynivalenol
ADONs	acetylated forms of deoxynivalenol
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFs	aflatoxines
ATP	adenosine triphophaste
BC	before Christ
BW	Body Weight
Caco-2	human colonic adenocarcinoma cell line
DOM-1	deepoxy-deoxynivalenol
DON	deoxynivalenol
DON15GlcA	deoxynivalenol-15-β-D-glucuronide
DON3G	deoxynivalenol-*3-β-D*-glucoside
DON3GlcA	deoxynivalenol-3-β-D-glucuronide
DON3S	deoxynivalenol-3-α-sulfate
DON-GlcA	deoxynivalenol-glucuronide
3-epi-DON	3-epi-deoxynivalenol
EFSA	European Food Safety Authority
ERK	extracellular signal-regulated kinases
EtOH	ethanol
F	fumonisin
FB1	fumonisin B1
FB2	fumonisin B2
FHB	Fusarium head blight
GSTs	glutathione *S*-transferases
HT2-Glc	HT-2 toxin
HT2-Glc	HT-2 toxin-glucoside
IARC	International Agency for Research on Cancer
IC_{50}	half maximal inhibitory concentration
Ig	immunoglobulin
IL	interleukin
ILSI	International Life Science Institute
IPEC-1	porcine intestinal epithelial cell line
IPEC-J2	porcine intestinal epithelial cell line derived from the jejunum
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOAEL	low observed adverse effect level
MAPK	mitogen-activated protein kinases
mRNA	messenger ribonucleic acid
NA	not available
NIV	nivalenol
NOAEL	no observed effect limit

NS	non significant
OTA	ochratoxine A
OVA	ovalbumin A
PAT	patulin
PCR	polymerase chain reaction
PCV2	porcine circo virus type 2
PHA	phytohemagglutinin
ppm	part per million
PRRSV	porcine reproductive and respiratory syndrome virus
PTC	peptidyl transferase center
q-PCR	quantitative-PCR
RIN	RNA integrity number
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	reverse transcriptase
SEM	standard error mean
T-2	T-2 toxin
T2-G	T-2 toxin-glucoside
TEER	trans epithelial electrical resistance
TCT	trichothecene
TDI	tolerable daily intake
Th	T helper cell
TNF-α	tumour necrosis factor-α
ZEN	zearalenone
ZEN14G	zearalenone-14-glucoside
ZEN14S	zearalenone-14-sulfate
ZEN16G	zearalenone-16-glucoside
α-ZEL	α-zearalenol
β-ZEL	β-zearalenol

List of figures and tables

List of figures and tables not included in paper or review: from Introduction, Chapter 2 and General Discussion & Perspectives parts.

INTRODUCTION

I. Context of the study

Plants and cereals are subject to numerous fungal contaminations occurring either in fields or during storage processes. Many species of fungi exist and are able to grow on various types of cereals (maize, wheat, barley, soybean, rice, rye…) or on food commodities (seeds, peanuts, fruits, spices, forages…) (Figure 1) (AFSSA 2009).

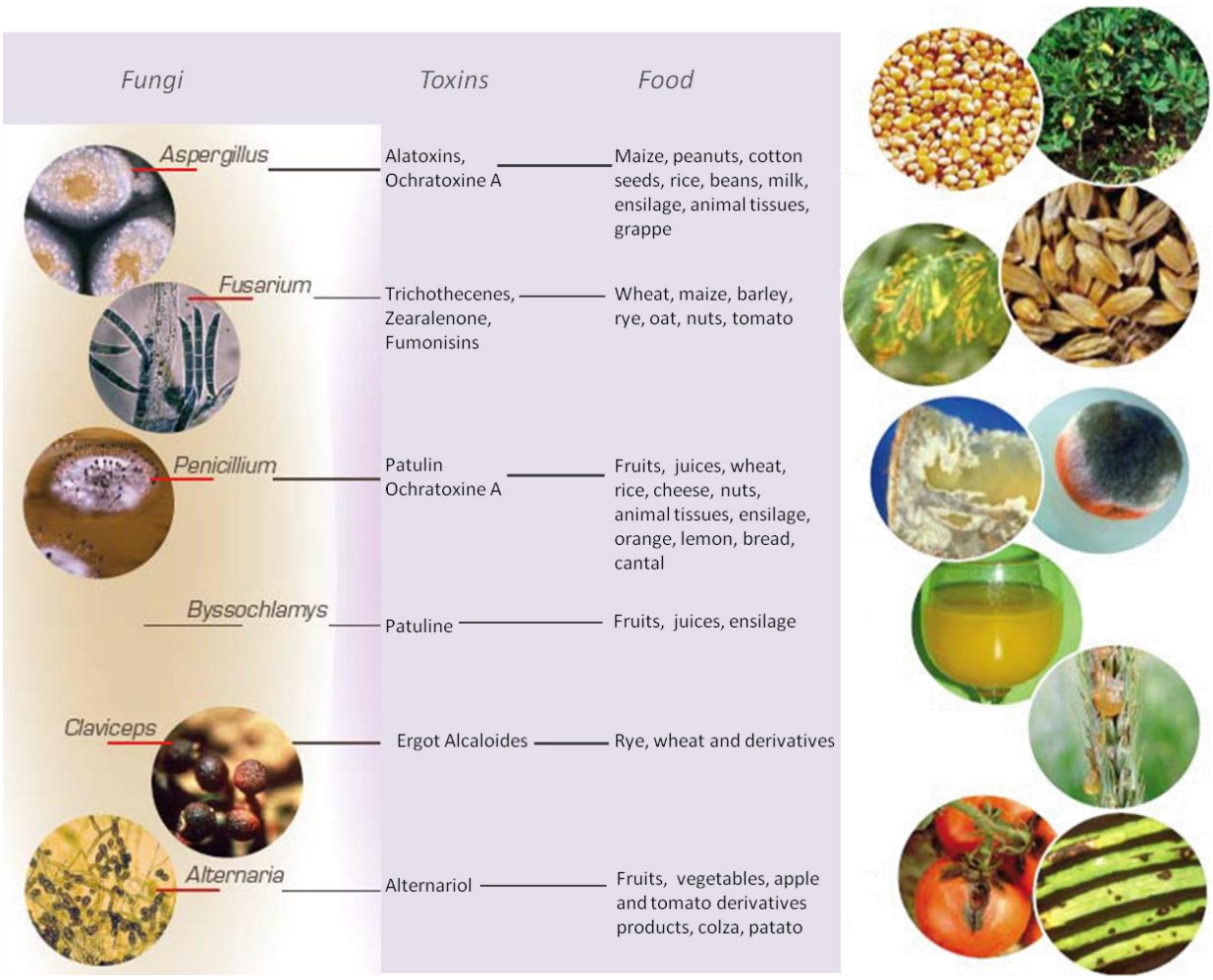

Figure 1 – Major mycotoxins produced by fungi and naturally found in several food products

These fungi are able to produce several toxic molecules, called mycotoxins (from Greek μύκης (mykes, mukos) ‘fungus’ and Latin (toxicum) ‘poison’) (Aiko and Mehta 2015). Mycotoxins are secondary metabolites; and unlike primary metabolites, they are not essential to the development and survival of the fungus but could constitute an advantage during the colonization of ecological niche when in competition with other microorganism. These molecules also discourage predators from eating the fungus (Keller et al. 2005).

Mycotoxins can be divided into polyketoacids, terpenes, cyclopepetides and nitrogen metabolites, depending on their origins and their structures (AFSSA 2009). They can also be classified according to their toxic effects. Mycotoxins considered important in terms of food safety are aflatoxins (AF), ochratoxins (OT) (in particular ochratoxin A (OTA)), patulin (PAT), fumonisins (FB), zearalenone (ZEA) and trichothecenes (TCT), especially deoxynivalenol (DON) (Figure 2) (Bennet and Klich 2003). Several factors control fungal growth and mycotoxin production, such as weather conditions, agricultural practices or storage conditions (Hesseltine 1976).

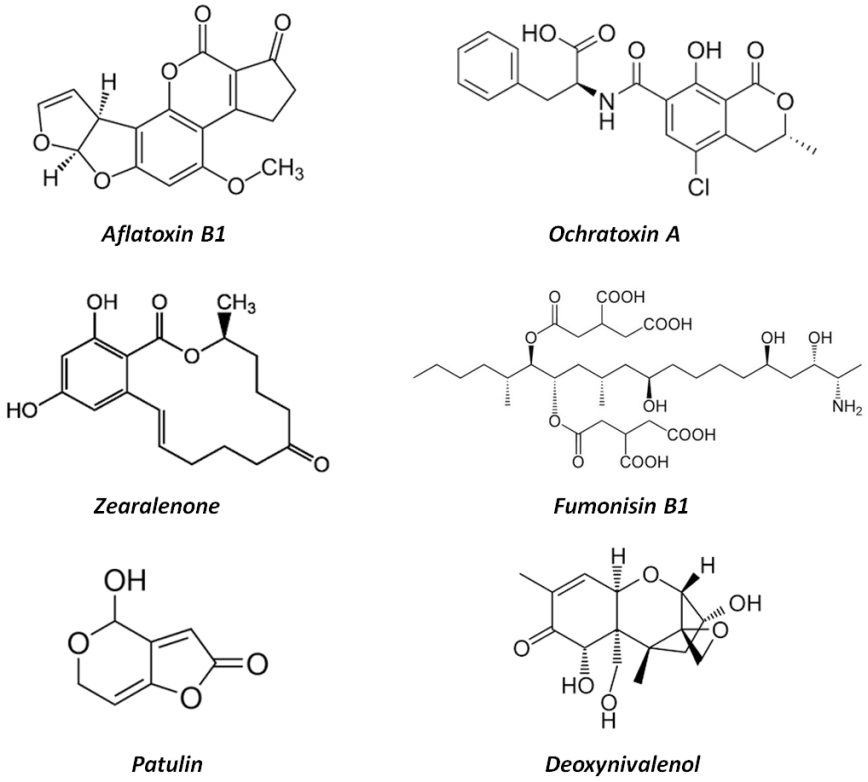

Figure 2 – Structural diversity of major mycotoxins

The toxic effects of moulds and fungi were already known in ancient times. Historically, many illnesses linked to mycotoxicoses have been reported (AFSSA 2009). The most famous case, which occurred in the Middle Ages, is known under the name of ignis sacer (sacred fire) or St Anthony's fire. It was caused by toxins of *Claviceps purpurea*, the ergot alkaloids of rye (Figure 3). Ergotism reached epidemic proportions, mutilating and killing thousands of people in Europe. Victims of ergotism suffered from delirium, prostration, acute pain, abscess and gangrene of the extremities, leading to serious and incurable infirmity. Epidemics occurred from the 8th to the 15th century due to the bad quality of food and contamination with fungal sclerotia. Similarly, fusariotoxins (toxin T2 and ZEA),

seem to have been involved in the decline of the Etruscan civilization 5 centuries B.-C (Richard 2003). "Yellow rice disease" or shoshin-kakke disease in Japan was also a mycotoxicosis caused by unhygienic conditions and practices, which is induced by the Citreoviridin, a metabolite produced by *Penicillium citreonigrum*. This fungus used to grow readily on rice during its storage (after harvest), especially in the colder regions of Japan. New hygiene measures applied, more rigorous than before, made it disappear (Udagawa and Tatsuno 2004). In 1960, the turkey X disease has been an important episode of mycotoxicosis on animals. It killed thousands of turkey, ducklings and other domestic animals in England. This allowed the discovery of aflatoxins, main mycotoxin produced by A*spergillus flavus*, and present in a high quantity in groundnut flour designed for poultry's food (Blount 1961).

Figure 3 – "Saint Antoine tentation" painted between 1512 – 1516 by Grünewald (on the left). Ears of rye contaminated with ergot (on the right).

Most mycotoxins have an acute toxicity, but nowadays it's exceptional to be exposed to such high doses in Europe (Européenne C. 2003). In this part of the world chronic contamination is the most threatening, due to the persistence of these mycotoxins in food and the repeated ingestion by animals for example. In 2004, a worldwide survey showed that 72% of more than 19000 samples analyzed contained detectable amounts of AF, FB, DON, ZEA or OTA (Figure 4). Among them, 38% represented a co-contamination by 2 or more mycotoxins (Schatzmayr and Streit 2013). Several toxic effects can be induced, depending of the mycotoxin and the organ targeting. At high doses, mycotoxins exposure can leads to general cytotoxicity, biochemical lesions and impact on early cellular functions in the cascade of events (Bryden 2012; Maresca and Fantini 2010). At low doses, various functions of tissues

and organs can be impaired. And some mycotoxins are also genotoxic, carcinogenic and teratogenic (Maresca and Fantini 2010).

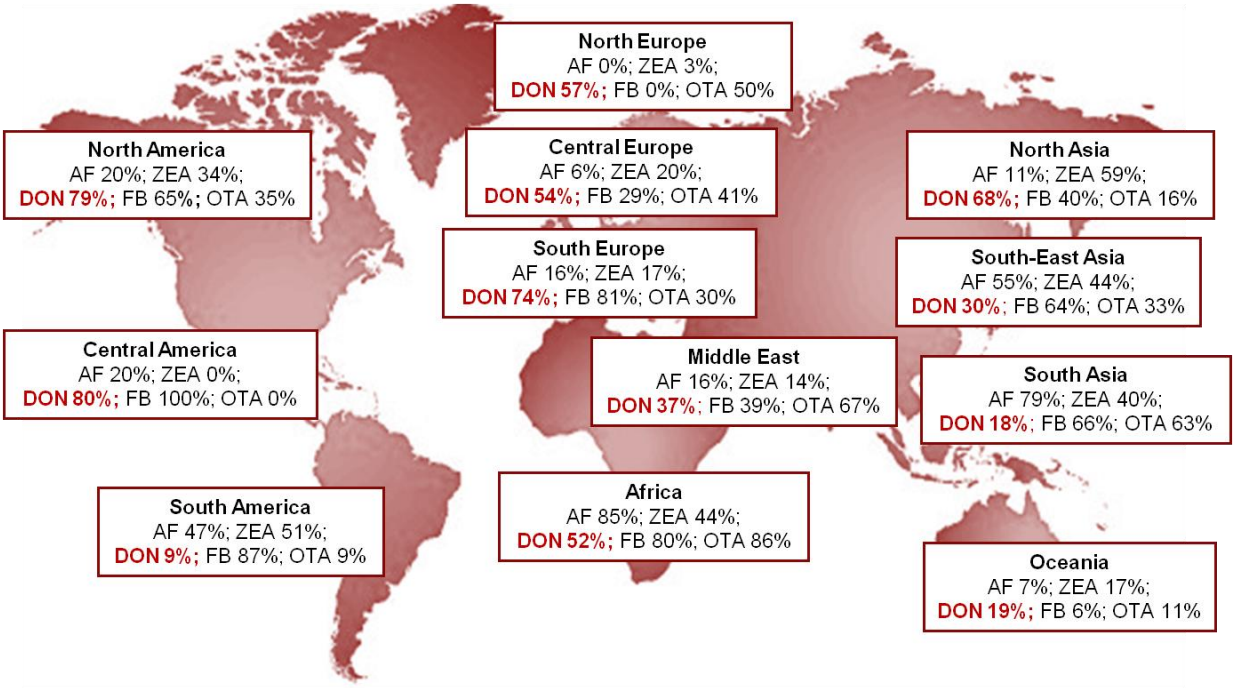

Figure 4 - Global mycotoxin prevalence in surveyed regions (adapted from Schatzmayr and Streit 2013). Aflatoxins (AF), zearalenone (ZEA), deoxynivalenol (DON), fumonisins (FB), ochratoxin A (OTA).

II. Problem of DON contamination

A. Occurrence

DON is produced by *Fusarium* fungi, one of the most common mycotoxin in the world. It and can be found in many cereals and raw materials, like wheat, barley, oat, rye, maize and sometimes on rice, sorghum and triticale. A worldwide survey to assess the contamination by mycotoxins in feed and feed raw materials, done on 19,000 samples, shows that DON was present in 56% of the tested samples (Schatzmayr and Streit 2013).

Fungal infection and DON production are difficult to predict and regulate; because largely dependent on the weather, high humidity and low temperature, and so vary greatly from year to year and between areas (Rotter et al. 1996). In developed countries, where storage conditions are well managed and controlled, DON contamination is especially a pre-harvest problem. While, in developing countries, DON can also be produced during the storage stage. So, DON can be commonly detected at low levels (< 1 ppm) and sporadically at higher levels (5 to 20 ppm) on cereals intended to be given to animals or humans (Abouzied et al. 1991). It

can also be present in end products, such as the cereal-based food for adults and infants or even at low levels in beer (Lombaert et al. 2003; Scott 1996).

Economic losses due to DON contamination are difficult to evaluate. Nevertheless a computer simulations evaluated that the annual costs for DON in the USA were $637 million in crop losses (mainly wheat and corn), $18 million in feed losses and $2 million in livestock losses (EFSA 2013).

B. Toxicity

The toxicity of DON is well known and numerous studies bring information on its toxic effects at high and chronic doses (Maresca 2013; Pestka 2010; Wang et al. 2014). A high concentration of DON causes effects and symptoms that are similar to those observed during an exposure to ionizing radiation, such as abdominal distress, salivation, discomfort, diarrhea, vomiting, leukocytosis and gastrointestinal bleeding. It also has high emetic and anorexic effects, that are equal or even higher to those of observed with the most toxic trichothecene B (Pestka and Smolinski 2005). Actually, the first name of DON was “vomitoxin” due to its emetic effects seen in pigs (Vesonder et al. 1973).

A chronic exposure can impact growth (by anorexia and disregulation of nutrients efficacy), immunity (increased or decreased) and reproduction in animals. At acute doses it can induces emesis, abdominal distress, malaise, diarrhea and increases the salivation (Pestka 2010). At low dose it impairs the growth and the immune function in human and interferes with nutritional efficiency on pigs (Rotter et al. 1996). At higher doses it causes diarrhea, emesis, leukocytosis, hemorrage, endotexemia and ultimately shock-like death (Ueno 1983).

C. Detoxification methods for DON

The effectiveness of detoxification methods of mycotoxins depends on several parameters, the nature of the food/feed, the environmental conditions such as moisture content, temperature, as well as the type of mycotoxin, its concentration and the extent of binding between mycotoxin and constituents (Grenier and Oswald 2011).

DON resist to most of the industrial processes; it is stable at high temperature, due to its high chemical stability and can be found in numerous final processed products (Hazel and Patel 2004). Actually DON is completely stable at 120°C, quite stable at 180°C and partially stable at 210°C (OMS 2001). At concentrations below 1mg/kg, DON is mainly found on the

seed surface but at higher concentrations, it can be found in the entire grain (Charmley and Prelusky 1994). To reduce the occurrence and the impact of mycotoxins and especially DON, several detoxifying strategies were established in the feed chain, including the prevention of fungal growth and the production of mycotoxin, strategies to reduce or eliminate mycotoxins from contaminated raw or finished materials or even in diverting contaminated product to low risk uses including animal feeds (Bryden 2012). However, the amount of information related to mycotoxins detoxifying methods is still limited. From the described detoxifying strategies there are three principal categories used: the physical, the chemical and the biological methods.

1. Physical methods

Some processes used to detoxify mycotoxins (such as milling, irradiation, ethanol fermentation or extrusion) were initially developed for other purposes, and some were specifically developed for the detoxification itself (such as sorting, cleaning or washing). These practices, are linked to the FAO guidelines, namely fulfilled: cheap and simple, no production of toxic metabolites, and no change in the nutritional value or properties of raw materials. However, all these approaches present some inconvenient. The standard processes, like milling and baking, do not allow the elimination of DON with efficacy (Abbas et al. 1985; Hart and Braselton 1983). Dry milling, permit an elimination that is up to 40% of DON present in the flour; sieving or cleaning can reduce the concentration in DON by over 60% (Pestka and Smolinski 2005). The problem of the milling and grain separation process, commonly used for human's food, is that it concentrates all the mycotoxins in bran and Germ, fractions will be used later for animal feed. However, in the sieving and cleaning procedures, an important loss of grains is reported. In their study, Trenholm et al. (1991) did observe a 73% reduction of DON, but they have also observed that up to 69% of the total weights of the corns was removed as well. And after flotation and washing, the cost of drying grains is high.

2. Chemical methods

Several chemical processes, using molecules like ammonia, calcium hydroxide, chlorine, hydrochloric acid, ozone, sodium bisulphate and sodium hydroxide are able to degrade DON. In fact ammoniation has been proved to reduce the aflatoxin levels but this process is not accepted in all countries and is quite expensive (Norred et al. 1991; Park and

Price 2001). With alkalization, DON can be transformed into different products, with various toxicity (Bretz et al. 2005; Bretz et al. 2006; Young et al. 1986).

In majority, the chemical methods can reduce mycotoxins' levels, but they can also severely damage the nutrient quality of the grains and can be health hazards on their own. Not only that, they can result in the formation of degraded products that might be constituted of new and unknown biologically active mycotoxins (Humpf and Voss 2004).

3. Biological methods

Two strategies are possible to manage DON, once present in plants and cereals. The first strategy consist on preventing the production of DON in infected crop by controlling the plant pathogens (*Fusarium* spp.). Equipping crops with DON detoxification activities can reduce the concentration of mycotoxin in grain and also increase the resistance against infection (Karlovsky 2011). It was shown that DON plays a role in the infection; host plants inoculated with fungal strain not able to produce TCT can't be able to infect the plant (Maier et al. 2006; Proctor et al. 1995). Another study shows that a major QTL responsible for the resistance of wheat to FHB co-segregated with the ability to detoxify DON by glycosylation (Lemmens et al. 2005); It has been proved that by selecting a plant naturally resistant to *Fusarium*, its capacity to glycosylate DON into D3G can be increased by 2.7 times more (Sasanya et al. 2008).

Some companies also tried to build transgenic plants, by transferring the 3-O-acetyltransferase gene issued from *F. sporotrichioides* to the plant in order to reduce the pathogenicity of *Fusarium* (Karlovsky 2011).

The second strategy consists in detoxifying DON that has been produced, by physical and chemical methods as we saw but more innovative by biological methods. The bio-detoxification of mycotoxins, by isolating microorganisms and/or enzymes that will degrade or metabolize the mycotoxins, is currently an innovative and promising strategy aiming to control mycotoxicoses in animals (Schatzmayr et al. 2006). (Cheng et al. 2010) obtained two Bacillus strains able to detoxify DON in wheat and maize. In another study, *Bacillus sp.* LS100, which transforms deoxynivalenol (DON) to a less toxic chemical de-epoxy DON (DOM-1) has been assessed. This intestinal bacteria, Genus novus species novus of family Coriobacteriaceae BBSH 797, isolated from digestive tracts, is able to de-epoxydize DON to DOM-1 (Fuchs et al. 2002). There is also the bacterial strain Devosia mutans 17-2-E-8, isolated from an alfalfa soil enriched with *F. graminearum*-infested corn that is able to highly

reduce DON level, in producing an epimer, the 3-epi-DON (He et al. 2015b; Zhou and He 2009, 2010).

Bringing the enzymatic kit to animals, by the use of bacteria, will allow them detoxify mycotoxins, and easily and effectively protect them against the toxic effects of mycotoxins. Definitely, since decontaminated or detoxified crops are cheaper (since they are considered as products of lower quality), they are mainly used for feed production and animal feeding (Grenier and Oswald 2011), in which explains why animals are very exposed. The need of feed additives preventing the absorption of mycotoxins and by that occurrence of their toxic effects in farm animals has increased significantly. Indeed, the adsorption is not a viable option regarding trichothecenes, zearalenone and ochratoxins, that's why the mycotoxin inactivation by biotransformation is a very promising strategy to detoxify these mycotoxins.
However, all the additives and bacterial products have to be tested before coming into the market to assure their efficiency and safety. *In vitro* and *in vivo* tests are mostly important to check and follow their scientific development and improvement. Sensitive parameters such as biochemistry, gross pathology, histopathology, immune parameters and animal performances have to be measured to evaluate their toxicity. This is why the aim of this thesis was to evaluate the toxicity of the products issued from biological detoxification and to assess the efficiency of this process in order to protect animals or humans from the toxic effects of DON.

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III. Literature review

The literature review consists on three reviews covering different aspects studied during this thesis. The first two reviews deal with the different effects caused by mycotoxins and the intestine or the immunity of pigs. The third review focuses on new forms of mycotoxins derived from these mycotoxins, the "masked" and "modified" mycotoxins.

To date, contamination by mycotoxins cannot be avoided. Mycotoxins can be present in several types of cereals (maize, wheat, barley, oats, rice ...) and end up in high concentrations due to cultural practices or storage. These mycotoxins can be found in co-contamination in pig feed. All these mycotoxins have toxic effects on pig that is particularly sensitive because of its simple digestive system and its high cereal rich diet. The first two reviews have a look on the two most affected parameters after a contamination by one or more mycotoxin, on the gut and the immune system of the pig. Mycotoxins contaminations, mainly by ingestion, cause many toxic effects on the digestive system and small intestine.
The first review presents the different mycotoxins that are found in pig's feed and their effects and consequences on the intestine and the general health of the pig. Mycotoxins have been also described as responsible of modifying important functions of the intestine (barrier function, mucus production, nutrient absorption…).
The second review reports the effect of mycotoxins on the immune system of pigs. Certainly, many mycotoxins have an immune-modulatory effect on the immune response and may affect the vaccine response as well as induce an increased susceptibility to infections or chronic infectious diseases.

Finally, the last review presents advances in terms of new analytical methods allowing the identification of new forms of mycotoxins, the mycotoxins called maskedand modified. It is important to study these new forms of mycotoxins to evaluate their impact on pig health and to assess whether they can represent an additional threat that will have to be taken into account in the overall management of the risk of mycotoxins.

A. Feed mycotoxins: impact on pig intestinal health

Nowadays, many mycotoxins can contaminate cereals and feeding stuffs designated to the pig consumption. These mycotoxins have several toxic effects on pigs, which are greatly impacted, due to their high sensibility and their cereals rich diet.

Due to the way of exposure, by ingestion, intestine is the major organ targeted by mycotoxins. This review summarizes the major effects induced by these mycotoxins on the intestine, on its integrity, its biological function and on its immune response. It also highlights the consequences of this contamination, which increases the translocation of bacteria and enhances the susceptibility to other diseases and thus impairs the global health of pigs.

This review is currently submitted to Porcine Health Management.

Mycotoxins in Feed: impact on pig intestinal health

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Abstract

Mycotoxins are secondary metabolites of fungi that grow on a variety of substrates. Due to their high consumption of cereals and their sensitivity, pigs are highly impacted by the presence of mycotoxins. Pigs can be exposed to different mycotoxins such as aflatoxins, ochratoxins, fumonisins, zearalenone, and trichothecenes especially deoxynivalenol. At the European level, regulations and recommendations exist for these mycotoxins in pig feed. The intestine is the first barrier to food contaminants and can be exposed to high concentrations of mycotoxins upon ingestion of contaminated feed. Mycotoxins target this organ, they alter the intestinal barrier, impair the immune response, reduce feed intake and weight gain. Among them, deoxynivalenol and fumonisin have been studied especially for their toxicity in the intestine. Their presence in feed increases the translocation of bacteria; mycotoxins can also impair the immune response and enhance the susceptibility to infectious diseases. In conclusion, because of their effect on the intestine, mycotoxins are a major threat to pig health, welfare and performance.

Keywords:

Pig, mycotoxins, feed contamination, intestine, barrier function, immune response

Introduction

Food safety is a major issue throughout the world. In this respect, much attention needs to be paid to the possible contamination of food and feed by fungi and the risk of mycotoxin production. Mycotoxins are secondary metabolites produced by filamentous fungi, mainly by species from the genus *Aspergillus*, *Fusarium* and *Penicillium*. They are produced on a wide variety of substrates before, during and after harvest. Mycotoxins are very resistant to technological treatments and difficult to eliminate, and therefore they can be present in human food and animal feed. The ingestion of mycotoxin-contaminated feed can induce acute diseases, and the ingestion of low doses of fungal toxins also causes damage in case of repeated exposure.

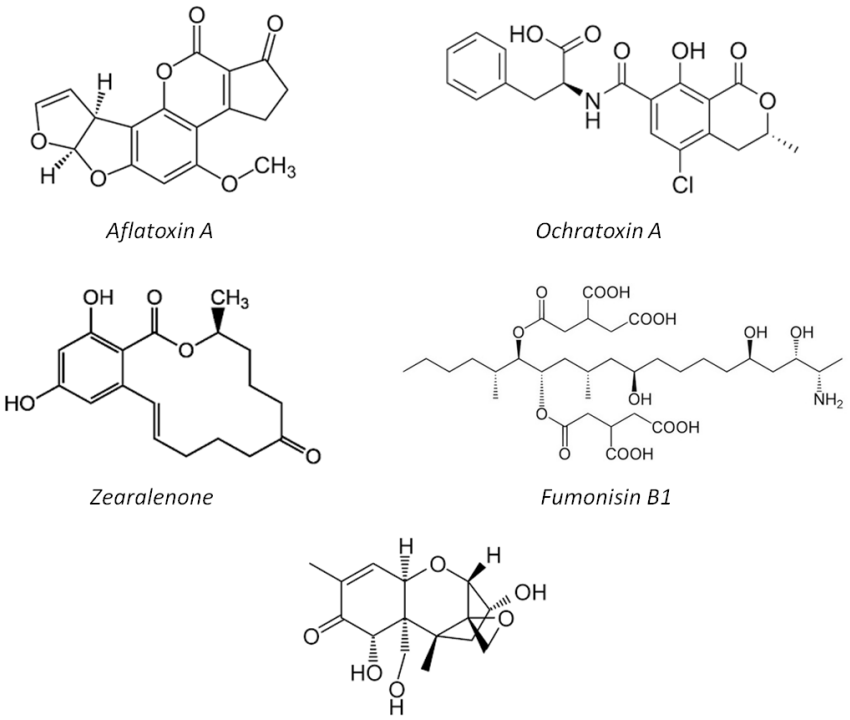
Monogastric livestock, pig and poultry, are particularly vulnerable to mycotoxins because of the high percentage of cereals in their diet and because they lack a rumen with a microbiota able to degrade mycotoxins before their intestinal absorption. From a pig health perspective, the most notorious mycotoxins (Fig.1) are aflatoxins (AF), ochratoxin A (OTA), fumonisins B (FB), zearalenone (ZEN), and trichothecenes, especially deoxynivalenol (DON) (CAST 2003).

This review will summarize the effect of mycotoxins on the intestine and analyze the consequences in terms of pig health.

I- Toxicity of the main mycotoxins in pig feed

The toxicity of mycotoxins varies according to several parameters such as the dose, the duration of exposure, the age and the sex of the animal, as well as nutritional factors (Andretta et al. 2012; Bryden 2007; Wild 2007). For example, the effects of AF, FB or DON on performance are greater in males and young pigs (Andretta et al. 2012; Marin et al. 2006). In the European Union, only AFs are regulated in animal feed; recommendations exist for OTA, DON, T2 and HT-2 toxins, FB1, FB2 and ZEN (Tab. 1).

Fig. 1. Chemical structure of the main mycotoxins present in pig feed.



The AFs are rapidly absorbed and metabolized in the liver (Haschek et al. 2002); they are hepatotoxic, and have some impacts on growth and on the immune response of the pig (Meissonnier et al. 2006). OTA is nephrotoxic and hepatotoxic and its oxydative metabolites are genotoxic (Aish et al. 2004; Pfohl-Leszkowicz and Manderville 2007). Among mycotoxins, pigs are very sensitive to DON, the most common mycotoxin of the type B trichothecene. Short exposure to high doses of DON induces vomiting and lower doses cause feed refusal (Haschek et al. 2002); chronic exposure is associated with weight loss, anorexia, immunomodulation and alteration of intestinal barrier functions (Haschek et al. 2002; Pestka 2010; Pinton and Oswald 2014). Type A trichothecene T2 and HT2 toxins have similar but more pronounced effects than DON. They also induce irritation of the intestine and the skin and increase the sensibility of pigs to diseases (Bryden 2012).

Table 1. Regulation and recommendations for the main mycotoxins present in pigs feed and feedstuffs. (EC Directive 2002/32/EC, and EC Recommendations 2006/576/EC and 2013/165/EU)

Mycotoxins	Pig feeds	Max. content mg/Kg (ppm)
AFB1+ B2	Cereals	60
	Complete and complimentary feeding stuffs for pigs, horse, rabbit and pets	0.5
OTA	Complete and complimentary feeding stuffs for pigs	0.05
DON	Cereals (without maize by-products)	8 (12)
	Complete and complimentary feedstuffs for pigs	0.9
ZEN	Cereals (without maize by-products)	2 (3)
	Complete and complimentary feeding stuffs: -for piglets and gilts -for sows and fattening pigs	 0.1 0.25
FB1+FB2	Cereals	60
	Complete and complimentary feeding stuffs for pigs, horse and rabbit	5
T2+HT2	Complete and complimentary feeding stuffs for animals -Oat milling products (husks) -Other cereals products -Compound feed, with the exception of feed for cats	 1 0.5 0.25

FB1 is the most prevalent toxin of the fumonisin family. It has a carcinogenic effect in humans and induces multiple toxic effects in different animal species. In pigs, this toxin induces pulmonary oedema (Haschek et al. 2002) and alters the immune response with a dysregulation of the T helper lymphocytes TH1/TH2 balance (Marin et al. 2006; Taranu et al. 2005). The last mycotoxin with a recommendation for pig feed is ZEN. This toxin has an impact on pig fertility and reproduction. ZEN and its principal derivatives, α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL) (more toxic than the other two), are non-steroidal oestrogens

inducing an oestrogenic response in animals (Fink-Gremmels and Malekinejad 2007). In pigs, especially young sows, ZEN induces red patching and tumefaction of the vulva, a prolapse of the vulva and sometimes of the rectum (Gaumy et al. 2001).

In terms of intestinal toxicity, the effects of DON and FB have been studied in detail in pigs; by contrast only few papers are concerned with the effect of OTA or AF on this organ.

II- Effects of mycotoxins on the pig intestine

The intestinal tract is the first target for mycotoxins following ingestion of contaminated feed. The intestinal epithelium is a single layer of cells lining the gut lumen that acts as a selective filter, allowing the translocation of dietary nutrients, essential electrolytes, and water from the intestinal lumen into the blood circulation. It also constitutes the largest and most important barrier to prevent the passage of harmful intraluminal substances from the external environment into the organism, including foreign antigens, microorganisms, and their toxins. Following the ingestion of mycotoxin-contaminated feed, intestinal epithelial cells may be exposed to high concentrations of toxins, potentially affecting intestinal functions (Alassane-Kpembi and Oswald 2015; Ghareeb et al. 2015; Grenier and Applegate 2013).

A. Effect on intestinal histomorphology

Consumption of mycotoxin-contaminated feed induces histological damage on intestinal tissue. Epithelial lesions in the intestine of pigs fed with a diet naturally contaminated with DON were observed (Bracarense et al. 2012; Eriksen and Pettersson 2004). Jejunal lesions, including shortened and coalesced villi, lysis of enterocytes, and edema, were also observed in an *ex-vivo* model of intestinal tissues after exposure to DON (Lucioli et al. 2013; Pinton and Oswald 2014). Exposure to FB also induces changes in intestinal villi morphology such as reduced villi height and villi fusion and atrophy (Bracarense et al. 2012).

A study on pigs showed that low doses of ZEN do not impair the morphology and ultrastructure of the small intestine (Obremski et al. 2005), in contrast to what has been observed in rats (Liu et al. 2014).

As far as AFB1 is concerned, no data on the effect of this toxin on the histomorphology of the pig intestine are available. Nevertheless, exposure of broiler chicken to AFB1 induced a

decreased jejunal villus height, villus height/crypt ratio, and shedding of epithelial cells on the tip of jejunal villi (Zhang et al. 2014).

B. Effect on intestinal digestion and nutrient absorption

The regressive intestinal lesions observed upon exposure to mycotoxins may explain, at least in part, the reduced absorption of nutrients and the impaired digestion observed after ingestion of mycotoxins. Pigs consuming corn culture extracts containing FB showed a markedly lowered activity of aminopeptidase N (Lessard et al. 2009). Likewise, exposure to 1.5mg/kg b.w. FB1 has been shown to induce sphingolipid depletion in pig intestinal epithelium, which can result in a deficiency of folate uptake (Grenier and Applegate 2013; Loiseau et al. 2007). The sodium-glucose dependent transporter (SGLT-1) activity is particularly sensitive to DON inhibition. SGLT-1 is the main apical transporter for active glucose uptake in the small intestine. Inhibition of SGLT-1 has nutritional consequences and could explain diarrhea associated with DON ingestion, since this transporter is responsible for daily absorption of water in the gut (Maresca 2013). Conversely, sodium-dependent glucose absorption might be up-regulated in pigs after acute or long term exposure to the mycotoxin FB1 (Lessard et al. 2009).

C. Effect on barrier function

Several mycotoxins are able to alter intestinal barrier functions (Ghareeb et al. 2015; Grenier and Applegate 2013). They affect the intestinal epithelium permeability through modulation of the tight junction complexes. A defective expression of occludin and E-cadherin has been observed in the ileum of piglets fed low doses of FB1 (Lucioli et al. 2013). The FB-induced alteration of the sphingolipid biosynthesis pathway and the associated lipid rafts could also contribute to impairing the establishment and maintenance of tight junctions. Likewise, the activation of MAP kinases by DON affects the expression and cellular localization of proteins forming or being associated with tight junctions such as claudins and ZO-1, which results in increased intestinal paracellular permeability (Pinton and Oswald 2014). Similarly to DON, T2-toxin, FB_1 and ZEN have been shown, in vitro and in vivo, to impair the pig intestinal barrier function and to promote oral absorption of antibiotics such as doxycycline, chlortetracycline and paromomycin (Goossens et al. 2012; Goossens et al. 2013).

D. Intestinal immune system

Some mycotoxins impact the systemic and/or the local immune response. At the intestinal level, they decrease the immunity leading to enhanced intestinal infections. They also have a direct or indirect proinflammatory effect (Cano et al. 2013; Maresca 2013). Indeed, the intestine is a major source of cytokines and chemokines, molecules involved in the regulation of the immune system. Among cytokines, IL-8, which is a chemoattractant cytokine, is of particular interest because it is involved in the recruitment of polymorphonuclear neutrophils at the site of infection, mediating a non-specific acute inflammatory response.

Ingestion of FB_1 specifically decreases expression of IL-8 mRNA in the ileum of exposed piglets while expression of other inflammatory cytokines is not affected. This decrease of IL-8 caused by FB_1 may lead to reduced recruitment of inflammatory cells in the intestine during infection, and may contribute to the observed increased susceptibility of FB_1-treated piglets to intestinal infections (Bouhet and Oswald 2007).

DON modulates intestinal innate immunity both directly (through activation of signal pathways) and indirectly (through crossing of luminal bacterial antigens, which was observed together with bacterial translocation following mucus layer alteration and tight junction opening) (Maresca et al. 2008). DON affects expression of proteins involved in epithelial innate immunity, including inflammatory cytokines, COX-2 and β-defensins (Cano et al. 2013; Lessard et al. 2015). Numerous studies have demonstrated that DON stimulates expression and secretion of IL-8 and thus potentially participates indirectly in the central effects of DON in terms of feed refusal and emesis. As described for immune cells (Pestka 2010), DON has a biphasic effect on the secretion of IL-8 by intestinal epithelial cells: Low doses of toxin cause a massive increase in secretion of IL-8, whereas higher doses inhibit it. Such a biphasic effect explains why DON acts: (i) as a proinflammatory toxin leading to intestinal inflammation at low doses; and (ii) as an inhibitor of intestinal immunity leading to higher susceptibility of animals to intestinal infections at higher doses (Maresca 2013).

The ability of ZEN to interact with the pig immune system has been poorly investigated. However, it is known that exposure to high concentrations of ZEN (5-250mg/Kg feed or 200-1000 µg/Kg b.w./day) induces chronic inflammation of the genital tract in females pigs (EFSA 2011; JECFA 2011). *In vitro* analyses also show that ZEN and its metabolites have

differential effects on synthesis of the inflammatory cytokines IL-8 and IL-10 in swine intestinal epithelial cells (Marin et al. 2015).

There is no report of OTA- induced impairment of local immunity. However, this mycotoxin decreases the level of inflammatory cytokines (TNF-alpha and IL-10) in the plasma of exposed pig (Bernardini et al. 2014).

E. Intestinal microbiota

The gut hosts an important microflora. Surprisingly, the impact of mycotoxins on the intestinal microflora has been poorly investigated. As far as pigs are concerned, only two studies have investigated the impact of mycotoxins on the intestinal microflora (Burel et al. 2013; Wache et al. 2009). The first study indicates that consumption of feed naturally contaminated with DON (2.8 mg/kg) for four weeks had a moderate effect on cultivable bacteria in the pig intestine, but changed the microflora (Wache et al. 2009). In the second study, pigs received feed contaminated with 12 mg FB/kg feed for 63 days. This diet transiently affected the balance of the digestive microbiota during the first four weeks of exposure; a co-infection with *Salmonella* typhimurium amplified this phenomenon (Burel et al. 2013).

Two recent studies performed on rats have also demonstrated an effect of OTA and AF on the intestinal microbiota (Guo et al. 2014; Wang et al. 2016). The effects of mycotoxins on the intestinal microbiota are not surprising; indeed other secondary metabolites produced by the same fungi, antibiotics, are well known for their effect on the gut flora. Recent advances in next-generation sequencing technologies and metagenomics should give us a comprehensive analysis of the effect of mycotoxins on the structure and function of gut microbial ecosystem in the near future.

III- Consequences of intestinal toxicity of mycotoxins for pig health

A. Impairment of zootechnical performance

All damage induced by mycotoxins on the intestine level and on the different functions lead to different symptoms expressed by the pig. Such symptoms are either directly associated with local toxicity in the intestine, or indirectly with a systemic effect, and with visible impact on the overall health of the pig.

The colloquial name of DON, vomitoxin, refers to its emetic effect observed both in field reports and in experimental intoxications where high doses of the toxin were given orally or intravenously to pigs. Complete feed refusal was observed at levels of 12 and vomiting at 20 mg DON/kg feed. Pig feeding trials with naturally or artificially contaminated diets have shown decreased feed consumption and weight gain at doses from 0.6 to 3mg DON/kg feed (Bracarense et al. 2012). A meta-analysis showed that deoxynivalenol reduced feed intake and weight gain by 26%; the same analysis also demonstrated a 16% reduction of feed intake in response to AFB1(Andretta et al. 2012).

Consumption of pure FB1 or FB1-contaminated feed also induces a slight reduction of body weight in piglets. Although FB are poorly absorbed and metabolized in the intestine, they induce intestinal disturbances (abdominal pain or diarrhea) and cause extra-intestinal organ pathologies (pulmonary edema in pigs, leukoencephalomalacia in horses, or neural tube defects in rodents).

Ingestion of ZEN and OTA doesn't alter zootechnical performance (Bernardini et al. 2014; Schoevers et al. 2012). However ZEN can induce a decrease in reproductive performance with a reduction of healthy follicles leading to premature oocyte depletion in adulthood and so leading to abortion (Schoevers et al. 2012).

B. Bacterial translocation

The intestinal disturbance induced by mycotoxins may lead to increased bacterial translocation across the intestine and increased susceptibility to enteric infections. The loss of tight junction integrity and resulting increased paracellular permeability can lead to entry of bacteria that are normally restricted to the gut lumen. Such an increase in bacterial passage through intestinal epithelial cells after mycotoxin exposure has major implications for pig health in terms of sepsis, inflammation and enteric infection.

Porcine ileal loops were used to reproduce *Salmonella typhimurium* induced intestinal inflammation. Co-exposure to bacteria and DON dramatically enhances the inflammatory response to *S. typhimurium* in the ileal loops, with a clear potentiation of expression of IL-1β, IL-8 or IL-6 (Vandenbroucke et al. 2011). It has been suggested that this potentiation coincided with significantly enhanced *Salmonella* invasion in and translocation over intestinal epithelial cells.

A higher susceptibility of the gastrointestinal tract to other bacteria was reported in pigs exposed to FB1. Two separate studies analyzed the effect of low to moderate doses of FB1 on intestinal colonization and mucosal response to pathogenic strains of *Escherichia coli* (Devriendt et al. 2009; Oswald et al. 2003). Besides, translocation of bacteria to the mesenteric lymph nodes and dissemination to the lungs, and to a lesser extent to liver and spleen, were observed in FB1-treated pigs in comparison to untreated animals (Oswald et al. 2003).

A study on human enterocytes exposed to low doses of DON or OTA showed an increase of translocation of commensal bacteria across the epithelium even without alteration of the intestinal permeability (Maresca et al. 2008). The mechanism involved in this increase is not elucidated, but this phenomenon could be due to an energetic modification of the cell status with a reduction of ATP levels (Grenier and Applegate 2013).

Conclusion

The intestine is a target for mycotoxins and as illustrated in this paper this may have some consequences in terms of pig health (fig.2). Regulations and recommendations exist for six mycotoxins (AF, FB, OTA, ZEN, T2/HT2 and DON) present in pig feed. Among them, DON and FB have been studied especially for their toxicity in the intestine. They are not only locally toxic for this organ, but also dysregulate many intestinal functions and impair the immune response. This results in systemic toxicity leading to many symptoms and impairment of zootechnical parameters. Feed contamination with mycotoxins also increases translocation of bacteria across the intestine and thus intestinal and systemic infections, and so aggravates pigs' condition. For AF, ZEN and OTA, little is known about their intestinal toxicity on pigs.

The increased performance of analytical methods reveals new toxins, especially emerging ones, as well as "masked" or "modified" forms. Occurrence and toxicity of these new mycotoxins are poorly documented (Broekaert et al. 2015; Pierron et al. 2015), and thus it still needs to be determined if they represent a new risk in pig production.

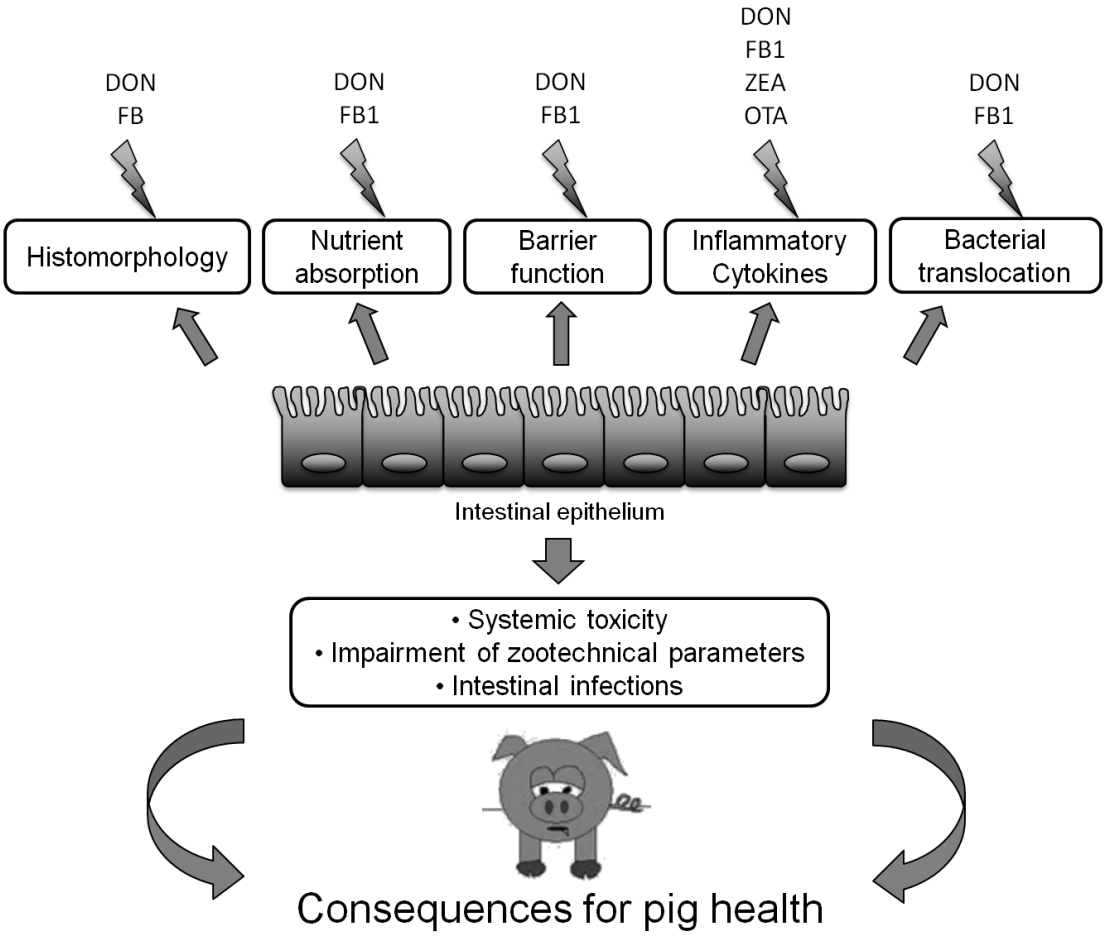


Fig.2: Summary of the intestinal toxicity of the main mycotoxins present in feed pig.

Global surveys indicate that animals are generally exposed to more than one mycotoxin (Streit et al. 2012). Indeed fungi are able to produce several mycotoxins simultaneously; and it is common practice to use multiple grains in animal diets. Unfortunately, the toxicity of mycotoxin mixtures cannot be predicted based on their individual toxicities. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive, or synergistic (Alassane-Kpembi et al. 2015). The data on combined toxicity of mycotoxins are limited and therefore, the health risk from exposure to a combination of mycotoxins is incompletely understood (Alassane-Kpembi et al. 2016; Grenier and Oswald 2011) and deserves further investigation.

List of abbreviations:

AF, Aflatoxin

AFB1, Aflatoxin B1

BW, Body Weight

DON, Deoxynivalenol

FB1, Fumonisin B1

Ig, Imunoglobulin

OTA, Ochratoxin A

OVA, Ovalbumin

PHA, Phytohemagglutinin

Th1, T helper 1

Th2, T helper 2

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors agree fully to the content of the review.

Acknowledgment

A. Pierron was supported by a fellowship from CIFRE (2012/0572, jointly financed by the BIOMIN Holding GmbH, Association Nationale de la Recherche Technique and INRA). This work was supported in part by the projects Liporeg (APP-ICSA 2015) and PHC STEFANIK 2016.

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B. Impact of mycotoxin on immune response and consequences for pig health

Many mycotoxins, alone or in co-exposure, can contaminate cereals and feeding stuffs for pig consumption. These mycotoxins induce different toxic effects on pigs and especially on the immune system. They are able to modulate or dysregulate the immune response.

This review summarizes the major effects induced by these mycotoxins on the immune response. It also highlights the consequences of these contaminations, which increase the susceptibility to infectious diseases or to chronic infection and can also decrease the vaccine efficacy. Moreover these mycotoxins can be found in co-contamination, with, as a consequence, a potential increase of the effects observed and an impairment of the global health of the pig.

This review was published this year in Animal Nutrition.

ARTICLE IN PRESS

Animal Nutrition xxx (2016) 1–6

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Review article

Impact of mycotoxin on immune response and consequences for pig health

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ARTICLE INFO

Article history:
Received 5 February 2016
Accepted 10 March 2016
Available online xxx

Keywords:
Pig
Mycotoxins
Feed contamination
Susceptibility to disease
Immunity
Vaccine efficacy

ABSTRACT

Mycotoxins are fungal secondary metabolites detected in many agricultural commodities, especially cereals. Due to their high consumption of cereals, pigs are exposed to these toxins. In the European Union, regulations and/or recommendations exist in pig feed for aflatoxins, ochratoxin A, fumonisins, zearalenone, and trichothecenes, deoxynivalenol and T-2 toxin. These mycotoxins have different toxic effects, but they all target the immune system. They have immunostimulatory or immunosuppressive effects depending on the toxin, the concentration and the parameter investigated. The immune system is primarily responsible for defense against invading organisms. The consequences of the ingestion of mycotoxin-contaminated feed are an increased susceptibility to infectious diseases, a reactivation of chronic infection and a decreased vaccine efficacy. In this review we summarized the data available on the effect of mycotoxins on the immune system and the consequences for pig health.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by various molds, such as *Aspergillus*, *Penicillium* and *Fusarium*, which may contaminate food and feed at all stages of the food/feed chain. Despite the improvement of good agricultural and manufacturing practices, mycotoxin contamination cannot be avoided and contaminants are virtually ubiquitous at some concentrations in the average human and animal diets (Bryden, 2012). A recent study performed on 1100 samples collected worldwide showed that about 70% of samples tested was contaminated (Streit et al., 2013). This result was confirmed on a smaller study realized on 83 feed ingredients sampled in China (Guan et al., 2011a).

The biological reactions following ingestion of mycotoxins vary from acute, overt diseases with high morbidity and mortality to chronic, insidious disorders with reduced animal productivity. Different mycotoxins target different organs, inducing various toxic effects. At high doses, mycotoxins exposure leads to general cytotoxicity, often related to macromolecule synthesis inhibition (Maresca and Fantini, 2010). Mycotoxins induce primary biochemical lesions and impact on early cellular functions/events in the cascade of events leading to toxic cell injury or cellular deregulation (Bryden, 2012). At low doses, mycotoxins affect the functions of various tissues and organs, such as the gastrointestinal tract, liver or kidney tissues, as well as the nervous, reproductive and immune systems. Some mycotoxins also have genotoxic, carcinogenic and teratogenic effects (Maresca and Fantini, 2010).

Mycotoxins contamination levels in pig feedstuffs are usually not high enough to cause an overt disease but may result in economical loss through changes in growth, production and immunosuppression (Bryden, 2012; Oswald et al., 2005; Wild and Gong, 2010).

Pigs are very sensitive to mycotoxins. Due to their high consumption of cereals, they are exposed to these toxins and to a chronical contamination. In Europe, regulation and/or recommendations exist for 6 mycotoxins that may be present in pig feed:

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Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

http://dx.doi.org/10.1016/j.aninu.2016.03.001

aflatoxins (AF), ochratoxin A (OTA), fumonisins (FB), zearalenone (ZEN) and trichothecenes (principally deoxynivalenol [DON], T-2 and HT-2 toxins) (Bennett and Klich, 2003).

This review summarizes the main effects induced by mycotoxins present in pig feed on immunity and determines the consequences of this immunomodulation in terms of susceptibility to infectious diseases, reactivation of chronic infection and vaccine efficacy.

2. Effect of major mycotoxins on the pig immune response

2.1. Aflatoxins

Aflatoxins are hepatotoxic and carcinogenic; they also display immunotoxic properties. These toxins impair both the innate and the acquired immune responses (Meissonnier et al., 2006; Weaver et al., 2013). The dysregulation of the antigen-presenting capacity of dentritic cells, which is starting from aflatoxin B1 (AFB1) low dose exposure, is deemed to be the mechanism by which the mycotoxin impairs cell-mediated immunity (Mehrzad et al., 2014). An exposition to AF increases the T-cell proliferation-inducing capacity of porcine monocyte-derived dentritic cells, thus enhances presenting capacity of cells (Mehrzad et al., 2015).

An alteration of the inflammatory response has been reported in pigs exposed to AF (Chaytor et al., 2011). A reduced synthesis of pro-inflammatory cytokines and an increase of anti-inflammatory ones was also demonstrated in weanling piglets fed for 4 weeks with low doses of AF (Marin et al., 2002). In utero exposure of piglets to this mycotoxin (through exposition of sows), the functional capacities of both macrophages and neutrophils were altered (Silvotti et al., 1997).

Experimentally, in a pig model vaccinated with a model antigen, which was ovalbumin (OVA), AFB1 exposure had no major effect on humoral immunity with unchanged plasma concentrations of total immunoglobulin A (IgA), IgG and IgM and the specific anti-OVA IgG. In these animals, the toxin exposure did not impair the mitogenic response of lymphocytes but delayed and decreased the OVA-specific proliferation, suggesting an impaired lymphocyte activation in pigs exposed to AFB1 (Meissonnier et al., 2008b). Similarly, in pigs vaccinated with *Mycoplasma*, the exposure to lower levels of AFB1 did not modulate the antigen-specific and total antibody response (Marin et al., 2002). Developing piglets are very susceptible to this mycotoxin. Indeed, after sows exposure to AF, the global piglets lympho-proliferative response upon mitogenic stimulation is reduced (Silvotti et al., 1997).

2.2. Trichothecenes

Type B trichothecenes, including DON, have the capacity to up- and down-regulate immune functions by disrupting intracellular signaling within leukocytes (Pestka, 2010). Depending on the dose, frequency and duration of exposure, DON will have either an immunostimulatory or immunosuppressing effect (Pestka et al., 2004). Deoxynivalenol is able to induce an inflammatory response by acting on the ribosome, inducing a Ribotoxic stress which actives the MAPK pathway, eliciting expression of inflammation-related genes as pro-inflammatory cytokines (Pestka et al., 2004; Pestka, 2010).

In mice, this toxin induced a pronounced elevation in serum IgA (Pestka et al., 2004). In pigs, a similar increase of IgA in the serum of animals receiving DON contaminated feed has been observed (Drochner et al., 2004; Pinton et al., 2008; Swamy et al., 2003). In animals immunized with OVA, the specific immune response was investigated during a DON exposure inducing no feed refusal or reduced body weight gain. Ingestion of DON increased the plasma concentration of total and anti-OVA IgA titers. Deoxynivalenol did not modulate lymphocytes proliferation after mitogenic stimulation, but the toxin had a biphasic effect on the OVA-specific lymphocyte proliferation: An up-regulation in the days after OVA immunization but a down-regulation in the weeks following (Pinton et al., 2008).

Another study on pigs immunized with OVA showed an increase of anti-OVA IgG titers, after 42 days of exposure to a DON contaminated diet. Simultaneously, the expressions of chemokines involved in inflammatory reactions (interleukin-8 (IL-8), chemokine (C-X-C motif) ligand 20 (CXCL20), interferon-γ (IFN-γ)) were up-regulated. Deoxynivalenol also up-regulated the gene of major antioxidant glutathione peroxidase 2 (GPX-2) and down-regulated expression of genes encoding enzymatic antioxidants including GPX-3, GPX-4 and superoxide dismutase 3 (SOD-3), involved in oxidative stress (Lessard et al., 2015).

Type A trichothecenes such as T-2 toxin are cytotoxic molecules and potent protein inhibitors. In pigs immunized with OVA, subclinical doses of T-2 toxin induced an early and transient increase of total IgA plasma concentration but a decrease in the anti-OVA IgG titer (Meissonnier et al., 2008a). For higher doses of exposure, T-2 toxin had been previously shown to decrease both the mitogenic and the antigen-specific lymphocytes proliferation following a horse globulin immunization (Rafai et al., 1995).

2.3. Fumonisins

Fumonisins induce various toxic effects depending on the animal species, and there is evidence for the carcinogenicity of these toxins (Stockmann-Juvala and Savolainen, 2008). In *in vitro* and *in vivo* experiments, fumonisin B1 (FB1) modifies the Th1/Th2 (T-helper 1/T-helper 2) cytokine balance in pigs similar to an impaired humoral response (Marin et al., 2006; Taranu et al., 2005). With pigs vaccinated against *Mycoplasma* and exposed to FB1 (8 mg/kg feed for 4 weeks), a sex-related difference in the specific immune response has also been observed. In male pigs but not for female ones, exposure to the toxin reduced the vaccine-specific antibody titer (Marin et al., 2006). However, ingestion of contaminated feed had no effect on the serum concentrations of total IgG, IgA, and IgM.

Studies have also demonstrated that FB1 influences the inflammatory response. For example, incubation of swine alveolar macrophages with FB1 led to a significant reduction of the number of viable cells and cell death by apoptosis (Liu et al., 2002). An *in vivo* experiment on pigs exposed to FB (6 mg/kg feed for 5 weeks) showed a decrease of IL-1β and IL-6 genes expression in spleen tissue (Grenier et al., 2011).

Fumonisin B1 also impairs on the maturation of antigen presenting cells *in vivo* by reducing the intestinal expression of IL-12p40 and decreasing the upregulation of major histocompatibility complex class II molecule (MHC-II) with a reduction of T cell stimulatory capacity upon stimulation (Devriendt et al., 2009).

2.4. Ochratoxin A

Ochratoxin A is mainly toxic for kidney and liver. Gilts fed OTA-contaminated had reduced cutaneous basophil hypersensitivity response to phytohemagglutinin, reduced delayed hypersensitivity to tuberculin, decreased stimulation index for lymphoblastogenesis, decreased interleukin-2 production when lymphocytes were stimulated with concanavalin A, and decreased number and phagocytic activity of macrophages. Ochratoxin A was shown to be toxic on purified lymphocytes of pigs with an half maximal inhibitory concentration (IC50), concentration producing 50% inhibition of cell proliferation, of 1.3 μM (Keblys et al., 2004).

Ochratoxin A show an impact on the cytokine expression. An experiment on weaned pigs that ingested an OTA contaminated

diet (181 ng/g of feed) has shown an increased level of TNF-alpha and IL-10 in plasma, with a decreased capacity to respond with cytokine expression to *ex vivo* challenge with lipopolysaccharides (LPS) (Bernardini et al., 2014). By contrast, OTA has no effect on total and specific immunoglobulin concentrations (Harvey et al., 1992).

2.5. Zearalenone

Zearalenone is best known for its toxic effect on reproduction and fertility (Zinedine et al., 2007); it induces an estrogenic activity on animal (Fink-Gremmels and Malekinejad, 2007). Pigs are particularly sensitive to ZEN, which can induce edematous swelling and reddening of vulva, prolapse of the vulva, ovarian follicle damage and abortions (Schoevers et al., 2012; Zinedine et al., 2007).

Only few papers described the effect of ZEN on immunity (Eriksen and Alexander, 1998). In pigs, exposure of intestinal epithelial cells ZEN (25 μM) has a tendency to increase the synthesis of the inflammatory cytokines IL-8 and IL-10 (Marin et al., 2015). Sows exposed to high concentration of ZEN (5–250 mg/kg feed or 200–1000 μg/kg BW per day) can develop a chronic inflammation of the genital tract (EFSA, 2011).

3. Consequence of mycotoxin induced immunomodulation for pig health

3.1. Susceptibility to infectious diseases

The broad immunosuppressive effect of mycotoxins may decrease host resistance to infectious diseases (Antonissen et al., 2014). Table 1 summarizes the data obtained in pigs.

In pigs, the consumption of feed contaminated with AF increases the severity of infection with *Erysipelothrix rhusiopathiae* (Cysewski et al., 1978). Similarly, during an experimental infection with *Brachyspira hydysenteriae*, the consumption of AF reduced the incubation time and increased the severity of diarrhea (Joens et al., 1981).

In presence of porcine circovirus type 2 (PCV2) virus, DON increases the severity of the viral infection, and in presence of the porcine reproductive and respiratory syndrome virus (PRRSV) it also increases the infection with more tissue lesions induced (Savard et al., 2014, 2015b). During a bacterial infection, DON enhances the inflammatory reaction with an increased production of pro-inflammatory cytokines (Vandenbroucke et al., 2011). The elevation of circulating IgA in presence of low quantity of DON may, by contrast, increase the resistance to certain pathogens. Indeed, IgA initiates rapid and transient up-regulation of many immune related genes (Pestka et al., 2004).

In pigs, FB1 ingestion can induce intestinal infections, with some intestinal functions affected (Burel et al., 2013; Devriendt et al., 2009; Oswald et al., 2003). The ingestion of FB1-contaminated feed was also associated with an increased susceptibility to pulmonary infection and an increase of the severity of the pathological changes with bacterial or viral pathogens (Devriendt et al., 2009; Halloy et al., 2005; Oswald et al., 2003; Posa et al., 2011, 2013; Ramos et al., 2010).

Ingestion of OTA contaminated feed also increases susceptibility to natural infectious disease. Indeed, salmonellosis arose spontaneously in all piglets receiving an OTA contaminated diet, and when the animals were vaccinated against salmonellosis, the consumption of contaminated feed leads to spontaneous *Brachyspira*

Table 1
Influence of mycotoxins on susceptibility to infectious diseases in pig.

Mycotoxin	Exposure dose	Exposure period	Pathogen	Effect compared with negative control	References
AFB1	0.07 and 0.14 mg/kg	32 days	*Brachyspira hyodysentariae*	↓ of incubation period for dysentery, ↑ diarrhea and dysentery time, ↑ death, visible clinical signs and lesions of dysentery at necropsy	Joens et al., 1981
AF	1.3 mg/kg feed	25 days	*Ersypelothrix rhusiopathiae*	↑ the severity of bacterial infection	Cysewski et al., 1978
DON	2.5 mg/kg feed	3 weeks	PCV2	↑ viremia and lung viral load no clinical effect	Savard et al., 2015b
DON	3.5 mg/kg feed	3 weeks	PRRSV	↓ weight gain, ↑ lung lesions and mortality, no effect on viral replication	Savard et al., 2014
DON	1 μg/mL	6 h	*Salmonella typhimurium*	synergistic ↑ gene expression *IL-12, TNF-α, IL-1β, IL-8, MCP-1 and IL-6*	Vandenbroucke et al., 2011
T-2 toxin	15 and 83 μg/kg feed	23 days	*Salmonella typhimurium*	↓ colonization of the cecum	Verbrugghe et al., 2012
FB1	10 mg/kg feed	3 days	*Bordetella bronchiseptica* & *Pasteurella multocida* (type D)	↑ extent and severity of the pathological changes	Posa et al., 2011
FB1	0.5 mg/kg BW	6 days	*Escherichia coli* (SEPEC)	↑ intestinal colonization; ↑ translocation to the mesenteric lymph node, lung, liver and spleen	Oswald et al., 2003
FB1	1 mg/kg BW	10 days	*Escherichia coli* (ETEC)	intestinal infection prolonged; impaired function of intestinal antigen presenting cells	Devriendt et al., 2009
FB1	25.4 mg/kg feed	42 days	*Mycoplasma hyopneumoniae*	↑ severity of the pathological changes	Posa et al., 2013
FB1	0.5 mg/kg BW	7 days	*Pasteurella multocida* (type A)	↓ growth rate and ↑ coughing; ↑ total number of cells, number of macrophages and lymphocytes in BALF; ↑ gross pathological lesions and histopathological lesion of lung	Halloy et al., 2005
FB1	12 mg/kg BW	18 days	PRRSV	↑ histopathological lesions of lungs	Ramos et al., 2010
FB1	11.8 mg/kg feed	9 weeks	*Salmonella typhimurium*	Modification of the microbiota profiles	Burel et al., 2013
OTA	3 mg/kg feed	3 weeks	*Brachyspira hyodysenteriae* & *Camphylobacter coli*	Salmonellosis arises spontaneously in animals fed the contaminated diet, clinical and patho-morphological changes (typical of salmonellosis), change of hematological and biological parameters	Stoev et al., 2000
OTA	75 μg/kg feed	42 days	PCV2	↑ PCV2 replication in serum and tissues	Gan et al., 2015

AFB1 = aflatoxin B1; AF = aflatoxins; DON = deoxynivalenol; FB1 = fumonisin B1; OTA = ochratoxin A; BW = body weight; PCV2 = porcin circovirus type 2; PRRSV = porcine reproductive and respiratory syndrome virus.

hyodysenteriae and *Campylobacter coli* infections (Stoev et al., 2000). During a PCV2 infection, OTA increases the viremia in sera and tissues (Gan et al., 2015).

To the best of our knowledge, there are no data available concerning the effect of ZEN on *in vivo* analysis on mice which were fed 10 mg/kg ZEA (1.5 mg/kg BW per day) during 2 weeks, infected with *Listeria monocytogenes, and* showed a decreased resistance to *Listeria* with in increasing trend of the splenic bacterial counts, compared with control animals (Pestka et al., 1987).

3.2. Reactivation of chronic infection

The effect of mycotoxin intoxication on the reactivation of chronic infection was also investigated. However, the experiment was not performed with pigs but with rodents. In the immunocompetent host, *Toxoplasma gondii* infection progresses to a chronic phase characterized by the presence of encysted parasites. Cyst rupture may occur, but infection remains latent and reactivation is prevented. In immunosuppressed animal and human subjects, such as patients infected with the human immunodeficiency virus, rupture is associated with the formation of new cysts and disease. Low and repeated doses of either AFB1 or T-2 toxin are able to accelerate *Toxoplasma* cyst rupture in previously infected mice (Venturini et al., 1996).

3.3. Vaccination efficacy

Immunity acquired through vaccination can also be impaired by mycotoxin ingestion (Table 2). For example, AFB1 interferes with the development of acquired immunity in swine following erysipelas vaccination with bacterin preparation (a suspension of killed bacteria) of *E. rhusiopathiae* (Cysewski et al., 1978). As already mentioned, ingestion of feed contaminated with AFB1 or T-2 Toxin reduced the vaccine response to the model antigen, ovalbumin, acting on the cellular and the humoral response respectively (Meissonnier et al., 2008a, 2008b). Ingestion of low doses of another mycotoxin, FB1, decreases the specific antibody response mounted during *Mycoplasma* vaccination in pigs (Taranu et al., 2005). In pigs exposed to OTA or FB1 and vaccinated against Aujesky disease (Suid Herpesvirus 1 [SuHV1]), the humoral immune response was greatly disturbed, with a strong decrease in antibody observed (Stoev et al., 2012). In diet contaminated with DON or FB1, pigs showed an alteration of the specific immune response upon vaccination with OVA (Grenier et al., 2011).

Likewise, feeding pigs a DON-contaminated diet was shown to inhibit the vaccination efficiency of PRRSV modified live vaccine by severely impairing viral replication (Savard et al., 2015a).

It should also been mentioned that the vaccine immune response is altered at mycotoxin doses that do not alter the global immune response (Meissonnier et al., 2008a, 2008b; Taranu et al., 2005). The breakdown in vaccine immunity and may lead to the occurrence of disease even in properly vaccinated flocks. These reactions are of considerable consequence in animals for which we rely on an effective vaccination program for disease prevention.

4. The problem of mycotoxins co-contamination

In the above paragraphs, the effects of single mycotoxin on immunity were described. However, mycotoxins often co-occur and animals are exposed to several mycotoxins at the same time. Indeed, raw materials can be contaminated by several fungi, which are able to simultaneously produce several mycotoxins, and in addition the diet of animal is composed of several commodities (Alassane-Kpembi et al., 2015, 2016). A worldwide survey on 7049 samples reported that 48% of feed and feedstuff samples are contaminated by 2 or more mycotoxins (Rodrigues and Naehrer, 2012). Other studies showed that 75%–100% of animal feed samples are contaminated with more than one mycotoxin (Guan et al., 2011a; Streit et al., 2012).

The toxicity of mycotoxins mixtures cannot always be predicted based upon their individual toxicities. It can be antagonistic,

Table 2
Influence of mycotoxins on vaccination efficacy in pigs.

Mycotoxin	Exposure dose	Antigen	Effect compared with negative control	References
AF	1.3 mg/kg feed	*Erysipelothrix rhusiopathiae*	Interfered on the development of acquired immunity,	Cysewski et al., 1978
AFB1	385–1807 μg/kg feed	OVA	Decreased and delayed cell-mediated immunity	Meissonnier et al., 2008b
DON	3.5 mg DON/kg feed	OVA	Increased OVA-primary IgG antibody response	Lessard et al., 2015
DON	2.5–3.5 mg/kg BW	PRRSV	Decreased PRRSV post-vaccinal viremia and reduced vaccinal efficacy	Savard et al., 2015b
DON	2.2–2.5 mg DON/kg feed	OVA	Increased concentration of OVA specific IgA and IgG	Pinton et al., 2008
DON	0.6–4.7 mg DON/kg	Human serum albumin, sheep red blood cells, paratuberculosis vaccine, tetanus toxoid and diphtheria toxoid	Significant dose-dependent reduction in secondary antibody response to tetanus toxoid	Overnes et al., 1997
DON + ZEN	2.1–3.2 mg DON/kg diet and 0.06–0.25 mg ZEN/kg diet	Parvovirus	No effect	Gutzwiller et al., 2007
DON or FB1	3 mg DON/kg feed or 6 mg FB1/kg feed	OVA	Reduced anti-OVA antibody production with a decrease of lymphocytes proliferation	Grenier et al., 2011
T-2 toxin	1324–2102 μg/kg feed	OVA	Reduced anti-OVA antibody production without significant alteration to specific lymphocyte proliferation	Meissonnier et al., 2008a
FB1	8 mg/kg BW	*Mycoplasma agalactiae*	Decreased specific antibody titer	Taranu et al., 2005
OTA	1 mg/kg feed	*Salmonella choleraesuis*	Immunosuppression and delayed response to immunization	Stoev et al., 2000
OTA or FB1	0.5 mg OTA/kg feed or 10 mg FB1/kg feed	Suid Herpesvirus 1 (Aujesky disease)	Decreased anti-SuHV1 antibody production after vaccination	Stoev et al., 2012

AF = aflatoxins; AFB1 = aflatoxin B1; DON = deoxynivalenol; ZEN = zearalenone; OTA = ochratoxin A; BW = body weight; OVA = ovalbumin; PRRSV = porcine reproductive and respiratory syndrome virus; OVA = ovalbumin.

additive or synergistic and increase the impact of each mycotoxin. The studies concerning the toxicity of mycotoxins mixture on pig immune response are scarce. Reduction of lymphocyte proliferation has been investigated in several pig *in vivo* studies, and different type of interaction were observed: additivity (co-exposure to AF and FB [0.05 and 30 mg/kg feed] for one month; co-exposure to OTA and T-2 toxin [2.5 and 8 mg/kg feed] for 30 days) or synergy (co-exposure to FB and DON [50 and 4 mg/kg feed] for 28 days) (Grenier and Oswald, 2011). In animals co-exposed to DON and FB (6 and 3 mg/kg of feed) for 35 days, synergistic interaction was observed on lymphocytes proliferation upon mitogenic stimulation, additive interaction on cytokines expression (IL-8; IL-1β, IL-6 and macrophage inflammatory protein 1β) and antagonistic interaction on levels of specific IgA and cytokine expression (Grenier et al., 2011).

In animals co-exposed to DON and FB (6 and 3 mg/kg of feed) for 35 days, additive interaction on specific IgG, on lymphocytes proliferation upon mitogenic stimulation and on cytokines expression (IL-8, IL-1β, IL-6 and macrophage inflammatory protein 1β) was observed, and antagonistic interaction on levels of specific IgA was observed (Grenier et al., 2011).

5. Conclusion

Mycotoxins can contaminate many raw materials and cause significant health risk to animals. Numerous strategies are used to minimize mycotoxins contamination throughout the feed chain. In the fields, resistant crops associated as well as agronomic control measures can be used. Similarly, during feed storage and processing, physical, chemical and biological methods can reduce mycotoxin contamination. However once mycotoxins are present in feed, it's difficult to reduce their concentrations and their toxicity due to the stability of these compounds (Bryden, 2009). The simultaneous presence of several mycotoxins, not sensitive to the same detoxification procedure, also increases the difficulty to control animals' exposure to mycotoxins (Bryden, 2012). Recently, new detoxification biological methods showed that the use of bacteria (Grenier et al., 2012, 2013; Guan et al., 2011b), feed additives such as arginine or glutamate were effective to decrease the toxic effects of mycotoxins in young pigs (Duan et al., 2014; Wu et al., 2013, 2015), even for exposition to mycotoxins mixtures (Yin et al., 2014; Grenier et al., 2013).

Pig, a species very sensitive to mycotoxins, is really exposed due to a cereal rich diet. At the European level, regulation or recommendations exist for 6 mycotoxins that are often present in pig feed. They are FB, AF, OTA, DON, T-2/HT-2 toxins and ZEN. Exposure to these toxins induces several toxic effects on pig, including a modulation of the immune response. This later effect increases the susceptibility and severity of infectious diseases, and reduces the efficacy of vaccines. This is of particular note for animal husbandry because during infection, nutrients are used for the immune system instead of growth and development (Klasing, 2007). Consequently, mycotoxin contamination also has an indirect effect on animal productivity (Klasing, 2007; Oswald et al., 2005).

The presence of new mycotoxins (emerging, masked, modified toxins, etc.) revealed by new analytical methods can also increase the risk for pig health. Currently, very few studies document the occurrence and toxicity of these toxins, thus there is a need to determine the risk they represent in pig production (Broekaert et al., 2015; Pierron et al., 2016).

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgment

Alix Pierron was supported by a fellowship from CIFRE (2012/0572, jointly financed by the BIOMIN Holding GmbH, Association Nationale de la Recherche Technique and INRA). This work was supported in part by the french project Tool4gutHealth, jointly financed by the Lallemand and the Région Midi-Pyrénées. Thanks are also due to Ryan Hines, Biomin, for language editing.

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C. Masked mycotoxins: a risk in pig production?

Currently, many mycotoxins induce toxic effects on pigs and are regulated in pig feed. New analytical methods of detection allow highlighting new types of molecules that derivate from these mycotoxins. There are also present in cereals and feed and their toxicity is not very well-known.

This review summarizes the knowledge on mycotoxins, their occurrence, their effects on pigs and their regulation in pig feed at the European level. It presents new forms of mycotoxins, the "masked" and the "modified" mycotoxins, which are derivated from these mycotoxins and which are recovered in co-occurrence with them in pig feed. It makes a statement on the way of production, the occurrence, the toxicity and the metabolization of these molecules in the pig. It summarizes the knowledge on these "masked" and "modified" mycotoxins, and talks about the necessity to take into account these molecules in the regulation of mycotoxins in pig feed.

This review was published in the book of the 48th days of the Journées de la Recherche Porcine (JRP), and as been presented as an oral synthesis at these same days of JRP in February 2016.

Original Version (French)

2016. Journées Recherche Porcine, 48, 331-340.

Les mycotoxines "masquées" : un nouveau risque en production porcine ?

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Les mycotoxines "masquées" : un nouveau risque en production porcine ?

Les mycotoxines sont des métabolites secondaires de moisissures qui peuvent contaminer différentes céréales et par conséquent l'alimentation du porc. Au niveau européen, des réglementations et des recommandations pour l'alimentation animale ont été édictées pour six mycotoxines dont la toxicité est documentée. Les avancées dans les techniques de détection ont permis de mettre en évidence des dérivés de ces mycotoxines "natives", appelés mycotoxines "modifiées" ou plus spécifiquement mycotoxines "masquées" lorsqu'elles sont issues d'une métabolisation par la plante.

Du fait de leur caractérisation récente, peu d'informations sont disponibles sur leur occurrence dans l'alimentation du porc et leur toxicité pour cette espèce. Les données préliminaires indiquent que ces toxines peuvent être présentes à de fortes concentrations dans les aliments. Le porc pourrait être une espèce cible également pour ces "nouvelles" mycotoxines, du fait de sa grande sensibilité à la présence de mycotoxines conventionnelles, et à son régime alimentaire composé en grande partie de céréales. Ces mycotoxines "modifiées" peuvent augmenter la somme de mycotoxines auquel le porc est exposé, si elles sont hydrolysées dans l'organisme de l'animal.

Cette revue recense les connaissances actuelles sur la toxicité des formes "modifiées" du déoxynivalénol, des toxines T2 et HT2, de la zéaralenone, de la fumonisine et de l'ochratoxine A pouvant se retrouver dans l'alimentation du porc. Nous nous attacherons à comparer le métabolisme et la toxicité des formes "modifiées" à celle de leurs précurseurs et à analyser la possible reconversion de ces formes "modifiées" par la flore intestinale ou les voies de métabolisation du porc.

Masked mycotoxins: a new risk in pig production?

Mycotoxins are secondary metabolites originating from mold, which contaminate many cereals and their by-products and so can be found in the pig's diet. Some recommendations and regulations for animal feed have been decreed in the EU for six mycotoxins for which the toxicity is well known. Recent detection methods have revealed new mycotoxins and new molecules that are derivates of these mycotoxins. They were originally called "Masked" mycotoxins because they are not detected by conventional analytical methods. Then, they are more generally called "Modified", and "masked" when they are metabolized by the plant.

So because of the difficulty in detecting them, there is little information about the toxicity of these molecules and they are not included in the current regulation on mycotoxin contamination in pig feed. Moreover, a high proportion of these modified mycotoxins can be found in co-contamination with the mycotoxins. Pigs are really sensitive to mycotoxins, and their high cereal-rich diet means that they are highly susceptible to mycotoxins and to these modified mycotoxins. These modified mycotoxins can potentially increase the amount of mycotoxins to which pigs are exposed if they are hydrolyzed in the animal.

This review summarizes recent knowledge about the toxicity of the modified mycotoxins of deoxynivalenol, T2 and HT2 toxins, zearalenone, fumonisin and ochratoxin A, and presents recent studies about the metabolization and toxic effects on the animals of these modified mycotoxins, and their potential impact on their health.

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INTRODUCTION

A l'heure actuelle, la contamination des denrées par les moisissures reste inévitable. Les conditions écologiques favorables aux moisissures (mauvaises conditions climatiques, humidité, forte chaleur, sensibilité de la plante…) rendent la gestion de la contamination des matières premières difficilement maîtrisable. Lors d'une contamination par des moisissures au champ ou plus tard pendant le stockage, des métabolites secondaires toxiques, les mycotoxines, sont produits et se retrouvent dans les grains. Ces toxines sont présentes sur de nombreuses céréales et dans leurs co-produits. Une enquête récente réalisée sur 1 100 échantillons d'aliments destinés aux animaux a indiqué qu'environ 70% des échantillons sont contaminés (Streit *et al.*, 2013). La présence de ces mycotoxines constitue une menace sérieuse en matière de santé (Bryden, 2007 ; Wild et Gong, 2010). Les syndromes dus à l'ingestion de doses fortes ou moyennes de mycotoxines sont bien caractérisés et vont de la mortalité aiguë à une croissance réduite ou à des problèmes de reproduction (Bryden, 2012). La consommation de quantités moindres de toxines peut conduire à une altération de la réponse immunitaire et diminuer la résistance aux maladies infectieuses (Oswald, 2007). Certaines mycotoxines ont une toxicité aiguë (exposition unique à une forte dose) très marquée, mais il est exceptionnel en Europe d'être exposé à des doses toxiques en une seule ingestion d'aliments contaminés. Les effets chroniques (exposition répétée à de faibles, voire très faibles doses) sont les plus redoutés en raison du régime alimentaire répétitif des animaux et de la rémanence de ces toxines souvent résistantes aux températures et aux procédés technologiques mis en œuvre dans l'industrie de l'alimentation animale.

Les avancées récentes dans le domaine des techniques analytiques ont mis en évidence de nouvelles mycotoxines ainsi que des formes de mycotoxines dites "masquées", non détectées par les méthodes conventionnelles. Actuellement, seules les mycotoxines "natives" sont réglementées et prises en compte dans le calcul de l'exposition totale dans les aliments bruts ou transformés. En effet, peu de données sont disponibles sur ces nouvelles molécules d'où le risque de sous-estimer la toxicité induite par ces composés non pris en compte dans la réglementation. Il devient donc important de mieux connaitre ces mycotoxines "masquées", pour permettre de mieux évaluer le risque qu'elles représentent pour l'homme et l'animal.

Après un rappel sur les mycotoxines conventionnelles habituellement détectées, cette revue propose une synthèse des connaissances actuelles sur les mycotoxines "masquées", leur identité, leur occurrence, leur métabolisme et leur toxicité. Elle pose un constat sur le danger éventuel que peuvent représenter ces toxines « masquées » sur la santé du porc.

1. LES MYCOTOXINES REGLEMENTEES DANS L'ALIMENTATION DU PORC

En alimentation animale, seules les aflatoxines (AF) font l'objet d'une réglementation au sens strict en Europe. Des recommandations (Tableau 1) ont été édictées pour cinq autres toxines, qui sont régulièrement présentes et qui sont connues pour leur toxicité sur le porc.

Il s'agit de l'ochratoxine A (OTA), du deoxynivalenol (DON), des toxines T2 et HT2, des fumonisines (FB_1, FB_2) et de la zéaralénone (ZEN) (Bennett et Klich, 2003) (Tableau 1).

Tableau 1 – Mycotoxines pour lesquelles une réglementation ou des recommandations ont été édictées pour l'alimentation du porc : type d'aliments et teneur maximales retenues (EC Directive 2002/32/EC, et EC Recommandations 2006/576/EC et 2013/165/EU) (adapté de Stoev, 2014).

Mycotoxines	Type d'aliment	Teneurs maximales, mg/kg d'aliment
AFB_1+ B_2	Tout type de céréales pour animaux	60
	Aliment complet pour porc, cheval, lapin et animaux de compagnie	0,5
OTA	Aliment complet et compléments alimentaires pour porc	0,05
DON	Tout type de céréales pour animaux (sauf co-produits de maïs)	8 (12)
	Aliment complet et compléments alimentaires pour porc	0,9
ZEN	Tout type de céréales pour animaux (sauf co-produits de maïs)	2 (3)
	Aliment complet et compléments alimentaires : -pour porcelet et cochette -pour truie et porc charcutier	 0,1 0,25
FB_1+FB_2	Tout type de céréales pour animaux	60
	Aliment complet et compléments alimentaires pour porc, cheval et lapin	5
T2+HT2	Produits céréaliers et aliments composés pour animaux : -produits de mouture d'avoine -autres produits céréaliers -aliments composés exceptés pour chat	 1 0,5 0,25

Abréviations : Aflatoxine B_1 (AFB_1), Aflatoxine B_2 (AFB_2), Ochratoxine A (OTA), Deoxynivalenol (DON), Zéaralénone (ZEN), Fumonisine B_1 (FB_1), Fumonisine B_2 (FB_2), Toxine T2 (T2) et toxine HT2 (HT2).

Ces composés appartiennent à différentes familles de mycotoxines, avec des structures chimiques très différentes, et donc des effets très divers sur le porc. Leur degré de toxicité dépend de nombreux paramètres, notamment la dose, la durée d'exposition, l'espèce concernée, l'âge, le statut de l'animal (sain ou malade) et son statut hormonal et nutritionnel (Bryden, 2007 ; Wild, 2007).

Le tableau 2 recense les principaux effets connus de ces mycotoxines sur la santé du porc.

Les AF sont rapidement absorbées et métabolisées au niveau du foie par le système microsomal qui active ou modifie les métabolites (Riley, 1998; Haschek *et al.*, 2002). Les AF altèrent la réponse immunitaire globale (innée et cellulaire) chez le porc (Meissonnier *et al.*, 2006).

L'OTA est principalement toxique pour le foie et les reins et induit des néphropathies chez le porc. L'OTA affecte le tubule proximal rénal (Krogh, 1987 ; Marquardt et Frohlich, 1992). De plus l'OTA acquiert un caractère génotoxique après sa métabolisation oxydative dans l'organisme (Aish *et al.*, 2004 ; Pfohl-Leszkowicz et Manderville, 2007 ; Steyn *et al.*, 2009). Le DON est le plus courant des trichothécènes B. Le porc est très sensible à cette mycotoxine, qui peut induire à faible concentration un refus de s'alimenter, et à plus forte concentration des vomissements (Haschek *et al.*, 2002). A des doses chroniques (faibles concentrations sur du long terme), il induit chez le porc une perte de poids, une anorexie, une immunomodulation et une modification de la fonction de barrière de l'intestin (Trenholm *et al.*, 1984 ; Rotter *et al.*, 1996 ; Haschek *et al.*, 2002 ; Pinton, Oswald, 2014).

Les toxines T2 et HT2 qui appartiennent à la famille des trichothécènes A présentent des effets de même nature mais plus prononcés que les trichothécènes B. Elles induisent des irritations au niveau du tube digestif et de la peau, et elles augmentent la sensibilité de l'animal aux maladies (Bryden, 2012).

Les fumonisines constituent un groupe de 12 composés parmi lesquels la fumonisine B_1 (FB_1) est la plus toxique et la plus étudiée. Les fumonisines induisent de multiples effets toxiques chez l'animal, avec un effet carcinogène reconnu. Chez le porc, la FB_1 affecte la réponse spécifique et la réponse humorale en modifiant la balance des lymphocytes T auxiliaires TH1/TH2 (Taranu *et al.*, 2005 ; Marin *et al.*, 2006). La FB_1 induit des œdèmes pulmonaires chez le porc (Haschek *et al.*, 2002). La zéaralénone (ZEN) a un effet important sur la reproduction et en particulier la fertilité dans l'espèce porcine. La α-zearalenol (α-ZEL) et la β-zearalenol (β-ZEL), issus de la réduction de la ZEN par les ketones-reductases de l'hôte, sont des œstrogènes non stéroïdiens qui induisent l'activité ostrogénique chez l'animal (Fink-Gremmels et Malekinejad, 2007). La ZEN et ses dérivés provoquent une rougeur et une tuméfaction de la vulve, un prolapsus vaginal et parfois un prolapsus rectal chez la truie. Chez les porcelets femelles, elles peuvent induire un gonflement important de la vulve (Gaumy *et al.*, 2001).

Tableau 2 – Effets associés à la présence de mycotoxines[1] dans l'aliment du porc

Effets[2]	Mycotoxines					
	AFB_1 AFB_2	OTA	DON	T2 HT2	FB_1 FB_2	ZEN
Anorexie	+	+	+++	+++	+	
Croissance	+++	+	+++	++	+	
Hépatotoxicité	+++	+			++	
Néphrotoxicité		+++			+	
Avortement					+	++
Infertilité						+++
Vulvovaginite						+++
Œdème pulmonaire					+++	
Immuno-modulation	+++		++	++	+++	+

[1]*Abréviations : Aflatoxine B_1 (AFB_1), Aflatoxine B_2 (AFB_2), Ochratoxine A (OTA), Deoxynivalenol (DON), Zéaralénone (ZEN), Fumonisine B_1 (FB_1), Fumonisine B_2 (FB_2), Toxine T2 (T2) et toxine HT2 (HT2).*

[2]*+, ++, +++ : effet faible, moyen, et fort de la (les) mycotoxine(s) sur le paramètre étudié*

2. LES MYCOTOXINES "MODIFIEES" DANS L'ALIMENTATION DU PORC

2.1. Présentation

Les avancées dans le domaine analytique ont permis l'identification de nouveaux métabolites secondaires fongiques, mais également des produits de transformation des mycotoxines.

Le terme de mycotoxines "masquées" a été introduit dès 1990 par Gareis pour décrire un glucoside de zéaralénone non détecté lors des analyses de routine, mais hydrolysé pendant la digestion (Gareis *et al.*, 1990).

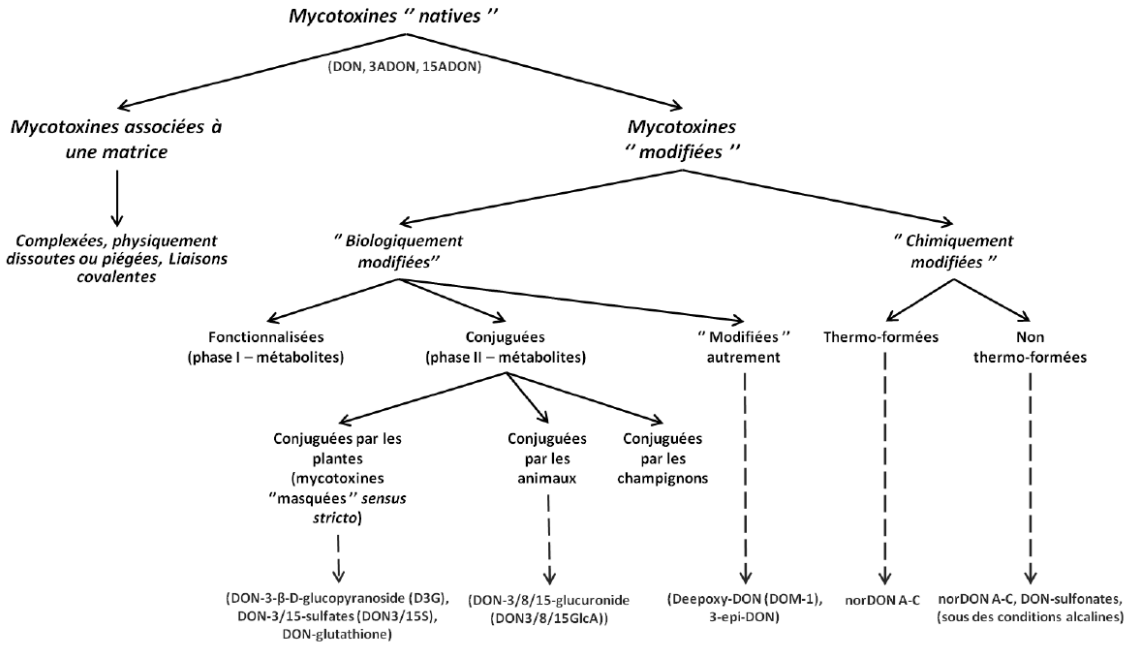

Figure 1 – Nomenclature des différentes formes de modification des mycotoxines: exemple du Déoxynivalénol (DON) (adapté de Rychlik *et al.*, 2014)

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Les mycotoxines peuvent en effet subir différentes modifications de leur structure qui les rendent indétectables par les techniques d'analyse classiques (Tableau 3). Il s'agit de modifications d'origine biologique (mises en œuvre par une plante, le champignon ou un organisme animal) ou d'origine chimique comme par exemple lors de la mise en œuvre de procédés thermiques de transformation alimentaire.

La dénomination de "mycotoxines masquées" a souvent fait l'objet d'une utilisation ambigüe, et récemment des auteurs ont proposé une terminologie plus précise pour les différentes formes de mycotoxines (Berthiller *et al.*, 2013 ; Rychlik *et al.*, 2014). Ces auteurs ont reprécisé la terminologie de "mycotoxines masquées" au sens strict et introduit la notion de "mycotoxines modifiées". La figure 1 illustre pour l'exemple du DON, l'ensemble des formes décrites de cette mycotoxine.

Les **mycotoxines dites "natives"** correspondent aux structures de base des mycotoxines formées par les moisissures. Les plus susceptibles d'être retrouvées dans l'alimentation du porc sont le DON, la ZEN, les fumonisines, les aflatoxines, ainsi que l'OTA.

Les **mycotoxines associées à une matrice** correspondent aux mycotoxines "natives" liées à une matrice c'est à dire physiquement dissoutes, et/ou piégées et/ou formant une liaison covalente avec cette matrice. Ainsi, les fumonisines sont capables de se lier aux polysaccharides ou aux protéines par leurs deux chaînes acides tricarballyliques, formant ainsi les fumonisines dites cachées (F cachées) ou liées à l'amidon (F liées à l'amidon) (Seefelder *et al.*, 2003).

En dehors de ces phénomènes de liaison à une matrice, les mycotoxines "natives" peuvent subir des transformations d'origine biologique ou purement chimique. Le terme de **"mycotoxine modifiée"** a été proposé pour désigner toute modification biologique ou chimique de la structure chimique d'une mycotoxine "native" (Rychlik *et al.*, 2014).

Les mycotoxines **"modifiées biologiquement"** désignent des composés issus d'une biotransformation par un organisme animal, végétal ou une moisissure. Les biotransformations sont divisées en deux types principaux : les réactions de phase I (oxydation, réduction ou hydrolyse) et les réactions de phase II (conjugaison). Généralement, la biotransformation permet une détoxification des toxiques, notamment en facilitant leur excrétion. Cependant dans certains cas, elle peut conduire à un composé plus toxique que la molécule mère. C'est par exemple le cas de l'aflatoxine B_1-époxyde qui est issue de l'oxydation de l'AFB_1 par les cytochromes P450 pendant les réactions de biotransformation de la phase I chez l'animal.

Les formes glucuronides (DON3-GlcA, ZEN14-GlcA, T2-GlcA, HT2-3/4-GlcA) sont issues de la phase II de biotransformation des mycotoxines "natives" correspondantes par l'animal, et représentent des exemples de mycotoxines dites **"biologiquement modifiées - conjuguées"**. Elles correspondent à des formes d'excrétion des mycotoxines natives de l'organisme animal.

Le DON-3-β-D-glucopyranoside (D3G), et la zéaralénone-14-β-D-glucopyranoside (ZEN14G) issus de la phase II de biotransformation par les végétaux respectivement du DON ou de la ZEN, en sont d'autres exemples. Par convention, la terminologie de **"mycotoxines masquées"** a été retenue pour les seules mycotoxines "biologiquement modifiées" issues d'une réaction de conjugaison dans une plante (Berthiller *et al.*, 2013). A l'heure actuelle, les quatre principales mycotoxines "masquées" au sens strict sont la ZEN14G, le D3G, la toxine T2-glucoside (T2-Glc) et la toxine HT2-glucoside (HT2-Glc) (Lattanzio *et al.*, 2012). Il est intéressant de souligner le cas du dérivé acétylé du 3ADON, un dérivé acétylé du DON. Ce composé peut être produit à la fois par le champignon, dans ce cas c'est une mycotoxine "native", et par les variétés transgéniques de riz, de blé et d'orge exprimant le gène de la 3-O-acetyltransférase, et donc considéré comme une mycotoxine "masquée". Le transfert du gène de l'3-O-acetyltransférase à des plantes est envisagé comme une stratégie d'avenir pour la réduction du pouvoir pathogène des fusarioses qui affectent certaines espèces végétales. Il est en effet établi que la conversion en 3ADON par la plante du DON produit par la moisissure, permet de limiter l'agressivité de la fusariose (Karlovsky, 2011).

Tableau 3 – Principales mycotoxines "modifiées" recensées (adapté de Broekaert *et al.*, 2015)

Mycotoxine "native"	Mycotoxine "modifiée"
Déoxynivalénol	15-acétyl-DON (15ADON) 3-acétyl-DON (3ADON) DON-3-O-glucoside (DON3O-Glc) DON-3/8/15-glucuronide (DON3/8/15-GlcA) 3-acetyl-DON-glucuronide (3ADON-GlcA) **DON-3-β-D-glucopyranoside (D3G)** DON-oligosaccharides Deepoxy-DON (DOM-1) 3-epi-DON 9-hydroxylmethyl DON lactone Nor-DON A-F DON-sulfonate (DON-S)
Zéaralénone	ZEN-14-glucuronide (ZEN14-GlcA) **ZEN-14-β-D-glucopyranoside (ZEN14G)** ZEN-14-sulfate (ZEN14S) α-zearalenol (α-ZEL) β-zearalenol (β-ZEL) α-zearalenol-14-α-D-glucopyranoside (α-ZEL14G) β-zearalenol-14-β-D-glucopyranoside (β-ZEL14G)
T2	T2-glucuronide (T2-GlcA) **T2-glucoside (T2-Glc)**
HT2	HT2-3/4-glucuronide (HT2-3/4-GlcA) **HT2-glucoside (HT2-Glc)**
Fumonisine	F N-(carboxymethyl) FB_1 N-(1-deoxy-D-fructos-1-µl) HFBx F-N-acetyl F-O-acetyl F cachées F liées à l'amidon
Aflatoxine	AFB_1-epoxide
Ochratoxine	OTA-oligossacharides 14-(R)-OTA 14-decarboxy-OTA

En gras : les mycotoxines masquées au sens strict.

D'autres mycotoxines peuvent être "biologiquement modifiées" par l'action d'un micro-organisme, et sont regroupées sous le vocable de "mycotoxines **modifiées différemment**". Le Deepoxy-DON (DOM-1) et le 3-epi-DON, issus de la transformation du DON par des bactéries extraites du microbiote humain ou animal, appartiennent à ce groupe (Eriksen *et al.*, 2002 ; Karlovsky, 2011 ; Gratz *et al.*, 2013).

Les mycotoxines **"chimiquement modifiées"** constituent le dernier groupe. Les modifications chimiques peuvent dépendre ou non de la chaleur.

Les mycotoxines **"chimiquement modifiées thermoformées"** apparaissent lors de la mise en œuvre des procédés de transformation alimentaire tels que la cuisson, le grillage, la congélation ou l'extrusion. Ces modifications thermo-dépendantes sont connues pour de nombreuses mycotoxines, notamment les fumonisines capables d'entrer dans une réaction de Maillard, du fait de la réduction des sucres, avec l'obtention par exemple de la fumonisine B_1 N-(1-deoxy-D-fructos-1-yl) et de la fumonisine N-(carboxymethyl) (Humpf et Voss, 2004). On peut citer également comme produits de dégradation thermique des dérivés du DON (norDON A-F et 9-hydroxymethyl DON lactone) dont certains peuvent être retrouvés dans des échantillons d'aliment du commerce (Bretz *et al.*, 2005).

Les mycotoxines **"chimiquement modifiées non thermo-formées"** proviennent quant à elles d'une variété de procédés, dont l'hydrolyse mise en œuvre avec les fumonisines (HFBx), la sulfatation du DON aboutissant au DON-sulfonate ou les produits de dégradation des ochratoxines par les rayons UV (Beyer *et al.*, 2010 ; Schmidt-Heydt *et al.*, 2012).

2.2. Occurrence des mycotoxines "natives" et "modifiées"

Certaines mycotoxines "modifiées", en particulier les formes "masquées" mais également les formes associées à la matrice et certaines formes chimiquement modifiées peuvent se retrouver dans les aliments destinés au porc. Le tableau 4 représente des données d'occurrence des principales mycotoxines et de leurs formes "modifiées" dans des échantillons de céréales (blé, orge, maïs, avoine et riz) sur la période de 2010 à 2014.

Les mycotoxines "natives" représentent la part majoritaire dans la contamination des aliments. Cependant, les autres formes sont également retrouvées de façon concomitante dans les aliments. Il est actuellement possible de détecter de nombreuses mycotoxines "modifiées", mais peu de données quantitatives sont disponibles, notamment en raison d'un manque de standards analytiques et de matériel de référence.

Le tableau 5 apporte de plus amples informations sur la proportion de certaines mycotoxines "modifiées", pour lesquelles quelques données sont disponibles, par rapport à leur forme "native". Pour certaines mycotoxines, comme la T2-Glc et la HT2-Glc, les données d'occurrence du tableau 4 ne proviennent que d'une seule étude. Leur présence a été rapportée pour la première fois en 2012 dans du blé et de l'avoine naturellement contaminés (Lattanzio *et al.*, 2012).

Pour le D3G, plus anciennement découvert, plus de données sont disponibles sur son occurrence et sur sa proportion par rapport au DON. La proportion de cette mycotoxine "masquée" est assez stable dans les aliments et correspond en moyenne à 20% du DON présent (Berthiller *et al.*, 2009). Cependant les ratios sont très variables selon la céréale, le génotype concerné, le pays et l'année de récolte et peuvent augmenter jusqu'à 46%. (Berthiller *et al.*, 2009 ; De Boevre *et al.*, 2012). De plus l'utilisation croissante de plantes résistantes à *Fusarium,* capables de glucosyler de façon importante le DON en D3G, pourrait encore augmenter le ratio D3G/DON. Certaines études sur ces plantes résistantes ont même trouvé jusqu'à 2,7 fois plus de D3G présent dans la plante que de DON (Sasanya *et al.*, 2008).

Les ZEN14S et ZEN14G sont aussi retrouvés en proportion assez stables, jusqu'à 30% de la ZEN présente (Scheneweis *et al.*, 2002 ; Streit *et al.*, 2013b).

Tableau 4 – Occurrence des trichothécènes et de la zéaralénone et de leurs formes "modifiées" dans des céréales (échantillons de blé, orge, maïs, avoine et riz issus de différents pays)

(adapté de Broekaert *et al.*, 2015)

Mycotoxines[1]	Nombre d'échantillons[2]	Incidence, %	Moyenne, µg/kg d'aliment
DON	5743	84	458
3ADON	2227	22	14,7
15ADON	686	31	36,6
D3G	529	55	85
NIV	3062	32	17,8
ZEN	2158	12	39,6
ZEN14G	36	25	37
ZEN14S	12	25	6
T2	321	45	16,7
HT2	321	54	61
T2G	15	73	2,4
HT2G	15	80	5,1

[1]*Abréviations: Nivalénol (NIV), voir également tableaux 1 et 3. Les mycotoxines "natives" sont indiquées en italique et les mycotoxines "modifiées" en gras.*

[2]*Pays d'origine des différentes céréales analysées : Allemagne, Autriche, Belgique, Chine, Danemark, Finlande, Italie, Nigeria, Norvège, République Tchèque et Suède.*

Pour ce qui est des fumonisines associées à la matrice (piégées physiquement), leurs proportions par rapport aux fumonisines libres sont plus variables. Leur présence a été montré après une étape d'hydrolyse des matières premières (Dall'Asta *et al.*, 2009). La proportion de ces formes physiquement piégées changent selon le génotype du maïs et selon les conditions de culture (Dall'Asta *et al.*, 2012).

En conclusion, plus de données d'occurrence, sur différentes céréales et dans différents pays, sont nécessaires pour évaluer correctement le risque associé à la présence de ces nouvelles mycotoxines.

2.3. Métabolisation et toxicité des mycotoxines "modifiées" chez le porc

L'occurrence des mycotoxines "modifiées" dans les denrées alimentaires et l'exposition des animaux à ces nouvelles toxines suscitent un certain nombre d'interrogations et le besoin d'investiguer la métabolisation et la toxicité de ces composés (EFSA, 2014). Il est en effet important d'étudier la toxicité intrinsèque de ces toxines, mais également de connaitre leur métabolisme et en particulier de déterminer si les mycotoxines "modifiées" sont reconverties en formes "natives" correspondantes.

Quelques récentes études se sont intéressées aux effets de ces mycotoxines "modifiées" sur le porc, sur des modèles *in vitro* ou *in vivo.* La majorité de ces études portent sur le métabolisme de ces molécules et peu sur leur toxicité.

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Tableau 5 - Proportion des mycotoxines "modifiées" présentes par rapport à leur mycotoxine "native" dans des matières premières naturellement contaminées

Matière première	Mycotoxines "modifiées"	Nombre d'échantillons	Proportion de mycotoxine "modifiée"/ "native", %	Références
Blé, Maïs	D3G	77	20% jusqu'à 46%	Berthiller *et al.* (2009)
Céréales	D3G	21	6-29%	Desmarchelier et Seefelder (2011)
Maïs, Blé, Avoine	D3G	11	jusqu'à 30%	De Boevre *et al.* (2012)
Maïs	ZEN14S	41	jusqu'à 30%	Streit *et al.* (2013)
Blé	ZEN14G	10	jusqu'à 30%	Scheneweis *et al.* (2002)
Blé	T2-Glc, HT2-Glc	9	jusqu'à 12%	Lattanzio *et al.* (2012)
Avoine	T2-Glc, HT2-Glc	9	2%	Lattanzio *et al.* (2012)
Maïs	Fumonisines associées à la matrice	31	jusqu'à 100%	Dall'Asta *et al.* (2010)
	Fumonisines associées à la matrice	97	jusqu'à 60%	Dall'Asta *et al.* (2010)
	Fumonisines associées à la matrice	120	jusqu'à 60%	Dall'Asta *et al.* (2012)

Abréviations: voir tableaux 1 et 3.

2.3.1. *Toxicité intrinsèque des mycotoxines "modifiées" pour le porc*

Les études consacrées à la toxicité des mycotoxines "modifiées" concernent en grande partie les formes "modifiées" du DON et de la ZEN. La majorité de ces études ont été réalisées *in vitro* sur des cellules humaines et seulement quelques-unes s'intéressent à la toxicité *in vivo* chez l'animal, notamment la souris et le porc.

La toxicité du DON a été comparée à celle de ses dérivés acétylés (3ADON et 15ADON) en prenant en compte la prolifération cellulaire, l'activation des MAPKs (Mitogen-activated protein kinases) et de l'expression des protéines de jonctions serrées, ainsi que l'expression des cytokines chez le porc (Pinton *et al.*, 2012). Les pourcentages de réduction de la viabilité cellulaire des cellules intestinales de porc (IPEC-1) incubées 24 heures en présence de DON, 3ADON et 15ADON sont respectivement de 60%, 13% et 69%. L'expression des protéines de jonction par ces cellules intestinales porcines est diminuée de 40% en présence du 15ADON, et est équivalente pour le DON et le 3ADON. Le 15ADON a également montré plus de toxicité que le DON et le 3ADON au niveau de l'activation des MAPKs, *in vitro* sur les cellules IPEC-1, *ex vivo* sur des cultures d'explants de jéjunum de porc ou *in vivo* dans le tissu jéjunal de porcelets (Pinton *et al.*, 2012).

Un des dérivés connu du DON est le DOM-1 issu d'une transformation bactérienne réduisant le groupement 12,13-epoxy qui est essentiel dans la toxicité du DON et des trichothécènes en général (Schatzmayr *et al.*, 2006 ; Zhou *et al.*, 2008). De ce fait le DOM-1 est considéré comme un métabolite non toxique du DON. Une étude *in vitro* a montré que le DOM-1 était 54 fois moins toxique que le DON au niveau de la synthèse d'ADN sur des fibroblastes de souris (Eriksen et Pettersson, 2004). La toxicité du DOM-1 sur les paramètres zootechniques a été évaluée *in vivo* sur des porcs. Les animaux recevant l'aliment contaminé avec du DON à 5mg/kg d'aliment et la souche bactérienne, capable de deépoxyder le DON, ne présentaient pas de diminution de la prise alimentaire ou de prise de poids (He *et al.*, 1993 ; Li *et al.*, 2011). Cependant l'évaluation de la toxicité de DOM-1 pur sur l'intestin et l'organisme du porc n'a pas été évaluée.

Comparé au DON, le D3G s'est avéré non toxique *ex vivo* sur des jéjunums de porcs, avec une incapacité du D3G à induire un stress ribotoxique et à activer la voie des MAPKs centrale dans la mise en œuvre de la réponse pro-inflammatoire observée avec le DON (Pierron *et al.*, 2016). Cette inaptitude du D3G contrairement au DON à induire une réponse pro-inflammatoire se confirme chez le rat avec une absence de sur-expression des cytokines et chemokines (Wu *et al.*, 2014a). Par ailleurs, le pouvoir émétique du D3G semble beaucoup plus faible que celui du DON (Wu *et al.*, 2014b).

Plusieurs études ont comparé les pouvoirs oestrogéniques de la ZEN et de ses deux dérivés α-ZEL et β-ZEL. L'oestrogénicité de ces molécules se classe ainsi comme suit : β-ZEL < ZEN < α-ZEL (Mukherjee *et al.*, 2014). Au niveau cellulaire, de plus fortes cytotoxicité et génotoxicité de la β-ZEN comparée à la α-ZEN ont été montrées sur des cellules endométriales de porc (Tiemann *et al.*, 2003 ; Othmen *et al.*, 2008) tandis que, l'α-ZEL s'est révélée plus toxique que la β-ZEL sur les oocytes de porc (Alm *et al.*, 2002). En résumé, la hiérarchie dans la toxicité des deux mycotoxines "modifiées", α-ZEL et β-ZEL, n'est pas clairement établie et elle dépend du type cellulaire considéré. Dans l'ensemble, leur toxicité est moindre que celle de la ZEN.

Les formes glucosylée et sulfatée de la ZEN, les ZEN14G et ZEN14S, semblent incapables de se lier aux récepteurs oestrogéniques et d'induire une toxicité *in vitro* (Poppenberger *et al.*, 2006; Berthiller *et al.*, 2009).

De façon globale, les composés issus des voies de détoxification des plantes, les formes "masquées" *sensu stricto* sont moins toxiques ou inactivés par rapport à la molécule "native". Les voies de biotransformation de la plante, qui sont similaires à celles des animaux (par exemple les réactions de conjugaison aux différentes molécules sulfate, glutathione ou acide glucuronique), augmentent la polarité de ces molécules, facilitant ainsi leur excrétion et diminuant leur toxicité (Yiannikouris et Jouany, 2002 ; Homolya *et al.*, 2003).

2.3.2. *Métabolisation des mycotoxines "modifiées" chez le porc*

Les interrogations sur une reconversion des mycotoxines "modifiées" en mycotoxines "natives" sont aussi vieilles que la découverte des premières mycotoxines "modifiées".

Très tôt en effet, il a été montré qu'en exposant oralement pendant 2 semaines un porcelet à du ZEN14G, il était possible de retrouver dans ses urines et ses fèces des quantités variables de ZEN et de son métabolite oestrogénique α-ZEL (Gareis *et al.*, 1990). Cette étude souligne le fait qu'une partie non négligeable de l'exposition du porc à une mycotoxine pourrait provenir de la conversion des mycotoxines "modifiées". Une telle reconversion peut être due à l'activité des enzymes digestives et du métabolisme de l'animal. Elle peut également résulter de l'activité enzymatique de la flore digestive suivie de la réabsorption de la mycotoxine "native" et/ou de ses métabolites. Le tableau 6 récapitule le devenir de certaines mycotoxines "modifiées" le long du tractus digestif et leur hydrolyse en leur forme "native".

Le porc est un animal physiologiquement très proche de l'homme, notamment pour ce qui est de la digestion. Dans un système *in vitro* humain mimant en étapes successives l'action du suc salivaire, du suc gastrique, du suc intestinal et de la bile, le D3G, la ZEN14G et la ZEN14S sont conservés respectivement à 99,5%, 97,3% et 98,6% (De Nijs *et al.*, 2012 ; Dall'Erta *et al.*, 2013).

Toutefois, le D3G qui n'est pas transformé par les enzymes présentes dans la salive et l'estomac des mammifères, pourrait être hydrolysé par l'acide lactique produit par certaines espèces bactériennes telles que *Enterococcus mundtii* et *Lactobacillus plantarum* présentes dans le tube digestif (Berthiller *et al.*, 2011).

Tableau 6 –Devenir de certaines mycotoxines "modifiées" le long du tractus digestif (adapté de Boevre *et al.*, 2015)[1,2]

Compartiment	Mycotoxines					
	D3G[1]	**3 ADON & 15ADON[3]**	**ZEA14G[3]**	**ZEA14S[4]**	**Fumonisines cachées[4]**	**Fumonisines liées[4]**
Salive	*Stable*	*Stable*	*Stable*	*Stable*	*Stable*	*Stable*
Estomac	*Stable*	11%DON (3ADON) 13% DON (15ADON)	Max.19% ZEN	*Stable*	*Stable*	*Stable*
Intestin grêle	*Max. 5% D3G détecté*	0% 3ADON 0% 15ADON	*Traces de ZEN14G détectées*	*Stable*	*Stable*	*Stable*
Colon	*Max. 2% D3G détecté* Fèces : DON+DOM-1	Déacétylation + glucuronidation	Fèces : 40% ZEN Fèces : 60% catabolites	Fèces : 40% ZEN Fèces : 60% catabolites	Max.99% fumonisines	*Stable*

[1]*Voir les tableaux 1 et 3 pour les abréviations.*

[2]*En italique: % de la mycotoxine "modifiée" retrouvée telle quelle après ingestion orale dans les différents compartiments du tractus digestif. En caractères normaux: % de la mycotoxine "modifiée" hydrolysée et retrouvée sous sa forme "native" dans les différents compartiments du tractus digestif. "Traces" si elle est très légèrement retransformée et "stable" si elle n'est pas retransformée dans ce compartiment.*

[3]*Données basées sur des expérimentations* in vitro *et* in vivo.

[4]*Données basées surdes expérimentations* in vivo.

Tout comme pour le DON, les formes "modifiées" de la ZEN peuvent être déconjuguées après une fermentation fécale par le microbiote humain, et la forme "native" de la mycotoxine relarguée dans la lumière intestinale (Dall'Erta *et al.*, 2013). Une augmentation de 30-50% de fumonisines détectables après digestion de la matrice alimentaire est également observée (Dall'Asta *et al.*, 2010). Ce constat suggère que les enzymes gastro-intestinales sont capables de détruire les interactions entre la matrice et les fumonisines, relarguant les formes "natives" des mycotoxines. Pour le DOM-1, issu d'une transformation bactérienne, il n'y a pas d'études *in vivo* où les animaux auraient reçu du DOM-1 pur.

La réaction d'épimérisation à l'origine de la formation du 3-epi-DON est une réaction irréversible (Karlovsky, 2011). Mais actuellement aucune donnée n'est disponible sur sa toxicité. Pour ce qui est des formes acétylés du DON, elles sont très vite déacétylés au sein de l'organisme en DON (Wu *et al.*, 2010).

Ces résultats obtenus *in vitro* montrent donc que certaines des mycotoxines "modifiées" peuvent être retransformées en leur mycotoxines "natives" dans des proportions variables, et suggèrent que cette transformation serait principalement due au microbiote intestinal.

Une étude s'est intéressée au devenir du DON ou du D3G administré oralement et aussi au D3G administré par voie intraveineuse à des porcelets (Nagl *et al.*, 2014). Le DON est excrété en très grande partie par voie urinaire, principalement dans les huit premières heures post-exposition, majoritairement sous forme "native" et minoritairement sous forme de DON-3-glucuronide (DON3-GlcA) et de DON-15-glucuronide (DON15-GlcA). Quant au D3G administré oralement, son excrétion urinaire, bien que majoritaire, est plus tardive, apparaissant entre la 8ième et la 24ième heure post-exposition. Seule une infime partie du D3G a été retrouvée non transformée dans l'urine, la majeure partie étant convertie en DON, et accessoirement en DON3-GlcA et en DON15-GlcA. Dans le cas du D3G administré par voie parentérale, presque toute la dose administrée était retrouvée sous forme inchangée dans les urines dans les huit premières heures. Cette étude démontre (i) que le D3G contaminant les aliments de porc peut être converti en DON dans le tube digestif, (ii) en raison de l'élimination urinaire tardive, que cette conversion a probablement lieu dans la portion basse du tube digestif, et (iii) que le DON provenant de l'hydrolyse microbienne du D3G pourrait très bien être réabsorbé et contribuer de façon significative à l'exposition totale en DON du porc.

3. EVALUATION DE L'EXPOSITION ET CARACTERISATION DU RISQUE POUR LES MYCOTOXINES "MODIFIEES" CHEZ LE PORC

Un travail d'évaluation de l'exposition du porc à certaines mycotoxines "modifiées" et de caractérisation du risque associé a été effectué par l'EFSA en 2014 (EFSA, 2014). Les mycotoxines concernées sont la ZEN, le nivalénol (NIV) (une mycotoxine de la famille des trichothécènes comme le DON), les toxines T2 et l'HT2 et les fumonisines.

Les calculs d'exposition ont été faits en traduisant en exposition cumulée, l'accroissement de l'exposition en une mycotoxine donnée qui résulterait de la conversion de la mycotoxine modifiée en mycotoxine "native".

Comme chez l'homme, cet accroissement a été estimé à 100% pour la ZEN, 30% pour le NIV, 10% pour T2 et HT2, et 60% pour les fumonisines.

Le tableau 7 présente les estimations d'exposition cumulée à ces mycotoxines "modifiées" et à leur forme "native".

Sur la base de la NOEL (No Observed Effect Level ; dose maximale sans effet nocif observé) de la ZEN fixée à 10 µg/kg poids vif/jour par rapport à ses effets oestrogéniques, l'EFSA a estimé que l'accroissement d'exposition liée à la prise en compte des formes "modifiées" n'était pas suffisant pour remettre en cause les recommandations en vigueur pour les valeurs limites de ZEN en alimentation porcine.

La LOAEL (Lowest Observed Adverse Effect Level ; dose minimale avec effet nocif observé) établie pour le NIV chez le porc est de 100 µg/kg poids vif/jour (EFSA, 2013). L'estimation de l'exposition cumulée en NIV natif et en NIV converti à partir des formes "modifiées" représenterait 2-3% de cette valeur (Tableau 7). Sur cette base, l'EFSA a estimé que la prise en compte des formes "modifiées" de NIV n'était pas de nature à remettre en cause la sécurité des aliments pour porc.

Tableau 7 - Estimations d'exposition des porcs à certaines mycotoxines (cumul des formes "natives" et "modifiées") selon deux hypothèses (h) haute et basse (adapté de EFSA, 2014)

Catégories d'âge	Poids vif, kg	Ingéré alimentaire, kg/jour	Niveau d'exposition, µg/kg poids vif/jour							
			ZEN "natif" &"modifié"		NIV "natif" &"modifié"		Toxines T2 + HT2 "natif" &"modifié"		Fumonisines "natif" &"modifié"	
			h basse	h haute	h basse	h haute	h basse	h haute	h basse	h haute
Porcelet	20	1	0,7	1	0,53	2,07	0,3	1,43	3,7	10,3
Porc à l'engraissement	100	3	0,6	0,9	0,31	1,21	0,31	0,96	7,4	11,1
Truie	200	6	2,2	2,5	0,32	1,26	0,33	0,92	4,6	11,9

Abréviations: voir tableaux 1, 3 et 4.

En partant des hypothèses les plus pessimistes, l'exposition cumulée en T2 et HT2, et en leur formes "modifiées" correspondrait à 9% de la LOAEL de groupe établie à 29 µg/kg poids vif/j pour ces trichothécènes du groupe A (EFSA, 2011). Sur cette base, l'EFSA a estimé que la prise en compte des formes "modifiées" de T2 et HT2 également, n'était pas de nature à remettre en cause le niveau de sécurité des aliments pour porc pour ces mycotoxines.

Dans les hypothèses hautes, l'exposition cumulée aux fumonisines "natives" et à leurs formes "modifiées" représenterait 25% de la LOAEL établie par l'EFSA en 2005 à 200 µg/kg poids vif/j. Pour ces mycotoxines également, la sécurité des aliments pour porc ne semble donc pas remise en cause.

En résumé, l'EFSA a rendu un avis concernant quatre mycotoxines ou groupes de mycotoxines (ZEN, NIV, T2- HT2 et fumonisines) pour lesquelles aucune préoccupation nouvelle ne semble émerger suite à la prise en compte de l'exposition aux formes "modifiées". Un avis sur le DON et ses formes "modifiées" est en cours de rédaction. De tels travaux de référence n'existent pas encore pour les aflatoxines et les ochratoxines qui sont deux familles des mycotoxines faisant respectivement l'objet d'une réglementation et d'une recommandation en alimentation du porc.

CONCLUSION

Les avancées en techniques analytiques et en toxicologie contribuent à augmenter les connaissances sur les dangers chimiques dans l'aliment. Elles ont mis en évidence de nouvelles formes de mycotoxines. Cependant, l'impact de ces mycotoxines "modifiées" et "masquées" sur la santé est encore peu connu. A l'heure actuelle, seulement quelques données *in vitro* et *in vivo* sont disponibles sur la métabolisation de ces formes "modifiées" au sein de l'animal. De plus amples études *in vivo* sur la biodisponibilité et sur la toxicité de ces mycotoxines "modifiées" chez l'animal seront nécessaires pour mieux comprendre leur impact sur la santé du porc et sur la santé des consommateurs.

La proportion de mycotoxines "modifiées" dans l'aliment et leur toxicité sont généralement inférieures à celles de leur forme "native". Ce qui veut dire que les effets toxiques observés suite à une contamination sont principalement dus aux mycotoxines "natives". Cependant, la possible hydrolyse des mycotoxines "modifiées" au niveau de l'intestin pose le problème d'une exposition totale augmentée, avec, si ces formes "modifiées" ne sont pas prise en compte, un risque sous-estimé pour l'animal.

Plusieurs mycotoxines "modifiées" sont retransformées en la mycotoxine "native". Pour cette raison il est important de prendre en considération les mycotoxines "modifiées" lors du calcul de l'exposition, alors qu'actuellement seules les mycotoxines "natives" sont prises en compte dans la réglementation. Les mycotoxines "modifiées" peuvent être en proportion importante dans les céréales par rapport à leur forme "native". Par exemple, les formes glucosylées comme le D3G peuvent aller jusqu'à 30% de la proportion de la mycotoxine "native". Les travaux récents de l'EFSA tendent à considérer la somme des mycotoxines "natives" et "modifiées" pour calculer les risques liés à l'exposition aux mycotoxines.

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Translated Version (English)

« Masked » mycotoxins : new risk in porcine production ?

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Les mycotoxines "masquées" : un nouveau risque en production porcine ?

Les mycotoxines sont des métabolites secondaires de moisissures qui peuvent contaminer différentes céréales et par conséquent l'alimentation du porc. Au niveau européen, des réglementations et des recommandations pour l'alimentation animale ont été édictées pour six mycotoxines dont la toxicité est documentée. Les avancées dans les techniques de détection ont permis de mettre en évidence des dérivés de ces mycotoxines "natives", appelés mycotoxines "modifiées" ou plus spécifiquement mycotoxines "masquées" lorsqu'elles sont issues d'une métabolisation par la plante.

Du fait de leur caractérisation récente, peu d'informations sont disponibles sur leur occurrence dans l'alimentation du porc et leur toxicité pour cette espèce. Les données préliminaires indiquent que ces toxines peuvent être présentes à de fortes concentrations dans les aliments. Le porc pourrait être une espèce cible également pour ces "nouvelles" mycotoxines, du fait de sa grande sensibilité à la présence de mycotoxines conventionnelles, et à son régime alimentaire composé en grande partie de céréales. Ces mycotoxines "modifiées" peuvent augmenter la somme de mycotoxines auquel le porc est exposé, si elles sont hydrolysées dans l'organisme de l'animal.

Cette revue recense les connaissances actuelles sur la toxicité des formes "modifiées" du déoxynivalénol, des toxines T2 et HT2, de la zéaralenone, de la fumonisine et de l'ochratoxine A pouvant se retrouver dans l'alimentation du porc. Nous nous attacherons à comparer le métabolisme et la toxicité des formes "modifiées" à celle de leurs précurseurs et à analyser la possible reconversion de ces formes "modifiées" par la flore intestinale ou les voies de métabolisation du porc.

Masked mycotoxins: a new risk in pig production?

Mycotoxins are secondary metabolites originating from mold, which contaminate many cereals and their by-products and so can be found in the pig's diet. Some recommendations and regulations for animal feed have been decreed in the EU for six mycotoxins for which the toxicity is well known. Recent detection methods have revealed new mycotoxins and new molecules that are derivates of these mycotoxins. They were originally called "Masked" mycotoxins because they are not detected by conventional analytical methods. Then, they are more generally called "Modified", and "masked" when they are metabolized by the plant.

So because of the difficulty in detecting them, there is little information about the toxicity of these molecules and they are not included in the current regulation on mycotoxin contamination in pig feed. Moreover, a high proportion of these modified mycotoxins can be found in co-contamination with the mycotoxins. Pigs are really sensitive to mycotoxins, and their high cereal-rich diet means that they are highly susceptible to mycotoxins and to these modified mycotoxins. These modified mycotoxins can potentially increase the amount of mycotoxins to which pigs are exposed if they are hydrolyzed in the animal.

This review summarizes recent knowledge about the toxicity of the modified mycotoxins of deoxynivalenol, T2 and HT2 toxins, zearalenone, fumonisin and ochratoxin A, and presents recent studies about the metabolization and toxic effects on the animals of these modified mycotoxins, and their potential impact on their health.

INTRODUCTION

Nowadays, contamination by fungi cannot be avoided. Ecological conditions allowing the development of fungi (bad weather, humidity, high heat, plant sensitivity…), makes the management of the contamination of raw materials difficult. During a fungi contamination, mycotoxins, which are secondary metabolites, are produced and found into the seeds. These toxins are present in numerous cereals and by-products. A survey realized on 1100 different samples of animals' feed show that about 70% of the samples are contaminated (Streit et al. 2013).

The presence of these mycotoxins is considered as a serious threat to the health (Bryden, 2007; Wild and Gong 2010). The syndromes caused by the ingestion of high or medium doses of mycotoxins are well characterized and can go from acute mortality to reduced growth or problems in reproduction (Bryden, 2012). Consuming smaller amounts of toxins can lead to an alteration in the immune response and decrease the resistance to infectious diseases (Oswald, 2007). Some mycotoxins have an acute toxicity (a single exposure at a high dose) that is very strong, but it is exceptional in Europe being exposed to toxic doses in a single ingestion of contaminated food. Chronic effects (repeated exposure to low or very low doses) are the most feared due to the repetitive diet of the animals and because of the persistence of these toxins that are often resistant to high temperatures and technological processes used in the animal feed industry.

Recent advances in analytical methods have revealed new forms of mycotoxins and "masked" mycotoxins, not detected by conventional analytical methods. Currently, only "native" mycotoxins are regulated and taken into account in the calculation of the total exposure in raw or processed food. Actually, only few data are available on these new molecules where the risk to underestimate the toxicity induced by these molecules is not included in the regulation. Therefore, it becomes important to better know the risk that these "masked" mycotoxins can pose to humans and animals.

After the presentation of the conventional mycotoxins usually detected, this review provides a summary of current knowledge on "masked" mycotoxins, their identity, their occurrence, their metabolism and toxicity. It concludes on the potential danger that these "masked" toxins can represent on the health of the pig.

1. THE MYCOTOXINS REGULATED IN PIG'S FEED

In animal feed, only aflatoxins (AF) are regulated in Europe. There are some recommendations (Table 1) for five other toxins, which occur regularly and which are known to be toxic to swine. There are the ochratoxin A (OTA), the deoxynivalenol (DON), the toxins T2 and HT2, the fumonisins (FB1, FB2) and the zearalenone (ZEN) (Bennett et Klich, 2003).

Table 1 – Reglementation and recommendation of mycotoxins in pig feed: different type of feed and maximum levels
(EC Directive 2002/32/EC, et EC Recommandations 2006/576/EC et 2013/165/EU) (adapted from Stoev, 2014).

Mycotoxins[1]	**Feed**	**Maximum levels, mg/kg of feed**
AFB1+ B2	Cereals for animal	60
	Complete food for pig, horse, rabbit and pets	0,5
OTA	Complete food and dietary supplement for pig	0,05
DON	Cereals for animals (excepted by-product of maize)	8 (12)
	Complete food and dietary supplement for pig	0,9
ZEN	Cereals for animals (excepted by-product of maize)	2 (3)
	Complete food and dietary supplement : -for piglet and young saw -for saw and feeder pig	 0,1 0,25
FB_1+FB_2	Cereals for animal	60
	Complete food and dietary supplement for pig, horse and rabbit	5
T2+HT2	Complete food and dietary supplement for animals : -product oat milling -other grain products -complete feed excepted for cat	 1 0,5 0,25

[1]*Abbreviations : Aflatoxin B1 (AFB1), Aflatoxin B2 (AFB2), Ochratoxin A (OTA), Deoxynivalenol (DON), Zearalenone (ZEN), Fumonisin B1 (FB1), Fumonisin B2 (FB2), Toxin T2 (T2) and toxin HT2 (HT2).*

These molecules belongs to different families of mycotoxins, with various chemical structures, and so various toxic effects on pig. The dose, time of exposure, the specie, the age and the status of the animal (Bryden, 2007; Wild, 2007).

The Table 2 lists the major known effects of these mycotoxins on pig health.

AF are quickly absorbed and metabolized in the liver by the microsomal system which actives or modifies the metabolites (Riley, 1998; Haschek et al., 2002). AF alters the global immune response (innate and cellular) in pigs (Meissonnier et al., 2006).

OTA is mainly toxic to the liver and kidneys and causes kidney diseases in pigs. OTA affects the renal proximal tubule (Krogh, 1987; Marquardt and Frohlich, 1992). Moreover OTA acquires a genotoxic effect after its metabolization in the body (Ash et al., 2004; Pfohl-Leszkowicz and Manderville, 2007; Steyn et al., 2009). DON is the most common trichothecenes B. Pig is very sensitive to this mycotoxin, which can induce at low concentration feed refusal, and in higher concentrations vomiting (Haschek et al., 2002). Chronicle doses of DON (low concentrations on the long term), induce in pigs weight loss, anorexia, immunomodulation and a modification of the intestinal barrier function (Trenholm et al, 1984;. And Rotter al., 1996; Haschek et al., 2002; Pinton, Oswald, 2014). Toxins T2 and HT2 that belong to the family of trichothecenes A have similar effects but more pronounced than Trichothecenes B. They induce irritation to the gastrointestinal tract and skin, and they increase the sensitivity of the animal disease (Bryden, 2012).

Fumonisins are constituted of 12 compounds including fumonisin B1 (FB1), which is the most toxic and most studied. Fumonisins induce multiple toxic effects on animals with a known carcinogenic effect. In pigs, the FB1 affects the specific and the humoral responses by altering the balance of helper T cells, TH1 / TH2 (Taranu et al., 2005; Marin et al., 2006)

FB1 induced pulmonary edema in pigs (Haschek et al., 2002). Zearalenone (ZEN) has a significant effect on reproduction and fertility especially in swine. The α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), from the reduction of ketones by ZEN-reductase of the host, are non-steroidal estrogens that induce estrogenic activity in the animal (Fink-Gremmels and Malekinejad, 2007). ZEN and its derivatives cause redness and swelling of the vulva, vaginal prolapse and sometimes rectal prolapse in sows. In young sows, they can induce a significant swelling of the vulva (Gaumy et al., 2001).

Table 2- Associated effects to mycotoxins' presence in pig feed

Effects[2]	Mycotoxins[1]					
	AFB_1 AFB_2	**OTA**	**DON**	**T2 HT2**	**FB_1 FB_2**	**ZEN**
Anorexia	+	+	+++	+++	+	
Growth	+++	+	+++	++	+	
Hepatotoxicity	+++	+			++	
Nephrotoxicity		+++			+	
Abortion					+	++
Infertility						+++
Vulvovaginite						+++
Pulmonary oedema					+++	
Immuno-modulation	+++		++	++	+++	+

[1]*Abbreviations : Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Ochratoxine A (OTA), Deoxynivalenol (DON), Zearalenone (ZEN), Fumonisin B1 (FB1), Fumonisin B2 (FB2), Toxin T2 (T2) and toxin HT2 (HT2).*

[2]*+, ++, +++ : low effect, middle, and high of mycotoxin (s) on the parameter studied*

2. « MODIFIED» MYCOTOXINS IN PIG FEED

2.1. Presentation

New analytical methods allowed putting in evidence new secondary metabolites and some molecules derivate from these mycotoxins. The term of "masked" mycotoxins" was introduced in 1990 by Gareis to describe a glucoside zearalenone not detected during routine analysis, but hydrolyzed during digestion (Gareis *et al.*, 1990).

Figure 1 - Systematic definition of "modified mycotoxins" (Rychlik et al. 2014)

1st level	2nd level	3rd level	4th level	Example
Free mycotoxins				DON, Aflatoxin B_1, 3-acetyl-DON, 15-acetyl-DON
Matrix-associated mycotoxins	Complexes, physically dissolved or trapped			
	Covalently bound			Fumonisines bound to starch, OTA- and DON-oligosaccharides
Modified mycotoxins	Biologically modified	Functionalised (phase 1–metabolites)		Aflatoxin B_1-epoxide
		Conjugated (phase 2 – metabolites)	Conjugated by plants (= masked according to ILSI)	DON-3-glucoside
			Conjugated by animals	DON-3/8/15-glucuronide, HT2-3/4-glucuronide
			Conjugated by fungi	ZEN-14-sulfate
		Differently modified		Deepoxy-DON (=DOM-1)
	Chemically modified	Thermally formed		norDON A-C, N-carboxy-methyl-FB_1, 14-(R)-OTA
		Non-thermally formed		DON-sulfonate, norDON A-C (under alkaline conditions)

Indeed, different changes can occur in the structure of mycotoxin, which make them undetectable by conventional analytical techniques (Table 3). There are biological changes (did by a plant, fungus or animal body) or chemical ones such as the ones caused during thermal food processing methods.

The name of "masked mycotoxin" has often been an ambiguous use, and recently some authors have proposed a more precise terminology for the various forms of mycotoxins (Berthiller et al, 2013;. Rychlik et al, 2014).

These authors have redefined the terminology of "masked mycotoxin" strictly and introduced the concept of "modified mycotoxins." Figure 1 shows for example, all the forms described for DON.

Mycotoxins called "native or free" correspond to the basic structures of mycotoxins formed by molds. Most likely to be found in the pig supply is DON, ZEN, fumonisin, aflatoxin and OTA.

Matrix-associated mycotoxins correspond to the "native" mycotoxins bound to a matrix, i.e. physically dissolved and / or trapped and / or forming a covalent bond with the matrix. Thus, Fumonisins are able to bind to polysaccharides or proteins by their two tricarballyliques acids chains, thus forming the hidden fumonisins (hidden F) or linked with starch (F related to starch) (Seefelder et al., 2003).

Excepted these binding phenomena in a matrix, "native" mycotoxins can undergo biological or purely chemical transformations. The term "**modified mycotoxin**" was proposed to describe any biological or chemical modification of the chemical structure of a "native" mycotoxin (Rychlik et al., 2014).

"**Biologically modified**" mycotoxins indicate compounds derived from biotransformation in an animal body, plant or a mold. Biotransformation are divided into two main types: Phase I reactions (oxidation, reduction or hydrolysis) and phase II reactions (conjugation).

Generally, biotransformation allows detoxification of toxics, for example in facilitating their excretion. However in some cases, it can lead to a more toxic molecule than the parent compound. This is for example the case of aflatoxin B1-epoxy which is derived from the oxidation of AFB1 by cytochromes P450 during the biotransformation reactions of stage I in animals. Glucuronide forms (DON3-GlcA, ZEN14-GlcA, T2-GlcA, HT2-3 / 4-GlcA) come from the Phase II biotransformation of the "native" mycotoxins corresponding by the animal,

and represent examples of mycotoxins called "**biologically modified - conjugated**". They correspond to the excretion of the native mycotoxins in animal body.

DON-3-β-D-glucopyranoside (D3G) and zearalenone-14-β-D-glucopyranoside (ZEN14G) are issued from DON or ZEN respectively after the phase II biotransformation of metabolization by plant. By convention, the terminology of "**masked mycotoxin**" was reserved only for the "**biologically modified**" mycotoxins from the conjugation reaction in a plant (Berthiller et al., 2013).

At present, the four major "hidden" mycotoxins in the strict sense are the ZEN14G, the D3G, T2 toxin-glucoside (T2-Glc) and HT2 toxin-glucoside (HT2-Glc) (Lattanzio et al. 2012). It is interesting to note the case of the acetylated derivative of 3ADON, an acetylated derivative of DON. This compound can be produced both by the fungus, in this case it is a "native" mycotoxin, and by transgenic varieties of rice, wheat and barley expressing the gene of the 3-O-acetyltransferase, and therefore considered as a "hidden"mycotoxin. Gene transfer of the 3-O-acetyltransferase to plants is a promising strategy to reduce the pathogenicity of Fusarium that affect some plant species. Indeed, it's established that the conversion of DON into 3ADON by the plant can limit the aggressiveness of Fusarium (Karlovsky, 2011).

Table 3- Major « modified » mycotoxins (adapted from Broaekart et al., 2015)

"Native"mycotoxin	**"Modified" mycotoxin**
Deoxynivalenol	15-acétyl-DON (15ADON) 3-acétyl-DON (3ADON) DON-3-O-glucoside (DON3O-Glc) DON-3/8/15-glucuronide (DON3/8/15-GlcA) 3-acetyl-DON-glucuronide (3ADON-GlcA) **DON-3-β-D-glucopyranoside (D3G)** DON-oligosaccharides Deepoxy-DON (DOM-1) 3-epi-DON 9-hydroxylmethyl DON lactone Nor-DON A-F DON-sulfonate (DON-S)
Zearalenone	ZEN-14-glucuronide (ZEN14-GlcA) **ZEN-14-β-D-glucopyranoside (ZEN14G)** ZEN-14-sulfate (ZEN14S) α-zearalenol (α-ZEL) β-zearalenol (β-ZEL) α-zearalenol-14-α-D-glucopyranoside (α-

	ZEL14G) β-zearalenol-14-β-D-glucopyranoside (β-ZEL14G)
T2	T2-glucuronide (T2-GlcA) **T2-glucoside (T2-Glc)**
HT2	HT2-3/4-glucuronide (HT2-3/4-GlcA) **HT2-glucoside (HT2-Glc)**
Fumonisin	F N-(carboxymethyl) FB_1 N-(1-deoxy-D-fructos-1-µl) HFBx F-N-acetyl F-O-acetyl F cachées F liées à l'amidon
Aflatoxin	AFB_1-epoxide
Ochratoxin	OTA-oligossacharides 14-(R)-OTA 14-decarboxy-OTA

In bold: Masked mycotoxins in the strict sense

Other mycotoxins may be "biologically modified" by the action of a microorganism, and are grouped under the term of "mycotoxins **differently modified**". The Deepoxy-DON (DOM-1) and 3-epi-DON, resulting from the transformation of DON by bacteria extracted from human microbiota or animal, belong to this group (Eriksen et al., 2002; Karlovsky, 2011; Gratz et al., 2013).

"**Chemically modified**" mycotoxins are the last group. The chemical modifications may or may not depend from the heat. "**Chemically modified- thermally formed**" mycotoxins appear during food processes such as baking, roasting, freezing or extrusion. These thermo-dependent changes are known for many mycotoxins, in particular fumonisins capable of entering into a Maillard reaction, due to the reduction of sugars with the production for example of fumonisin B1 N- (1-deoxy D-Fructos-1-yl) and fumonisin N- (carboxymethyl) (Hmph and Voss, 2004).

We can also mention the derivatives of DON (Nordon A-F and 9-hydroxymethyl DON lactone) as thermal degradation products; some of which can be found in commercial food samples (Bretz et al., 2005). "**chemically modified – non-thermally formed**" mycotoxins are formed by different processes, including hydrolysis carried out with fumonisins (HFBx),

sulfation of DON leading to DON-sulfonate or the degradation products of ochratoxins by UV rays (Beyer et al, 2010;. Heydt-Schmidt et al, 2012.).

2.2. Occurrence of “natives” and “modified” mycotoxins

Some "modified" mycotoxins, particularly the "hidden" forms but also the “matrix-associated” forms and some chemically modified forms can be found in pig feed. Table 4 shows data of occurrence of major mycotoxins and their "modified" forms in cereal samples (wheat, barley, corn, oats and rice) over the period from 2010 till 2014.

The "native" mycotoxins represent the major part in food contamination. However, other forms are also concomitantly found in foods. It is currently possible to detect many "modified" mycotoxins, but few quantitative data are available, particularly because of a lack of analytical standards and reference materials.

Table 5 provides more information on the proportion of certain "modified» mycotoxins for which few data are available, compared to their "native" form. For some mycotoxins, such as T2-HT2-Glc and Glc, the occurrence data are from only one study. Their presence was reported for the first time in 2012 in wheat and oats naturally contaminated (Lattanzio et al, 2012).

For the D3G, discovered earlier, more data are available on its occurrence and its ratio to DON. The proportion of this "masked" mycotoxin is stable in food and corresponds, to almost, 20% of the DON present (Berthiller et al, 2009). However, the ratios vary depending on the cereal, genotype concerned, the country and the year of harvest and can increase up to 46%. (Berthiller et al, 2009; De Boevre et al, 2012). Also the increasing use of *Fusarium* resistant plants, able to glucosylate DON in D3G, could increase the ratio D3G / DON. Some studies of these resistant plants have even found up to 2.7 times more D3G present in the plant than DON (Sasanya et al, 2008).

Table 4 - Occurrence of trichothecenes and ZEN and occurrence of their "modified" forms in cereals (samples of wheat, barley, oat, maize and rice from different countries) (adapted from (Broekaert et al. 2015)

Mycotoxins[1]	**Number of samples**[2]	**Incidence, %**	**Mean, µg/kg of feed**
DON	5743	84	458
3ADON	2227	22	14,7
15ADON	686	31	36,6
D3G	529	55	85
NIV	3062	32	17,8
ZEN	2158	12	39,6
ZEN14G	36	25	37
ZEN14S	12	25	6
T2	321	45	16,7
HT2	321	54	61
T2-Glc	15	73	2,4
HT2-Glc	15	80	5,1

[1] *Abbreviations: Nivalenol (NIV), see also Tables 1 and 3. The "native" mycotoxins are in italics and "modified" mycotoxins in bold.*

[2]*Country of different grains analyzed: Austria, Belgium, China, Denmark, Finland, Italy, Nigeria, Norway, Czech Republic and Sweden.*

In terms of matrix- associated fumonisins (physically entrapped), their proportions compared to the ones of free fumonisins are more variable. Their presence has been shown after a hydrolysis step of raw materials (Dall'Asta et al., 2009). The proportion of physically trapped forms change according to the genotype of corn and according to the culture conditions (Dall'Asta et al., 2012).

In conclusion, more occurring data on different crops and in different countries are needed to properly assess the risk associated with the presence of these new mycotoxins.

2.3. Metabolization and toxicity of "modified" mycotoxins on pig

The occurrence of "modified" mycotoxins in feed and animal exposure to these new toxins raise a number of questions and the need to investigate the metabolism and toxicity of these compounds (EFSA, 2014). It is important to study the intrinsic toxicity of these toxins, but

also to know their metabolism and in particular to determine if "modified" mycotoxins are converted into their "native" forms.

Some recent studies were interested in the effects of these "modified" mycotoxins on the pig, on *in vitro* or *in vivo* models. Most of these studies focus on the metabolism of these molecules and few about their toxicity.

Table 5 - "Modified" mycotoxin proportion compared to their "native" mycotoxins in raw materials naturally contaminated

Raw materials	"Modified" mycotoxins[1]	Number of samples	Mycotoxins proportion "modified"/ "native", %	References
Wheat, Maize	D3G	77	20% up to 46%	(Berthiller et al. 2009)
Cereals	D3G	21	6-29%	(Desmarchelier and Seefelder 2011)
Maize, Wheat, Oat	D3G	11	up to 30%	De Boevre *et al.* (2012)
Maize	ZEN14S	41	up to 30%	(Streit et al. 2013)
Wheat	ZEN14G	10	up to 30%	(Scheneweis et al. 2002)
Wheat	T2-Glc, HT2-Glc	9	up to 12%	(Lattanzio et al. 2012)
Oat	T2-Glc, HT2-Glc	9	2%	(Lattanzio et al. 2012)
Maize	Fumonisins associated to matrix Fumonisins associated to matrix Fumonisins associated to matrix	31 97 120	up to 100% up to 60% j up to 60%	(Dall'Asta et al. 2010) (Dall'Asta et al. 2010) (Dall'Asta et al. 2012)

[1]*Abbreviations: see tables 1 et 3.*

2.3.1. Inherent toxicity of "modified" mycotoxins for pigs

Studies on the toxicity of "modified" mycotoxins mainly concern the "modified" forms of DON and ZEN. The majority of these studies were conducted *in vitro* on human cells and only a few are interested *in vivo* toxicity in animals, including mice and pigs.

The toxicity of DON were compared to those of its acetylated derivatives (3ADON and 15ADON) taking into account the cell proliferation, activation of MAPKs (mitogen-activated protein kinase) and expression of tight junction proteins as well that the expression of cytokines in pigs (Pinton et al., 2012). Percentages of cell viability of the pig intestinal cells (IPEC-1) incubated for 24 hours in the presence of DON, 3ADON and 15ADON were decreased to 60%, 13% and 69%. The expression of the junction proteins by these porcine intestinal cells is decreased by 40% in the presence of 15ADON, and is equivalent to the

DON and 3ADON. The 15ADON also showed more toxicity than DON and 3ADON on the activation of MAPKs, *in vitro* on IPEC-1 cells, *ex vivo* on explant cultures of porcine jejunum or *in vivo* in the tissue jejunal piglets (Pinton et al., 2012).

One of the known derivatives of DON is the DOM-1 coming from a bacterial transformation reducing the 12,13-epoxy group, which is essential in the toxicity of DON and trichothecenes in general (Schatzmayr et al., 2006; Zhou et al. 2008). Thus the DOM-1 is considered as a non-toxic metabolite of DON. An *in vitro* study showed that the DOM-1 was 54 times less toxic than DON level of DNA synthesis in mouse fibroblasts (Eriksen and Pettersson, 2004). The toxicity of DOM-1 on production parameters was evaluated in vivo in pigs. Animals receiving the contaminated food with DON 5mg / kg feed and the bacterial strain capable of deepoxydise DON, showed no decrease in food intake or weight gain (He et al., 1993; Li et al., 2011). However the *in vivo* evaluation of the pure DOM-1 toxicity on intestine and on the pig organism has not been evaluated.

Compared to DON, the D3G was nontoxic on pig jejunum *ex vivo* study with an inability to induce a D3G ribotoxic stress and to activate the MAPK pathway central, in the implementation of the observed pro-inflammatory response with DON (Pierron et al., 2016). This inability of D3G to induce a pro-inflammatory response, on the contrary to DON, is confirmed in the rat with an absence of an over-expression of cytokines and chemokines (Wu et al., 2014A). Moreover, the power of the emetic D3G seems much lower than that of DON (Wu et al., 2014b).

Several studies have compared estrogenic powers of the ZEN and its two derivatives α- ZEL and β-ZEL. Estrogenicity of these molecules thus ranks as follows: β-ZEL <ZEN <α-ZEL (Mukherjee et al, 2014.). At the cellular level, higher cytotoxicity and genotoxicity of β-ZEN compared to the α-ZEN were shown on endometrial pig cells (Tiemann et al., 2003; Othmen et al, 2008), while the α- ZEL was more toxic than the β-ZEL on pig oocytes (Alm et al., 2002). In summary, the hierarchy in the toxicity of these two "modified" mycotoxins, α-β-ZEL and ZEL, is not clearly established and it depends on the cell type considered. Overall, their toxicity is lower than ZEN.

Generally, glycosylated and sulfated forms of ZEN, as ZEN14G and ZEN14S, seem unable to bind to estrogen receptors and induce *in vitro* toxicity (Poppenberger et al., 2006; Berthiller et al., 2009). However, Plasencia and Mishra (1991) found in our study that ZEN4S was able to induce the same estrogenic activity than ZEN in the rat uterus enlargement bioassay.

Overall, the compounds from the detoxification pathways of plants, "masked" forms *sensu stricto* are less toxic or inactivated with respect to the "native" molecule. The metabolic pathways of the plant which are similar to those of animals (e.g. conjugation reactions to different molecules sulfate, glutathione or glucuronic acid), increase the polarity of these molecules, thereby facilitating their excretion and decreasing their toxicity (Yiannikouris and Jouany 2002; Homolya et al, 2003).

2.3.2. Metabolization of “modified” mycotoxins in pig

Questions about the conversion of "modified" mycotoxins into "native" mycotoxins are as old as the discovery of the first "modified" mycotoxin.

Very early, it has been shown that exposing orally a piglet for 2 weeks to the ZEN14G, it was possible to find in his urine and feces varying amounts of ZEN and its estrogenic metabolite α-ZEL (Gareis and al., 1990). This study highlights the fact that a significant part of the pig exposure to mycotoxin could come from the conversion of "modified" mycotoxins. Such conversion may be due to the activity of digestive enzymes and metabolism of the animal. It may also result from the enzymatic activity of the digestive flora followed by reabsorption of the "native" mycotoxin and / or its metabolites. Table 6 summarizes the features of some “modified” mycotoxin along the digestive tract and their hydrolyzed to “native” form.

Pig is physiologically very close to man, particularly in terms of gastrointestinal tract. In *an in vitro* system mimicking human in successive stages, the action of salivary juice, gastric juice, intestinal juice and bile, D3G, ZEN14G and ZEN14S are retained respectively to 99.5%, 97.3% and 98.6% (De Nijs et al, 2012;. Dall'Erta et al, 2013.). However, D3G that is not converted by the enzymes present in saliva and mammalian stomach, may be hydrolyzed by lactic acid produced by certain bacterial species such as *Enterococcus mundtii* and *Lactobacillus plantarum* present in the gastrointestinal tract (Berthiller et al., 2011).

Table 6 – Becoming of some "modified" mycotoxins along the digestive tract (adapted from De Boevre et al., 2015)[2]

Compartment	Mycotoxins[1]					
	D3G[3]	**3 ADON & 15ADON[3]**	**ZEA14G[3]**	**ZEA14S[4]**	**Hidden Fumonisins[4]**	**Bound Fumonisins[4]**
Saliva	*Stable*	*Stable*	*Stable*	*Stable*	*Stable*	*Stable*
Stomach	*Stable*	11%DON (3ADON) 13% DON (15ADON)	Max.19% ZEN	*Stable*	*Stable*	*Stable*
Small intestine	*Max. 5% D3G detected*	0% 3ADON 0% 15ADON	*Traces of ZEN14G detected*	*Stable*	*Stable*	*Stable*
Colon	*Max. 2% D3G detected* Feces : DON+DOM-1	Deacetylation + glucuronidation	Feces : 40% ZEN Feces : 60% catabolites	Feces : 40% ZEN Feces : 60% catabolites	Max.99% fumonisins	*Stable*

[1]*Abbreviations: see tables 1 and 3*

[2]*In italic:% of "modified" mycotoxin found after oral ingestion in different parts of the diigestive tract. In normal character: % of "modified" mycotoxin found in his 'native" form in the different parts of the digestive tract. "Traces" if the molecule is slightly transformed and "stable" if the molecule is not transformed in its"native" form in the compartment.*

[3]*Based on in vitro and in vivo experiments*

[4] *Based on in vivo experiments*

As for DON, "modified" forms of ZEN can be deconjugated after fermentation in presence of human feces, and so the "native" forms can be released (Dall'Erta et al, 2013). An increase of 30-50% of detectable fumonisins is observed, after food matrix digestion (Dall'Asta et al, 2010). This suggests that the gastrointestinal enzymes are capable of destroying the interactions between the matrix and fumonisin, salting-out forms of "native" mycotoxins. For the DOM-1, from a bacterial transformation, there is no *in vivo* study with pure DOM-1.

The epimerization forming the 3-epi-DON, is an irreversible reaction (Karlovsky, 2011). But currently there is no data available on its toxicity. In terms of acetylated forms of DON, they are quickly deacetylated in DON within the organism of animal (Wu et al., 2010).

These *in vitro* results therefore show that some "modified" mycotoxins can be transformed back into their "native" mycotoxins in variable proportions. This suggest that this transformation would be mainly due to intestinal microbiota.

One study looked at the becoming of DON or D3G orally and intravenously administered to piglets (Nagl et al., 2014). DON is excreted largely in the urine, mainly in the first eight post-exposure hours by mainly "native" form and to a minor form of DON-3-glucuronide (DON3-GlcA) and DON-15-glucuronide (DON15-GlcA). Urinary excretion of D3G administered orally, although a majority, appearing between the 8th and the 24th hour post-exposure. Only a tiny fraction of D3G was found unprocessed in the urine, most of which is converted into DON, and as well in DON3 GlcA-and-DON15 GlcA. In the case of D3G parenteral administered, most of the administered dose was recovered unchanged in the urine within the first eight hours. This study demonstrates that (i) the contaminant D3G pig feed can be converted to DON in the digestive tract, (ii) due to the delayed urinary excretion, this conversion takes place probably in the lower portion of the digestive tube, and (iii) the DON from the D3G of microbial hydrolysis may well be absorbed and contribute significantly to the total exposure of pig to DON.

3. ASSESSMENT OF THE EXPOSURE AND CHARACTERIZATION OF THE RISK FOR THE “MODIFIED” MYCOTOXINS IN PIG

In 2014, EFSA performed a work of assessing exposure of pig to certain "modified" mycotoxins and on the characterization of the risk associated to these molecules (EFSA, 2014). Mycotoxins concerned are ZEN, nivalenol (NIV) (a mycotoxin of the family of trichothecenes such as DON), T2 toxin and HT2 and fumonisins.

Exposure calculation traduces in cumulative exposure the increase of exposure of one mycotoxin due to the conversion of “modified” forms into “native” mycotoxin. As in human, the increase was estimated at 100% for ZEN, 30% for NIV, 10% for T2 and HT2, and 60% for fumonisins.

Table 7 presents the calculation of estimated exposure to these “modified” and “natives” mycotoxins.

Based on NOEL (No Observed effect Level) of ZEN fixed at 10µg/Kg BW per day for these estrogenic effects, EFSA has estimated that the increase exposure, linked to the “modified” forms, was not enough to overcome the regulation of ZEN in pig feed.

The LOAEL (Lowest Observed Adverse Effect Level) established for NIV in pigs is 100 µg/Kg BW/day (EFSA, 2013). The calculation of estimated exposure in NIV and NIV from the "modified" forms retransformed will represent 2-3% of the LOAEL (Table 7). So, EFSA estimated that the addition of "modified" forms of NIV, was not likely to jeopardize the safety of pig feed.

Table 7 - Pigs exposure estimation for certain mycotoxins ("native" accumulated forms and "modified") in two hypotheses (h) high and low (adapted from (EFSA 2014)[1]

Age	Weight, kg	Feed daily intake (Kg/day)	Level of exposure, µg/kg feed daily intake/day							
			ZEN "native" &"modified"		NIV "native" &"modified"		Toxins T2 + HT2 "native" &"modified"		Fumonisins "native" &"modified"	
			h low	h high	h low	h high	h low	h high	h low	h high
Piglet	20	1	0,7	1	0,53	2,07	0,3	1,43	3,7	10,3
Feeder Pig	100	3	0,6	0,9	0,31	1,21	0,31	0,96	7,4	11,1
Saw	200	6	2,2	2,5	0,32	1,26	0,33	0,92	4,6	11,9

[1]*Abbreviations: see tables 1, 3 and 4.*

Starting from the most pessimistic hypothesis, the cumulative exposure in T2 and HT2, and their "modified" forms correspond to 9% of the LOAEL group established at 29 µg/kg BW/day for these trichothecenes of group A (EFSA, 2011). On this basis, the EFSA considered that the inclusion of "modified" forms of T2 and HT2 also was not likely to jeopardize the level of food safety for pigs for these mycotoxins. In the highest hypothesis, cumulative exposure to "native" fumonisin and their "modified" forms represent 25% of the LOAEL established by EFSA in 2005 at 200 µg/kg BW/day. For these mycotoxins the safety of food for pigs does not appear compromised.

To sum up, the EFSA issued an opinion on four mycotoxins or groups of mycotoxins (ZEN, NIV, T2/HT2 and fumonisins) for which no new concern seem to emerge following the consideration of exposure to "modified" forms. One advice for DON and its "modified" forms is being written. Such advices of reference are not yet available for aflatoxins and ochratoxin, which are two families of mycotoxins respectively subject to regulation and recommendation in pig feed.

CONCLUSION

New methods in toxicology and in analysis allow to increase the knowledge on the hazards present in food and to put in evidence new types of mycotoxins. However, effects of these “modified” and “masked” mycotoxins on the health is not well known. Actually, only few studies *in vivo* and *in vitro* are available on the metabolization of these molecules into the animal. More studies are necessary to better understand their impact on the health of pigs and humans.

The percentage of “modified” mycotoxins are generally lower than the one of “native” mycotoxins which means that the observed toxic effects are mainly due to “native mycotoxins”. However, the feasible hydrolyze of “modified” mycotoxins in the intestine, could increase the total amount of mycotoxins to which pigs are exposed, and so minimize the real risk of exposure for the animal.

Many “modified” mycotoxins are retransformed into their “native” mycotoxins. For this reason, it’s important to take into account both “modified” and “natives” mycotoxins in the risk assessment of exposure. Actually, only “natives” mycotoxins are regulated, but “modified” forms can be found in important proportion in cereals. Indeed, for example, glucosylated forms, as D3G can reach in proportion 30% of the “native” mycotoxins.

The EFSA recent works tend to consider the amount of “natives” and “modified” mycotoxins in order to calculate the risk linked to the exposure to mycotoxins.

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AIMS OF THE THESIS

Aims of the thesis

The *Fusarium* mycotoxin deoxynivalenol (DON) is one of the most frequently widespread mycotoxin worldwide, in cereals and feed raw materials. DON is known for its toxic effects on animals, and causing big economic losses. Due to its high structural stability of the DON makes its elimination difficult, once present in cereals or feed materials. Several strategies were then developed to manage mycotoxins and DON contamination, like physical (cleaning, sieving), chemical (ammoniation) and biological (binding agents, feed additives) treatments.

Thereby, in the context of use of new strategies of detoxification, some metabolites are formed during the biological transformation of DON. In this context my work deals with the toxicity of biological degradation product of mycotoxins the deepoxy-deoxynivalenol (DOM-1) and the 3-epi-deoxynivalenol (3-epi-DON) from bacterial degradation and the deoxynivalenol-3-β-D-glucopyranoside (D3G) transformed into the plant.

The **general objective** of this thesis was to **assess the toxicity of three derivatives of DON: D3G, DOM-1 and 3-epi-DON**.

Indeed, one of the major questions associated with these metabolites of DON is related to their toxicity: are they toxic by themselves? Indeed, little is known about the toxicity, of these masked and modified forms of DON, compared to the extensively investigated DON. As part of this thesis, I assessed the toxicity of these DON derivatives with a special emphasis on DOM-1 with an *in vivo* experiment.

Intestine is the first exposed organs to xenobiotics or mycotoxins, present in food, and so constitutes the first barrier upon exposure of toxins and could be exposed to high level of mycotoxins (Rotter et al. 1996). Intestinal viability cells could be directly damage or it is the barrier function that can be impaired, and so promote the entry of contaminant in the blood and in all the organism. Thus, analysis focused on intestinal tissue.

Moreover, DON is well known for its immunomodulatory effects and for its great impact on the intestine, the first organ targeted following the ingestion of mycotoxins. DOM-1, 3-epi-DON and D3G derivatives of DON may also have an impact on these two functions. Therefore, it becomes important to know whether these transformations of DON will lead to less toxic molecules for animal or whether they will induce other effect on the animal.

The **specific aims** of this research were:

- **to assess the toxicity of the masked form of DON, D3G, and to determine its molecular mode of action using *in vitro, ex vivo* and *in silico* approaches** (Chapter I, Part 1).
- **to assess the toxicity of two microbial derivatives of DON, DOM-1 and 3-epi-DON and to determine their mode of action using *in vitro, ex vivo* and *in silico* models. In the case of DOM-1, *in vivo* experiment was also performed to assess its toxicity and anorectic effect in piglets** (Chapter I, Part 2 & Chapter II).
- **as DON was always included as a positive control, the experiments allowed us to get more insight on intestinal toxicity of DON, especially at the transcriptional level** (Chapter I).

The aim of this thesis was to assess the toxicity of several DON derivatives, formed either in the plant (D3G) or after microbial transformation (DOM-1 and 3epiDON). D3G is formed by a natural way of defense in plant, aiming to manage xenobiotics and their excretion. Microbial transformation, is the use of isolated and stabilized microorganism able to transform mycotoxin.

The use of *in silico, in vitro,* and *ex vivo* models, allow the comparison of the intestinal and immune toxicity of D3G, DOM-1 and 3-epi-DON to the one of DON. *In silico* analysis allowed to draw molecules in three dimensions, to determine the interaction of the molecules and the ribosome and to understand the molecular mechanism of toxicity. *In vitro* model using cell line is a powerful enabling tool for the exploration of fundamental questions regarding cell and the study of drug delivery dynamics and kinetics.

To study the toxicity of DON and its derivatives on intestinal epithelial cells, the choice was to use the human intestinal cell line Caco-2. It has advantages to express characteristics markers of adults' intestinal cells and to be a reference line in toxicology studies, and to be largely used to study intestinal absorption (Sambruy et al. 2001). The explant model was also chose to enable directly testing the toxicity of molecules on intestinal living tissue keeping its polarity and extracellular integrity (Gonzalez-Vallina et al. 1996). Moreover, in the context of 3R, it limits the use of animals, and to test many conditions with limited number of animals. *In vivo* pig model allowed studying more deeply the comparative toxicity of DON and DOM-

1, on the intestine and on the immune response. Therefore in the context of risk assessment, *in vivo* experiment allows to investigate the metabolism of modified mycotoxin into the animal.

Experimental animal phases were conducted on pigs at the ToxAlim laboratory. From an agronomic point of view, breeding a monogastric animal such a pig is particularly vulnerable to mycotoxins due to the importance of share of cereals in its diet. In addition, pig is very sensitive to mycotoxins, due to the absence of ruminal tank, known to contain microorganisms capable of degrading toxins before their intestinal absorption. Finally, considering the similarity in the immune and digestive systems of pigs and humans, the use of pig model permit also to extrapolate data to man. For the choice of doses and time of exposure, this work fits in the current dynamic of a toxicological study, for chronical exposure going from low doses to moderate ones, not inducing major clinical manifestations. Finally, performing microarray analysis on all the genome of the pig allowed identifying genes and biological pathways impacted by DON as well as demonstrating the absence of toxicity of the DON derivatives.

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EXPERIMENTAL STUDIES

CHAPTER I

Chapter I

Part 1: Intestinal toxicity of the masked mycotoxin DON-3-glucoside (D3G)

Arch Toxicol
DOI 10.1007/s00204-015-1592-8

BIOLOGICS

Intestinal toxicity of the masked mycotoxin deoxynivalenol-3-β-D-glucoside

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Received: 5 June 2015 / Accepted: 31 August 2015

Abstract Natural food contaminants such as mycotoxins are an important problem for human health. Deoxynivalenol (DON) is one of the most common mycotoxins detected in cereals and grains. Its toxicological effects mainly concern the immune system and the gastrointestinal tract. This toxin is a potent ribotoxic stressor leading to MAP kinase activation and inflammatory response. DON frequently co-occurs with its glucosylated form, the masked mycotoxin deoxynivalenol-3-β-D-glucoside (D3G). The toxicity of this later compound remains unknown in mammals. This study aimed to assess the ability of D3G to elicit a ribotoxic stress and to induce intestinal toxicity. The toxicity of D3G and DON (0–10 µM) was studied in vitro, on the human intestinal Caco-2 cell line, and ex vivo, on porcine jejunal explants. First, an in silico analysis revealed that D3G, contrary to DON, was unable to bind to the A-site of the ribosome peptidyl transferase center, the main targets for DON toxicity. Accordingly, D3G did not activate JNK and P38 MAPKs in treated Caco-2 cells and did not alter viability and barrier function on cells, as measured by the trans-epithelial electrical resistance. Treatment of intestinal explants for 4 h with 10 µM DON induced morphological lesions and up-regulated the expression of pro-inflammatory cytokines as measured by qPCR and pan-genomic microarray analysis. By contrast, expression profile of D3G-treated explants was similar to that of controls, and these explants did not show histomorphology alteration. In conclusion, our data demonstrated that glucosylation of DON suppresses its ability to bind to the ribosome and decreases its intestinal toxicity.

Keywords Glucosylation · Trichothecenes · Modified mycotoxins · Gut · *Fusarium* · Wheat

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Electronic supplementary material The online version of this article (doi:10.1007/s00204-015-1592-8) contains supplementary material, which is available to authorized users.

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Published online: 24 September 2015

Introduction

Mycotoxin contamination is a worldwide problem. Mycotoxins are toxic secondary metabolites produced by various molds, such as *Aspergillus*, *Penicillium or Fusarium* (Bennett and Klich 2003). Crops, at all stages of the food chain, are frequently contaminated by these fungi and so by mycotoxins. Worldwide surveys indicate that 72 % of all agricultural commodities are contaminated with mycotoxins (Streit et al. 2013).

Among mycotoxins, deoxynivalenol (DON) produced by *Fusarium* spp. is commonly detected in cereals and grains, particularly in wheat, barley, maize and their by-products. It is one of the most prevalent mycotoxins in food and feed (EFSA 2013). The toxicity of DON on mammals is well documented. Acute exposure induces abdominal pain, anemia, diarrhea, vomiting and circulatory shock that can result in death (Pestka 2010; Rocha et al. 2005). At chronic low doses, DON induces anorexia, growth retardation and causes neuroendocrine changes and immune modulation (Pestka and Smolinski 2005). Molecular mechanisms of action of DON are well understood. DON binds to the A-site of the peptidyl transferase center of the 60S subunit of ribosomes in eukaryotic cells and interferes with protein translation (Garreau de Loubresse et al. 2014; Pestka 2010). A ribotoxic stress response is then induced and results in the activation of mitogen-activated protein kinases (MAPKs). These signaling pathways are involved in regulation of multiple biological processes including development, apoptosis and immunity (Joshi and Platanias 2012). Upon DON exposure, MAPK activation mediates apoptosis and aberrant up-regulation of pro-inflammatory cytokines and chemokines (Pestka 2010; Wu et al. 2014a).

Upon ingestion of contaminated food, the gastrointestinal tract is particularly impacted by DON. This toxin alters the intestinal histomorphology, barrier function and nutrient absorption (Ghareeb et al. 2015; Maresca 2013; Pinton et al. 2012). DON alters the integrity of the intestine, resulting in a loss of mucosal integrity and a translocation of commensal and pathogenic bacteria across the epithelium (Maresca 2013; Pinton and Oswald 2014). DON also disturbs the intestinal immune responses: The toxin induces the local production of pro-inflammatory cytokines and potentiates existing intestinal inflammatory processes (Cano et al. 2013; Van De Walle et al. 2010; Vandenbroucke et al. 2011).

Associated with DON, DON-3-β-D-glucoside (D3G) is a "masked" mycotoxin produced from the enzymatic conjugation of glucose to DON in the plant (Berthiller et al. 2005; Poppenberger et al. 2003). The glucosylation reaction of DON is considered as an important detoxification mechanism occurring in plants to protect against *Fusarium*-related diseases (Lemmens et al. 2005). Thanks to the improvement in detection methods for mycotoxins, many publications investigated D3G contamination in recent years (Berthiller et al. 2013). D3G was found in cereals and cereal products all over the world at concentrations that can reach nearly half that of DON (Berthiller et al. 2009; De Boevre et al. 2012; Malachova et al. 2011).

Despite its frequent occurrence, the toxicity of D3G remains largely unknown. To date, only one group investigated the toxicity of D3G in mammals (Wu et al. 2014a). The authors observed that D3G was ineffective in evoking splenic cytokine or chemokine mRNA responses. Because D3G is poorly absorbed, the intestine can be a target for this toxin (De Nijs et al. 2012; Nagl et al. 2012, 2014).

The purpose of this study was to investigate the toxicity of D3G on the intestine. An in silico analysis indicates that D3G is unable to bind to the ribosome and suggests a lack of toxicity of this compound. The toxicity of D3G was further assessed on intestinal cells and explants; physiological, morphological and transcriptomic analysis confirmed the absence of adverse effects of D3G. Our paper provides the first insight into the toxicity of this plant conjugate on the small intestine.

Materials and methods [additional data are available in Online resource (Online resource 1)]

Toxins

Purified DON was purchased from Sigma-Aldrich (St Louis, MO, USA). D3G was isolated from DON-treated wheat (Berthiller et al. 2005) or enzymatically produced from DON (Michlmayr et al. in preparation). Mycotoxins were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at −20 °C until use.

Caco-2 cell culture and analysis

Caco-2 cells were maintained in Dulbecco's modified Eagle medium enriched with glutamine (Gibco, Cergy-Pontoise, France) supplemented with 10 % heat-inactivated fetal bovine serum, 1 % nonessential amino acids (Sigma-Aldrich) and 0.5 % gentamicin (Eurobio, Courtaboeuf, France). Caco-2 cells were spontaneously differentiated from confluent monolayer after 21 days of culture with medium changed every 2 days (Sambuy et al. 2005; Pinton et al. 2012).

Cell viability was assessed on proliferating or differentiated cells seeded 96-well microtiter plates using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, USA) according to manufacturer's instructions. Trans-epithelial electrical resistance (TEER) was measured on differentiated cells with a Millicell-ERS voltohmmeter

(Millipore, Saint-Quentin-en-Yvelines, France) as already described (Pinton et al. 2012). Expression of non-phosphorylated and phosphorylated MAPK P38 and JNK was assessed on differentiated cells by immunoblotting (Meissonnier et al. 2008) using specific rabbit antibodies (Pinton et al. 2012). The expression of the proteins was estimated after normalization to GAPDH.

Intestinal jejunal explants, preparation and analysis

Jejunal explants were obtained from 5-week-old crossbred castrated male piglets as described previously (Kolf-Clauw et al. 2009). They were treated for 4 h with toxins (DON or D3G) or diluent (DMSO) and fixed in 10 % formalin for histological analysis or stored at −80 °C for RNA extraction.

For histological analysis, explants were embedded in paraffin wax; sections (5 µm) were stained with hematoxylin and eosin for histopathological evaluation (Bracarense et al. 2012).

For gene expression analysis, total RNA was extracted in lysing matrix D tubes (MP Biomedicals, Illkirch, France) containing guanidine thiocyanate–acid phenol (Eurobio). Quality of these samples was assessed (Agilent RNA 6000 Nano Kit Quick, Agilent Bioanalyzer 2100); the mean RNA integrity number (RIN) of these mRNAs was 6.32 ± 0.83.

Reverse transcription and RT-qPCR steps were performed as already described (Halloy et al. 2005; Gourbeyre et al. 2015). Primers are indicated in Table (Online resource 2). Amplification efficiency and initial fluorescence were determined by the ΔCt method. Obtained values were normalized using two reference genes, ribosomal protein L32 (RPL32) and cyclophilin A. mRNA expression levels were expressed relative to the mean of the control.

The 60 K microarray (Voillet et al. 2014) was used as described in Supplemental Material (see Supplemental Material, Part Methods). The experimental details are available in the Gene Expression Omnibus (GEO) database under accession GSE66918 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=olixoosedvmdnqr&acc=GSE66918). Microarray data from Feature Extraction software was analyzed with R using Bioconductor packages.

Statistical analysis

The Fisher test on equality of variances, one-way ANOVA and Bonferroni's post hoc test were used to compare the effect of DON and D3G on Caco-2 cells and explants; p values <0.05 were considered significant.

For microarray data, statistical analysis was performed with R 3.0.1 software.

Results

In silico analysis of the interaction of DON and D3G with ribosome

A recent paper shows that DON binds to the 60S subunit inside the A-site of the peptidyl transfer center of yeast ribosome (Garreau de Loubresse et al. 2014). The crystallographic data (4UJX) were used to compare the ability of DON and D3G to interact with the ribosome (Fig. 1, Online resources 3 and 4). DON is able to fit in the pocket of the A-site of the ribosome 60S subunit. In this position, the 3-hydroxyl group of DON is associated with a magnesium atom and stabilized by other nucleotides. The alignment of the common backbone of D3G and DON allows us to generate a model of interaction with the ribosome and to study the sterical hindrance of glucoside inside the A-site pocket. Even in the absence of the magnesium atom, the size of the pocket is too small for the glucosyl group, showing that D3G cannot sterically bind to the ribosome 60S subunit A-site pocket.

Inability of D3G to activate MAPK

As other ribosome-binding translational inhibitors, DON rapidly activates MAPK. As expected (Pinton et al. 2012), 1 h of exposure of differentiated Caco-2 monolayers to 10 µM DON significantly increased the expression of phosphorylated p38 and phosphorylated JNK but did not change the expression of the non-phosphorylated MAPK. By contrast, the activation of these MAPKs upon treatment with D3G was similar to untreated control monolayers (Fig. 2a). Taken together, our data indicate that D3G does not bind to ribosome and does not activate MAPK. The lack of MAPK activation is indicative of an absence of toxicity of D3G and was further tested.

D3G does not impair cell viability and barrier function of human intestinal cells

Comparative toxicity of DON and D3G was evaluated both on proliferating (Fig. 3) and differentiated (Fig. 2b, c) Caco-2 human cells. After 48 h of exposure to DON, the viability of the proliferating cells was decreased in a dose-dependent manner, and 50 % inhibition was observed at 1.30 µM DON. Exposure to 10 µM DON for 48 h reduced cell viability by approximately 80 %. By contrast, no cytotoxicity was observed on Caco-2 cells treated with 0–10 µM D3G (Fig. 3) or higher concentrations (up to 100 µM, data not shown).

The comparative toxicity of D3G and DON was also performed on differentiated Caco-2 cells. As already described

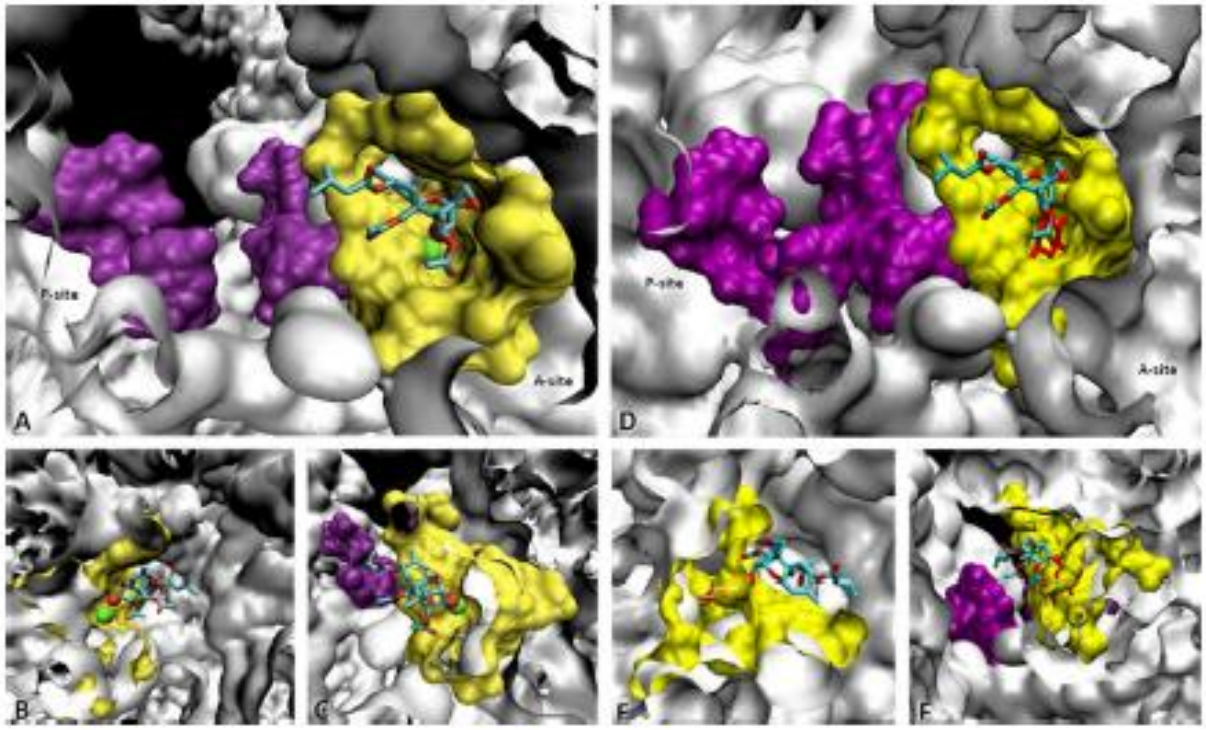

Fig. 1 Interaction between the ribosome 60S subunit binding site and DON or D3G (*front view* **a**, **d**, *right orthogonal view* **b**, **e**, *left orthogonal view* **c**, **f**. P and A-sites of the yeast ribosome 60S subunit are colored in *purple* and *yellow*, respectively). **a**, **b** and **c** detailed views of the co-crystal (4UJX) of DON inside the A-site. The 3-hydroxyl group of DON has been pointed out being in *red* CPK representation. The magnesium atom inside the A-site pocket has been pointed out in *green* CPK representation. **d**–**f** Detailed of the interaction model of D3G inside the A-site after alignment of the backbone of D3G over the DON. The 3-O-glucosyl group of D3G has been *colored in red*. Videos showing 360 °C view of DON and D3G inside the A-site of the ribosome 60S subunit are available as Supplemental Material Figures S1 and S2 (color figure online)

(Bony et al. 2006; Vandenbroucke et al. 2011), differentiated cells are fairly resistant to DON and toxin concentration of 30 µM or higher is needed to induce a significant decrease in viability (Fig. 2c). At 10 µM, a non-cytotoxic concentration, DON induced a significant decrease in TEER (Fig. 2b). The decrease was time dependent, and the cell viability reached 69, 42, 30 and 19 % after 2, 4, 6 and 8 days of exposure to DON, respectively. By contrast, cell treatment with D3G did not decrease TEER.

D3G does not induce histological and functional alterations in intestinal explants

In order not to restrict the observations to an intestinal cell line, the comparative toxicity of DON and D3G was also performed on whole intestinal tissue, using porcine jejunal explants.

Control intestinal explants displayed normal villi lined with columnar enterocytes and goblet cells (Fig. 4a). As already described (Pinton et al. 2012), the main histological changes observed on explants after 4-h incubation with 10 µM DON were multifocal to diffuse villi atrophy, multifocal villi fusion, necrosis of apical enterocytes and cellular debris (Fig. 4b). Conversely, explants exposed to D3G showed normal villi height lined with columnar enterocytes. However, mild cellular debris in the apical surface and lymphatic vessel dilation were also observed in D3G-treated explants (Fig. 4c).

D3G in contrast to DON does not alter gene expression profile in intestinal explants

To complete the investigations on the intestinal toxicity, gene expression was analyzed by RT-qPCR and microarray.

Exposure to 10 µM DON for 4 h significantly increased the expression of IL-1α, TNFα, IL-1β, IL-8, IL-17A and IL-22 (3.5- to 17.4-fold increase, Fig. 3b). By contrast, no increased expression of these pro-inflammatory cytokines was observed in D3G-treated explants, demonstrating that this toxin was not able to induce an intestinal inflammation.

To deeper investigate the extent of DON and D3G on gene expression, a 60 K microarray covering the whole transcriptome was used (Voillet et al. 2014). Principal component analysis, Venn diagram and heat map indicate a similar expression pattern of D3G-treated and control explants (Fig. 5). In contrast, two clusters were distinguished in DON-treated samples: cluster 1

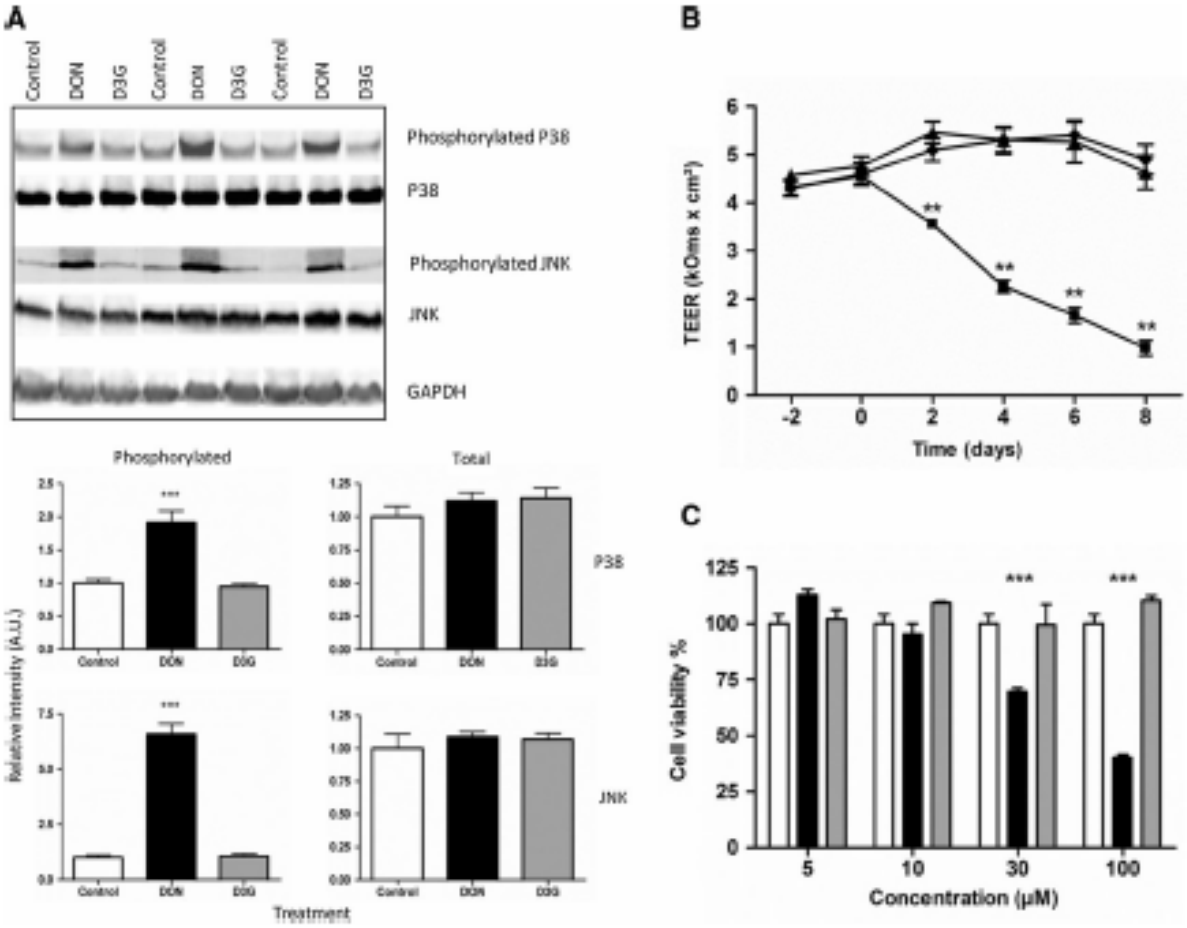

Fig. 2 Effects of DON and D3G on differentiated human intestinal epithelial cells. **a** Effect on the activation of MAPK. Caco-2 cells, differentiated on inserts, were treated for 1 h with 10 µM toxins and analyzed by Western blot for expression of total and phosphorylated p38 and JNK and GAPDH as a protein loading control. Results are expressed as mean ± SEM of 3–4 independent experiments, $***p < 0.001$. **b** Effect on trans-electrical epithelial resistance. Caco-2 cells, differentiated on inserts, were treated at day 0 with 10 µM of diluent (*filled diamond*), D3G (*filled triangle*) or DON (*filled square*) and TEER was measured. Results are expressed as mean ± SEM of 3–4 independent experiments, $**p < 0.01$. **c** effect on cytotoxicity. Caco-2 cells, differentiated in plate, were incubated with increasing concentrations of diluent, D3G or DON for 8 days. Cell viability evaluated by measurement of ATP is expressed as % of control cells. Results are expressed as mean ± SEM of 3–4 independent experiments, $***p < 0.001$

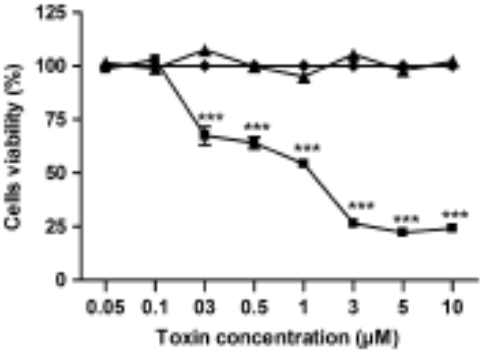

Fig. 3 Toxicity of DON and D3G on proliferating human intestinal epithelial cells. Proliferative Caco-2 cells were incubated with increasing concentrations of diluent (*filled diamond*), D3G (*filled triangle*) or DON (*filled square*) for 48 h. Cell viability evaluated by measurement of ATP is expressed as % of control cells. Results are expressed as mean ± SEM of 3–4 independent experiments, $***p < 0.001$

contains 303 genes (corresponding to 681 probes) that are up-regulated by the mycotoxin; cluster 2 includes 33 down-regulated genes (corresponding to 66 probes). As expected, the 6 pro-inflammatory cytokines tested in RT-qPCR were also up-regulated in the gene expression microarray analysis and a linear regression revealed a strong correlation between these two techniques ($R^2 = 0.96$). Besides pro-inflammatory cytokines, DON treatment also up-regulates (fold change >2.4) genes involved in inflammation and immune response such as *CCL20*, *CXCL2*, *PRDM1*, *AREG*, *CSF2* and *FOSL1* as well as genes involved in oxidative stress, NFkB activation pathway, cell cycle regulation and apoptosis. Down-regulated genes concerned molecular transport (*ABCC2*, *SLC15A1* and *SLC9A2*) and mitochondrial functions (*CPT1A*).

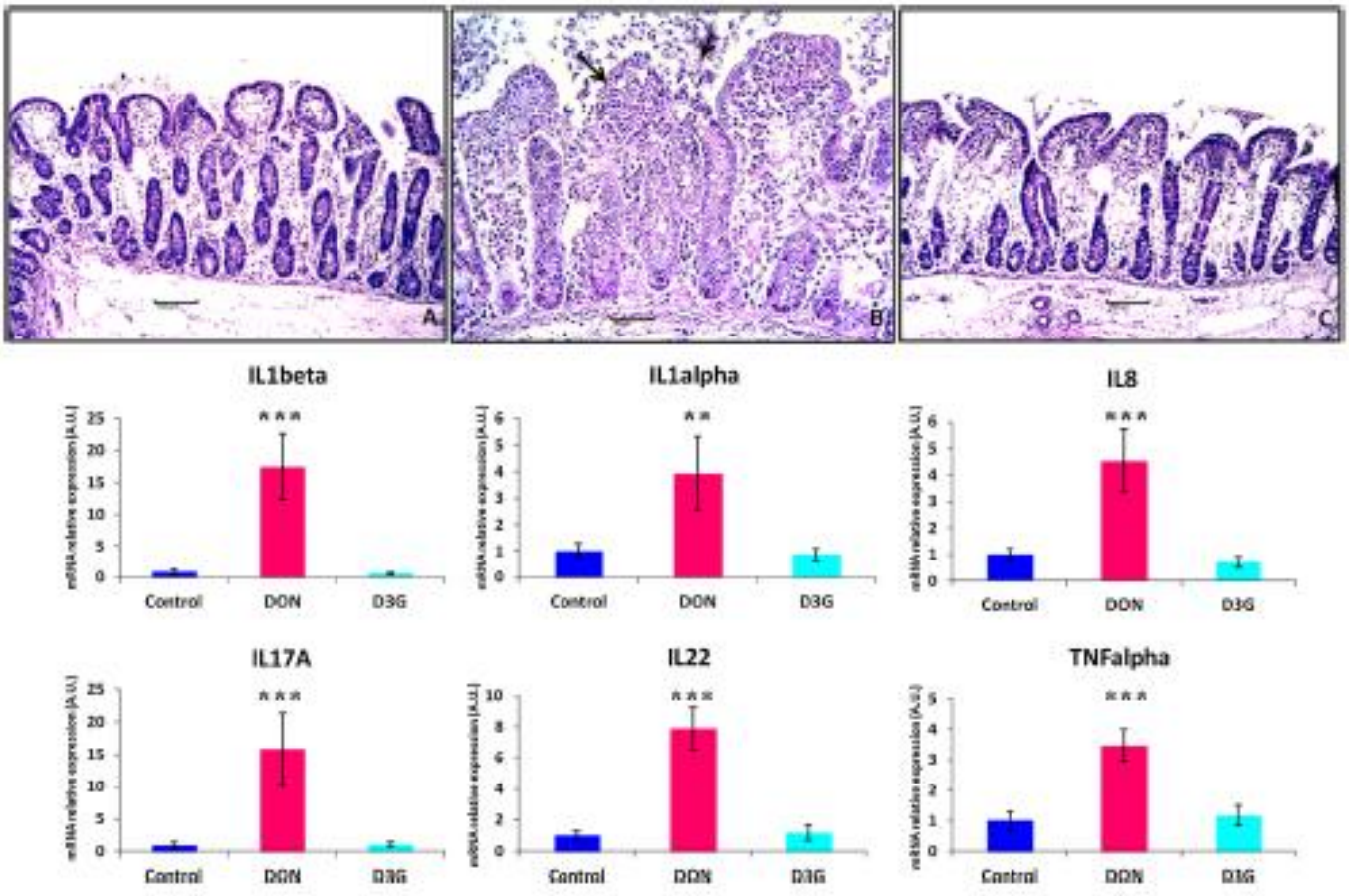

Fig. 4 Effects of DON and D3G on intestinal explants: Morphology (*upper panels*) and inflammation (*lower panels*) *Upper panels* Jejunal explants were exposed for 4 h to diluent (3a), 10 µM DON (3b) or 10 µM D3G (3c) and stained with hematoxylin and eosin. *Arrowheads* indicate necrosis of apical enterocytes and cellular debris. *Bar* 100 µm (3a and 3c), 50 µm (3b). *Lower panels* Jejunal explants were exposed for 4 h to diluent or 10 µM toxins, and relative expression of mRNA encoding for pro-inflammatory cytokines was measured by RT-qPCR. Data are normalized to diluent and expressed, in arbitrary unit, as mean ± SEM of explants from 6 animals, $^{**}p < 0.01$; $^{***}p < 0.001$

Discussion

Food safety authorities such as JECFA (WHO–FAO) and EFSA recognized the need for regulating masked mycotoxins. However, scientific data especially concerning the toxicity of these toxins are scarce (JECFA 2011). The aim of our work was to assess the toxicological relevance of D3G on intestinal gut health in comparison with the parent molecule DON. The intestine is the first line of defense protecting against harmful luminal food contaminants. It acts as physical barrier limiting penetration of agents and releases chemical and immunological mediators that contribute to the development of innate immune responses and maintenance of intestinal homeostasis (Pinton and Oswald 2014; Maresca 2013).

DON induces several pathophysiological effects in experimental animals and in cell cultures that are attributable, in part, to ribotoxic stress and the activation of MAPK (Pestka 2010; Pinton and Oswald 2014). In silico analysis demonstrates that glucoside residue of D3G hinders interaction of this masked mycotoxin with the ribosome. This result suggests an absence of ribotoxic stress, and literature data report a reduced inhibitory activity of D3G on wheat ribosomes (Poppenberger et al. 2003). We further observed that D3G, in contrast to DON, did not induce phosphorylation of JNK and P38 proteins in Caco-2 cells. These results suggest an absence of toxic effect of D3G on mammalian cells as recently described for microbial cells (Suzuki and Iwahashi 2015). This hypothesis was further tested, and the impact of DON and D3G was compared on the Caco-2 intestinal cell lines and intestinal explants. Mycotoxins were tested at 10 µM, a concentration corresponding to food contaminated at 3 mg/kg (Sergent et al. 2006). We confirmed that DON induced cytotoxicity and alters intestinal barrier integrity in human intestinal epithelial cells (Alassane-Kpembi et al. 2013; Sergent et al. 2006). In

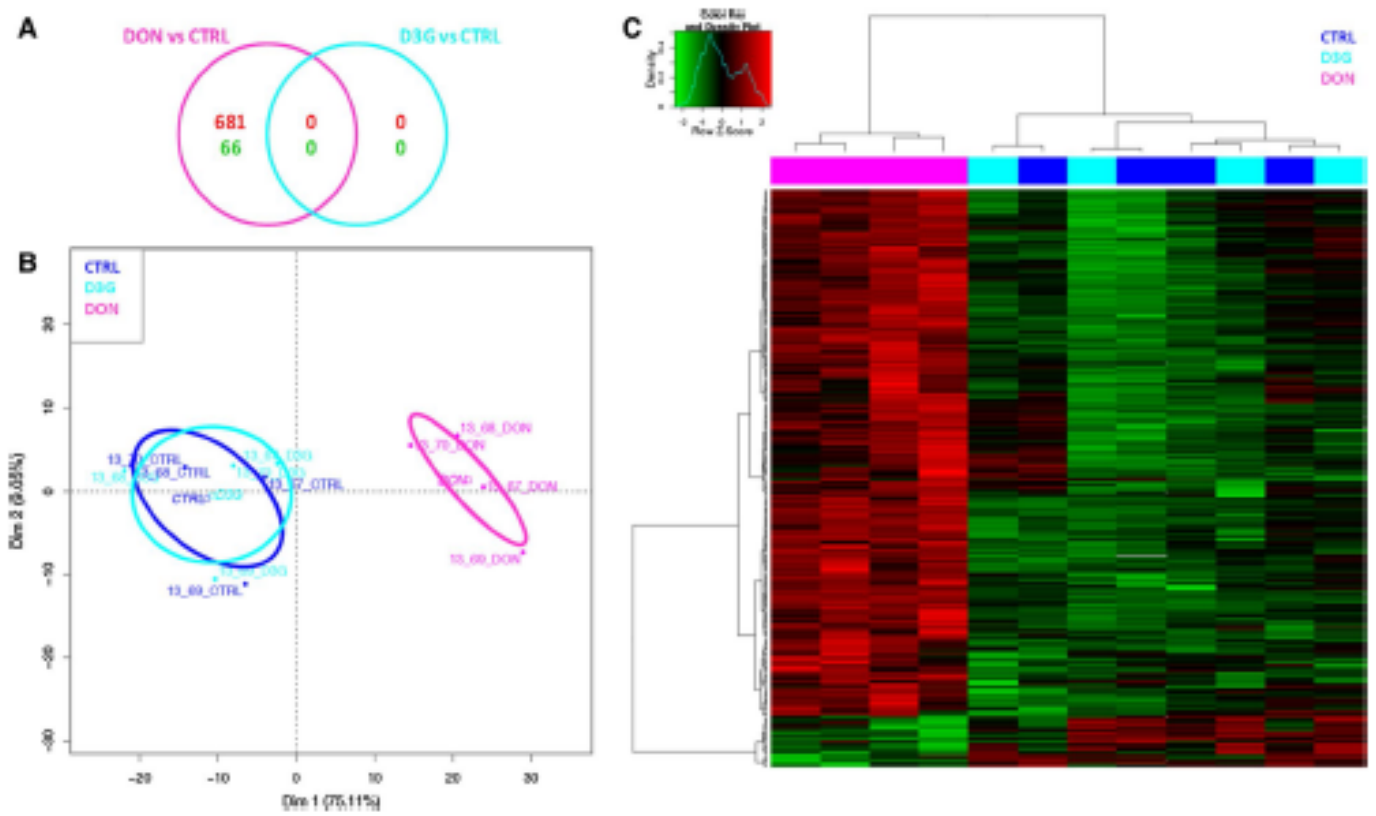

Fig. 5 Gene expression profile of intestinal explants exposed to DON or D3G. Jejunal explants from 4 different animals were exposed for 4 h to diluent or 10 µM toxins, and gene expression was analyzed with a 60 K microarray. **a** Venn diagram illustrating the overlaps between the probes significantly up- or down-regulated in response to DON or D3G treatment. **b** Principal component analysis of differentially expressed probes between DON/D3G and control (747 with BH adjusted *p* value <0.05). **c** Heat map representing differentially expressed probes between DON, D3G and control explants. *Red* and *green colors* indicate values above and below the mean (average Z-score), respectively. *Black color* indicates values close to the mean (color figure online)

contrast, D3G was not cytotoxic on intestinal cells and did not impair trans-epithelial electrical resistance.

The impact of DON and D3G was further assessed on porcine jejunum tissue. Pig can be regarded as a good model of extrapolation to humans (Helke and Swindle 2013). DON induces morphological injury (Osselaere et al. 2013) and inflammation on porcine jejunum explants (Cano et al. 2013). Cytokines and chemokines are sensitive biomarkers for DON exposure (Pestka 2010; Wu et al. 2014a). Indeed, pro-inflammatory response upon DON exposure has been described both in vitro in cell lines from murine, human and porcine origins and in vivo in several organs (Cano et al. 2013; Pestka 2010). In contrast to DON, D3G did not induce any histological damage and was largely incapable of evoking ribotoxin-induced cytokine production on pig jejunum confirming data obtained in vivo in mouse spleens (Wu et al. 2014a). The lack of induction of gene expression by D3G was further demonstrated using a pan-genomic DNA array containing 62,976 probes. Indeed, the transcriptomic analysis revealed that no probes were differentially expressed between control explants and the one treated with D3G.

As already mentioned, data concerning the metabolism and toxicity of D3G are very scarce. Only one study has been performed in mice that indicated that D3G was ineffective in evoking inducing the expression of cytokine or chemokine mRNA in the spleen (Wu et al. 2014a). To the best of our knowledge, our experiment provides the first data on the lack of toxic effect of D3G on human cells and on the intestine. It has been described that D3G is considerably less bioavailable in mammals when compared to DON. Indeed, following oral administration, only small percentage of applied dose of D3G was found in urine in rats and piglets (Nagl et al. 2012, 2014). Moreover, in a human volunteer consuming a diet naturally contaminated with D3G, the masked mycotoxin could not be detected in urine (Warth et al. 2013). Interestingly, De Nijs et al. (2012) observed that D3G is less absorbed by human intestinal Caco-2 cells than DON. In fact, because of its higher molecular weight and its increased polarity, D3G would have a reduced ability to enter the cells by lipid diffusion or through membrane transporters if such transporters are involved.

In vitro data indicated that D3G can be cleaved to DON by lactic acid bacteria and human feces (Berthiller et al. 2011; Dall'Erta et al. 2013; Gratz et al. 2013). These findings correlate well with those of Nagl et al. (2012, 2014), underlining that the majority of oral administrated D3G was recovered as DON in feces. In vivo, the conversion of D3G to its toxic aglycone may occur in the distal part of the gut, while ingested DON is absorbed in the proximal part of the small intestine (Maresca 2013). Hence, ingestion of D3G-contaminated food might lead to a release of DON in the distal part of intestine, shifting the toxic effect of D3G in the distal part of the intestine as already described for ingestion of DON-contaminated food in the presence of this adsorbing agent (Osselaere et al. 2013). Nevertheless, acute ingestion of high doses of D3G does not induce inflammatory reaction in mice and mink (Wu et al. 2014a) nor emetic effect in mink (Wu et al. 2014b). Of note, D3G causes an anorectic response in mice, suggesting that this later effect is mediated through a different mechanism than ribotoxic stress involving CCK and PYY release from enteroendocrine cells via GPCRs and TRP channels (Zhou and Pestka 2015).

As demonstrated in this study, exploring intestinal explants by microarray analysis offers a promising approach for assessing the intestinal toxicity of mycotoxins. Using intestinal explants allows integrating the overall complexity of the gastrointestinal tract including their cytoarchitectures, intercellular interactions and the presence of target cells. The microarray analysis allows having a view of the modulated genes. Using this approach, we demonstrated the lack of effect of D3G while confirming the interference of DON with the immune response. Effects of DON using microarray analysis have previously been described in vitro in human T-lymphocyte cell line (Jurkat cells) and human peripheral blood mononuclear cells (PBMCs) or in vivo in murine thymus, exposed to DON (Katika et al. 2012; van Kol et al. 2011). We also observe the interference of DON with genes involved in other biological functions such as oxidative stress, cell cycle molecular transport and mitochondrial functions. As recently described in vivo (Alizadeh et al. 2015), a decreased expression of mRNA levels of efflux transporters was also observed in intestinal explants exposed to DON. Studies reported that DON is a substrate for ABC transporter (Videmann et al. 2007); thus, a decrease expression of efflux protein would decrease uptake of DON by intestinal cells and therefore decrease its toxicity.

The approach combining explant and microarray can be used to investigate the effect of other contaminants or additives on the intestine and may represent an interesting tool for health risk assessment.

This study significantly extends the current knowledge about intestinal toxicity of D3G. D3G is not toxic by itself but may pose a risk of gut health through its reconversion into its parent mycotoxin DON.

Acknowledgments The authors thank A.M. Cossalter, J. Laffitte and P. Pinton, INRA Toxalim, for sample collection and western experiments. Thanks are also due to R. Hines, BIOMIN, for language editing, and to P. Pinton and I. Alassane-Kpembi for critical reading of the manuscript. A. Pierron and L.S. Murate were supported by fellowship from CIFRE (2012/0572, jointly financed by the BIOMIN Holding GmbH, Association Nationale de la Recherche Technique and INRA) and CAPES (Brazil), respectively. The Austrian Federal Ministry of Science, Research and Economy, the Austria National Foundation for Research, Technology and Development and BIOMIN Holding GmbH are acknowledged for funding the Christian Doppler Laboratory for Mycotoxin Metabolism. The enzymatic production of D3G was performed within the Vienna Science and Technology Fund project WWTF LS12-012 by Dr. Herbert Michlmayr and Prof. Gerhard Adam.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest during the realization of the experimental work submitted.

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Chapter I

Part 2: Intestinal toxicity of the microbial derivatives : the deepoxy-deoxynivalenol (DOM-1) and the 3-epi-deoxynivalenol (3-epi-DON)

Microbial biotransformation of DON: molecular basis for reduced toxicity

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Running title: Microbial detoxification of DON

Keywords:

trichothecenes, modified mycotoxins, gut, *Fusarium*, wheat

Acknowledgments

The authors are grateful to A.M. Cossalter, J. Laffitte and P. Pinton (INRA Toxalim) for sample collection and western-blot experiments. The authors thank Drs L. Huc (INRA, Toxalim), F. Smih-Rouet and M. Barutaut (I2MC INSERM UMR 1048) for Bioenergetic measurements. Thanks are also due to Catherine Anne Moll for language editing. A. Pierron and L.S. Murate were supported by fellowship from CIFRE (2012/0572, jointly financed by the BIOMIN Holding GmbH, Association Nationale de la Recherche Technique and INRA) and CAPES (Brazil) respectively. The authors declare no conflicts of interest during the realization of the experimental work submitted.

Abstract (250 words)

The contamination of cereals with deoxynivalenol (DON), the most prevalent mycotoxin in the world, cannot be avoided however biotransformation can be used to reduce its toxicity. Bacteria are able to de-epoxidize or epimerize DON to deepoxy-deoxynivalenol (deepoxy-DON or DOM-1) or 3-epi-deoxynivalenol (3-epi-DON), respectively. Using *in silico*, *in vitro* and *ex vivo* approaches, the intestinal toxicity of DON, deepoxy-DON and 3-epi-DON was compared and the molecular basis for the reduced toxicity investigated. In human intestinal epithelial cells, deepoxy-DON and 3-epi-DON were not cytotoxic, did not change the oxygen consumption or impair the barrier function. In intestinal explants, exposure for 4 hours to 10µM DON induced intestinal lesions not seen in explants treated with deepoxy-DON and 3-epi-DON. A pan-genomic transcriptomic analysis was also performed on intestinal explants treated with DON and its biotransformation metabolites. 747 probes, representing 323 genes, involved in immune and inflammatory responses, oxidative stress, cell death, molecular transport and mitochondrial function, were differentially expressed, between DON-treated and control explants. By contrast, no differentially expressed genes were observed between control, deepoxy-DON and 3-epi-DON treated explants. Both DON and its biotransformation products were able to fit into the pockets of the A site of the ribosome peptidyl transferase center. In this position DON forms three hydrogen bonds with the A site and activates MAPKinases (mitogen-activated protein kinases). By contrast deepoxy-DON and 3-epi-DON only form two hydrogen bonds and do not activate MAPKinases. Our data demonstrate that bacterial de-epoxidation or epimerization of deepoxy-DON modified their interaction with the ribosome, leading to an absence of MAPKinase activation and toxicity.

Introduction

Mycotoxins are toxic secondary metabolites produced by various molds, such as *Aspergillus*, *Penicillium* and *Fusarium* which may contaminate food and feed at all stages of the food/feed chain (Bennett and Klich 2003; Frisvad et al. 2006). Despite the improvement of agricultural and manufacturing practices, mycotoxin contamination cannot be avoided and still represents a permanent health risk for both humans and animals. It is thus important to develop decontamination strategies (Awad et al, 2010). Among mycotoxins, deoxynivalenol (DON) produced by *Fusarium* species, is commonly detected in cereal crops, including wheat, barley, and maize. It is the most abundant trichothecene in food with a frequent occurrence at toxicologically relevant concentrations worldwide (EFSA 2013, CAST 2003).

DON causes acute and chronic disorders in humans and animals, with the gastrointestinal tract being an organ sensitive to its adverse effects (Pestka 2010a). DON affects the intestinal histomorphology, impairs barrier function and nutrient absorption (Maresca 2013 ; Pinton and Oswald 2014). DON also disrupts the local intestinal immune response; it triggers and potentiates intestinal inflammation (Cano et al. 2013; Vandenbroucke et al. 2011). At the cellular and subcellular level, DON binds to the ribosome, inhibits protein and nucleic acid synthesis and triggers ribotoxic stress (Shifrin and Anderson 1999; Pestka et al, 2004; Garreau de Loubresse et al. 2014) leading to the activation of kinases, MAPKs and their downstream signaling pathways (Pestka 2010b).

Several strategies have been developed to limit DON toxicity (Zhou et al. 2008), among them, bacterial biotransformation which depends on the ability of microorganisms to generate DON metabolites with reduced toxicity. De-epoxidation is a reductive chemical reaction opening the 12,13-epoxy ring transforming DON into its de-epoxide metabolite de-epoxy-deoxynivalenol (deepoxy-DON or DOM-1) (Fig.1) (Sundstol Eriksen et al. 2004). Several microbial strains are capable of DON de-epoxidation (Schatzmayr et al. 2006; Zhou et al. 2008). Several *in vitro* studies demonstrated the reduced toxicity of deepoxy-DON. *In vivo* trials on farm animals receiving feed contaminated with DON have also shown a beneficial effect of the bacteria able to de-epoxidize DON, according to zootechnical parameters and immune response (Grenier et al. 2013). The hydroxyl on carbon 3 also seems to be significant for the toxic activity of DON and a detoxification strategy targeting this part of the C3-OH, leading to the formation of 3-epi-DON, was recently proposed (Karlovsky 2011). Four bacterial strains, all isolated from soil, have been described to epimerize DON into 3-epi-DON (Karlovsky 2011; He et al. 2015a). Only one paper has investigated the effect of 3-epi-

DON and demonstrated the lack of toxicity, both *in vitro* and *in vivo*, of this DON metabolite (He et al. 2015b).

The aim of the current study was to assess the efficacy of microbial transformation through analysis of the intestinal toxicity of deepoxy-DON and 3-epi-DON. Using physiological, histological and transcriptomic analysis, we have observed reduced toxicity of deepoxy-DON and 3-epi-DON, both for human intestinal epithelial cells and pig intestinal explants. We have further demonstrated that these microbial metabolites of DON fit into the ribosome pocket but do not elicit ribotoxic stress or activate the MAPKinase pathway. Our paper provides the first molecular insight for the reduced toxicity of deepoxy-DON and 3-epi-DON.

Experimental procedures

Toxins

Purified DON was purchased from Sigma-Aldrich (St Louis, MO, USA). DOM-1 was obtained by transformation of crystalline DON (Romer Labs, Tulln, Austria), dissolved in medium 10 (Caldwell and Bryant, 1966) at a concentration of 2 mg/ml, by inoculation with BBSH 797, *Gen. nov. sp. nov. of family Coriobacteriaceae* in sterile medium, at 37°C for six days. Biotransformation of DON to deepoxy-DON was confirmed by LC-MS/MS, and deepoxy-DON was purified by solid phase extraction and preparative HPLC (Schwartz-Zimmermann et al., 2014). The purity of the deepoxy-DON preparation was 99%, based on chromatograms recorded at 220nm.

3-epi-DON was produced by microbial transformation of DON (He et al. 2015a). Briefly, DON was co-incubated with the bacterial strain, *Devosia mutans* 17-2-E-8, in corn meal broth medium at 28 °C for 48 h. High-speed counter-current chromatography (HSCCC) and preparative high performance liquid chromatography (prep-LC) were applied to separate 3-epi-DON. The obtained product was analyzed by liquid chromatography (LC) and identified by congruent retention time and UV/Vis spectrum and mass spectrometric (MS) data. Nuclear magnetic resonance (NMR) experiments such as correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and nuclear overhauser effect (NOE) were conducted for structural characterization of 3-epi-DON. The 3-epi-DON used in the experiment had a purity of 96.8%.

Toxins were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -20°C until use.

Caco-2 cell culture

Caco-2 cells (passages 99 - 106) obtained from the TC7 were cultured in 75-cm^2 culture flasks (Cellstar cell culture flasks, Sigma-Aldrich) in Dulbecco's Modified Eagle Medium enriched with glutamine (Gibco, Cergy-Pontoise, France), supplemented with 10% of heat inactivated fetal bovine serum, 0.5% of gentamycine (Eurobio, Courtaboeuf, France) and 1% of non-essential amino acids (Sigma-Aldrich). Cells were maintained at 37° C in an atmosphere of 5% CO^2 and 90% relative humidity. The medium was changed every 2 days. Cells were passaged once a week. The partially confluent cell monolayers were trypsinized with Trypsin-EDTA (Eurobio).

Cell viability assay

Cell viability assay was performed with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, USA) according to manufacturer's instruction. This test measures the quantity of ATP, proportional to the quantity of cells. Cells were seeded at the density of $1.56x10^5$cells/cm^2 in 96-well microtiter plates. Cells were grown for 24 hours and exposed to DON, deepoxy-DON or 3-epi-DON, or corresponding concentrations of DMSO, for 48 hours. Luminescence was measured with a spectrophotometer (TECAN Infinite M200, Männedorf, Switzerland).

Trans-epithelial electrical resistant measurements

To assess the integrity of individual monolayers, trans-epithelial electrical resistance (TEER) was measured as already described (Pinton et al. 2012). Cells (1.34×10^5 cells/cm^2) were grown until differentiation on polyethylene terephthalate membrane inserts (surface area 0.3 cm^2, pore size 0.4 µm) in 24-well format (Becton Dickinson, Pont de Claix, France). The medium was changed every two days. Differentiated cells were exposed to 10µM of diluent or toxins, DON, deepoxy-DON or 3-epi-DON. The culture medium in the apical side of differentiated cells in each well was replaced every two days with medium containing toxin. The TEER was measured for each well daily for 11 days using a Millicell-ERS Voltohmmeter (Millipore, Saint-Quentin-en-Yvelines, France). TEER values were expressed as % of initial values.

Oxygen consumption measurements

The acute effect of toxins on oxygen consumption of Caco-2 cells was assessed using an XF24 extracellular flux analyzer (Seahorse Bioscience, North Billerica, USA). The procedure was performed according to the manufacturer's instructions. Briefly, cells were cultured in XF24 cell culture microplates at $1.5x10^4$ cells per well and maintained as described above. Oxygen consumption rates (OCR) were measured in both differentiated and undifferentiated proliferated Caco-2 cells in non-buffered DMEM (Seahorse Bioscience) supplemented with 10 mM glucose, 2 mM sodium pyruvate (Sigma-Aldrich) and 2mM glutamine (Eurobio) and adjusted to pH 7.4. OCR was monitored every 20 minutes before (basal level) and after injection of diluent or toxins (10µM). OCR values from each well were normalized against viable cell counts (calculated with a Malassez cell) and expressed as a percentage of the baseline value.

Intestinal jejunal explants

Jejunal explants were obtained from 5 week old crossbred castrated male piglets as described previously (Lucioli et al. 2013). The experiment was conducted under the guidelines of the French Ministry of Agriculture for animal research. Two authors (I.P.O. and A.P.) have an official agreement with the French Veterinary Services permitting animal experimentation. Explants were treated for 4 hours at 39°C with 10µM of toxins (DON, deepoxy-DON or 3-epi-DON) or diluent (DMSO) in complete medium. After incubation, treated explants were fixed in 10% formalin (Sigma-Aldrich) for histological analysis or stored at -80°C for RNA extraction.

Histological assessment

Explants fixed with 10% formalin for 24 hours were dehydrated and embedded in paraffin wax (Labonord, Templemars, France) according to standard histological procedures. Sections of 5 µm were stained with haematoxylin and eosin (Sigma-Aldrich) for histopathological assessment. Histological findings were scored based on histological changes and the severity of lesions as previously described (Lucioli et al. 2013).

Gene expression analysis of explants by RT-qPCR

For the gene expression analysis, total RNAs were extracted in lysing matrix D tubes (MP Biomedicals, Illkirch, France) containing guanidine thiocyanate-acid phenol (Eurobio). The quality of these RNA was assessed (Agilent RNA 6000 Nano Kit Quick, Agilent Bioanalyzer 2100); the mean RNA Integrity Number (RIN) of these mRNA preparations was 6.32 ± 0.83. Reverse transcription and RT-qPCR steps were performed as already described (Gourbeyre et al. 2015; Halloy et al. 2005) with previously published primers (Pierron et al. 2015). Amplification efficiency and initial fluorescence were determined by the ΔCt method. Obtained values were normalized using two reference genes, ribosomal protein L32 (RPL32) and cyclophilin A (CycloA). Gene expression levels of treated explants were expressed relative to the mean of the control explants.

Gene expression analysis by microarray

The microarray GPL16524 (Agilent technology, 8 x 60K) used in this experiment consisted of 43,603 spots derived from the 44K (V2:026440 design) Agilent porcine specific microarray. This was enhanced with 9,532 genes from adipose tissue, 3,776 genes from the immune system and 3,768 genes from skeletal muscle (Pierron et al. 2015). For each sample, Cyanine-

3 (Cy3) labeled cRNA was prepared from 200 µg of total RNA using the One-Color Quick Amp Labeling kit (Agilent) according to the manufacturer's instructions, followed by Agencourt RNAClean XP (Agencourt Bioscience Corporation, Beverly, Massachusetts). About 600 ng of Cy3-labelled cRNA were hybridized on SurePrint G3 Porcine GE microarray (8X60K) following the manufacturer's instructions. Slides were scanned immediately, after washing on an Agilent G2505C Microarray Scanner with Agilent Scan Control A.8.5.1 software. All experimental details are available in the Gene Expression Omnibus (GEO) database under accession GSE66918 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=olixoosedvmdnqr&acc=GSE66918). The differentially expressed (DE) genes (adjusted p-value $\leq$ 0.05) were hierarchically clustered and visualized in heat maps. Functional analysis of DE genes was performed using the Ingenuity pathway Analysis tool (IPA, http://www.ingenuity.com) to identify pathways and processes affected by toxins.

Immunoblotting

Expression of the phosphorylated MAPK p38 and JNK (Jun amino-terminal kinases) was assessed on differentiated Caco-2 cells and jejunal explants by immunoblotting as previously described (Pinton et al. 2012). Cells differentiated on 24-well inserts or explants were treated with 10µM of diluent (DMSO) or toxins, DON, deepoxy-DON or 3-epi-DON for 1 hour. Proteins were extracted, quantified and a total of 15 µg of protein was separated by SDS-PAGE. The membranes were probed with rabbit antibodies (Cell Signaling Technology, Danvers, USA) specific for: phospho-SPAK/JNK or phospho-p38 diluted at 1:500 or GAPDH diluted at 1:1000. After washing, the membranes were incubated with 1:10,000 CF^{TM}770 goat anti-rabbit IgG (Biotium, Hayward, USA) for the detection. Antibody detection was performed using an Odyssey Infrared Imaging Scanner (Li-Cor Science Tec, les Ulis, France) with the 770nm channel. The expression of the proteins was estimated after normalization with GAPDH signal.

Molecular modeling.

All the compounds tested were modeled with NAMD under VMD1.9. The structures of deepoxy-DON and 3-epi-DON were built using molefacture from the DON structure included in PDB-file 4U53, after adding hydrogen chirality and atom verification for minimization. Docking of deepoxy-DON and 3-epi-DON was done in VMD based on the carbon backbone of DON. To relax structures and to evaluate the interaction of H-bonds between ribosome and

ligand, a semi-rigid energy minimization was calculated by NAMD using AMBER topology and parameter files with a fixed ribosome structure and a flexible structure for the ligand.

Statistical analysis

Data are expressed as a mean ± SEM of values. The results were analyzed using the Fisher test on equality of variances, one-way ANOVA and Bonferroni as a test post-hoc; p-values < 0.05 were considered significant.

Microarray data from Feature Extraction software was analyzed with R using Bioconductor packages and the limma lmFit function as previously described (Pierron et al. 2015). Probes with adjusted p-value ≤ 0.05 were considered differentially expressed between treated and control conditions. Hierarchical clustering was applied to the samples and the probes using 1-Pearson correlation coefficient as distance and Ward's criterion for agglomeration.

Results

Deepoxy-DON and 3-epi-DON do not impair proliferation of human intestinal cells

Comparative effects of deepoxy-DON, 3-epi-DON and DON were first evaluated on proliferating human Caco-2 cells. The cell viability was assessed by the quantification of ATP using the luminescent cell viability assay. As shown in figure 2, 48 hours exposure to deepoxy-DON or 3-epi-DON at concentrations up to 30µM had no significant impact on cell viability. By contrast DON markedly decreased the viability of proliferating cells in a dose-dependent manner; exposure to 10µM of DON for 48 hours reduced cell viability by approximately 70%. The IC50 was calculated at 1.30µM.

Deepoxy-DON and 3-epi-DON do not impair cell viability and TEER of differentiated human intestinal cells

V. The comparative toxicity of deepoxy-DON, 3-epi-DON and DON was also performed on differentiated Caco-2 cells through the measurement of TEER. As already described (Pierron et al. 2015), differentiated cells are more resistant to DON and at least 30µM are needed to induce a significant decrease in viability. At 10µM of DON, a significant decrease of TEER in differentiated Caco-2 cells at a non-cytotoxic dose was observed (Figure 3). The decrease was time-dependent and reached 25% after 2 days and about 90% at 10 days. By contrast, cells treated with 10µM deepoxy-DON or 3-epi-DON didn’t show any decrease in TEER.

Deepoxy-DON and 3-epi-DON do not affect oxygen consumption in Caco-2 cells

The impact of deepoxy-DON, 3-epi-DON and DON on bioenergetic function in Caco-2 cells was evaluated using extracellular flux analyses. As shown in figure 4, DON linearly decreased the rate of oxygen consumption in a time-dependent manner starting at the 40 minute stage. Approximately 3 hours after DON exposure, oxygen consumption values were 32% less than the base value. By contrast, deepoxy-DON and 3-epi-DON had no effect on the cellular oxygen consumption of proliferating Caco-2 cells and displayed bioenergetics profiles comparable to that of control cells. Similar data were obtained with differentiated Caco-2 cells (data not shown).

Deepoxy-DON and 3-epi-DON do not induce histological alterations of intestinal explants

In order not to restrict the observations to an intestinal cell line, experiments were also performed on jejunal explants, a model developed to assess short-term effects of mycotoxins (Lucioli et al. 2013). The effects on intestines of deepoxy-DON, 3-epi-DON and DON were first compared with histology (Figure 5). Lymphatic vessel dilation was observed at different intensities in all groups. Control explants displayed normal villi lined with columnar enterocytes (Figure 5A). Explants exposed to deepoxy-DON (Figure 5C) and 3-epi-DON (Figure 5D) presented similar features but mild interstitial edema and cell debris on apical surface (arrow) were also observed. By contrast, multifocal to diffuse villi atrophy, multifocal villi fusion (arrows), necrosis of apical enterocytes and cellular debris (arrowhead, Figure 5B) were observed after 4 hours of explant incubation with 10µM of DON.

Deepoxy-DON and 3-epi-DON do not induce intestinal inflammation

To complete the analysis of the intestinal toxicity of deepoxy-DON and 3-epi-DON, their effects on the expression of inflammatory genes were analyzed by RT-qPCR. As already described (Cano et al. 2013), a strong intestinal inflammatory response was observed in jejunal explants in the presence of DON and a significant increase in expression of IL-1α, TNFα, IL-1β, IL-8, IL-12p40 Il-17A and IL-22 was also observed (Table 2). By contrast, no induction in the expression of these genes was observed in deepoxy-DON and 3-epi-DON treated explants, demonstrating that microbial transformation of DON to DEEPOXY-DON or 3-epi-DON led to decreased inflammatory response in intestinal explants (Table 2).

DON but not deepoxy-DON and 3-epi-DON changes gene expression profile in intestinal explants

The effects of DON, deepoxy-DON and 3-epi-DON were investigated beyond the inflammatory response, through a genome wide transcriptomic analysis. Exposure to DON resulted in differential expression of 747 probes; 681 and 66 probes corresponding to 303 and 33 genes were up- and down-regulated respectively (Figure 6A). By contrast, no genes were differentially expressed in deepoxy-DON and 3-epi-DON treated explants when compared to control explants, indicating that microbial transformation of DON to deepoxy-DON or 3-epi-DON abolishes the toxicity of the mycotoxin.

DON differentially expressed genes were then selected to perform principal component analysis (PCA) (Figure 6B) and cluster analysis (Figure 6C). Two clusters were distinguished, indicating up-regulated and down-regulated genes (supplementary table 3). The most significantly up-regulated genes in DON-treated explants, with a change of more than 2.4 fold compared to control, were immune genes such as *CCL20, CXCL2, PRDM1, AREG, CSF2, FOSL1* (Table 3). As expected, the 6 pro-inflammatory cytokines already tested in RT-qPCR analysis were also up-regulated in the DNA array analysis; a strong correlation between the two methods of analysis was observed (coefficient R^2=0.96). DON also increased the expression of the ER heat shock protein HSP70 gene (*HSPA2)*, genes of ubiquitination pathway (*HSPA2, BIRC2, NEDD4L, BIRC3*) and genes of metallothioneins (*MT1A, MT1M* and *MT2B*). DON decreased expression of the *CHAC1* gene, genes for molecular transport including *ABCC2*, *SLC15A1*, *SLC9A2,* the *CCL24* gene which is thought to play a role in the immune response, the *MLEC* gene which is connected to protein misfolding under conditions of endoplasmic reticulum (ER) stress, and other genes (table 3).

Pathway analysis of differentially expressed genes exposed to DON was performed using Ingenuity Pathway Analysis software (IPA). The top 10 scored pathways are listed in Table 4. DON disturbed pathways related to immunity/inflammation, such as cytokines regulations (IL-17 axis, IL-10 signaling), leukocytes functions (diapedesis), iNOS and NFkB signaling. DON affected other pathways associated with cell cycle regulation, apoptosis and ER stress response. Moreover, the results underlined the effects of DON on PXR/RXR, FXR/RXR signaling pathways and mitochondrial L-carnitine Shuttle Pathways.

In silico analysis of the interaction of deepoxy-DON and 3-epi-DON with ribosomes and their inability to activate MAPKs

The above data indicate that microbial transformation of DON into deepoxy-DON or 3-epi-DON abrogates its toxicity. The last step of this study was to investigate the underlying mechanism and more specifically to determine the ability of DON, deepoxy-DON and 3-epi-DON to bind to the A site of the ribosome peptidyl transferase center and to activate the MAPKs.

As expected, after 1 hour of exposure to 10µM DON, MAPKs were activated in both differentiated monolayers Caco-2 cells and jejunal explants (Figure 7). In cells, DON significantly increased phosphorylated p38 (3.24 ± 0.75 vs. 1 ± 0.09) compared to the control (relative intensity in arbitrary unit (A.U.); p<0.05; n=3) and phosphorylated SapKjunk (7.28 ± 0.64 vs. 1 ± 0.12 for control A.U.; p<0.05; n=3). By contrast, deepoxy-DON and 3-epi-DON

346 were not able to activate these MAPKs in Caco-2 cells (Figure 7 panel A). Similar trends
347 were observed in jejunal explants (Figure 7 panel B).
348 The last step was to investigate the ability of DON and its bacterial metabolites, deepoxy-
349 DON and 3-epi-DON, to bind to the 60S sub-unit inside the A-site of the peptidyl transferase
350 center of the ribosome. The crystallographic data (4U53.pdb) obtained for DON and yeast
351 ribosomes were used (Garreau de Loubresse et al. 2014). As shown in Figure 8, (panel A)
352 DON is able to fit in the pocket of the A-site of the ribosome 60S subunit. Within the pocket,
353 the 3-hydroxyl group of DON is associated with a magnesium atom and stabilized by other
354 nucleotides. In this position, DON forms 3 hydrogen bonds with the A-site. The first one is
355 between the oxygen of the DON epoxy group on C12 and one hydrogen of the sugar of the
356 uracil U2873; the second one is between the oxygen of the C15 group CH2OH and one
357 hydrogen of the guanine basis G2403; and the last one is between the hydrogen of the C3
358 group and one oxygen of the uracil U2869. The *in silico* analysis revealed that both deepoxy-
359 DON and 3-epi-DON were also able to fit into the pocket of the peptidyl transferase center of
360 the ribosome. However, because of the absence of the epoxy group or the isomeric change,
361 these two metabolites were only able to form 2 hydrogen bonds with the A sites of the
362 peptidyl transferase center. Deepoxy-DON and 3-epi-DON didn’t form the bond with U2873
363 and U2869, respectively.

364

Discussion

Despite good agricultural practices, contamination by mycotoxins cannot be avoided. Several strategies have been developed to reduce mycotoxin exposure. Among them, microbial transformation is of interest but requires demonstration of the absence of toxicity of the metabolites produced. The aims of the present study were (i) to analyze the intestinal toxicity of two bacterial metabolites of DON, deepoxy-DON and 3-epi-DON and (ii) to investigate the molecular basis for their reduced toxicity.

Through the action of bacteria, DON can be epimerized on the hydroxyl group of C3 or de-epoxidized on the C12-C13 epoxide (Karlovsky 2011). Epimerization is an aerobic irreversible transformation that may require two enzymatic activities of partially overlapping substrate specificity which occur together in sequence: oxidation of DON to 3-keto-DON and then conversion to 3-epi-DON. To date only four bacterial strains have been described to epimerize DON to 3-epi-DON (Karlovsky 2011). A very recent paper shows that this bacterial metabolite is substantially less toxic than DON when tested *in vitro* on proliferating human Caco2 cells, as well as *in vivo* when given orally to mice for 14 days (He et al. 2015b). Deepoxy-DON is obtained after de-epoxidation of DON; several bacterial species and enzymes are able to catalyze this reaction (Karlovsky 2011). The lower toxicity of deepoxy-DON, as compared to DON has been demonstrated *in vitro* on Swiss mouse 3T3 fibroblasts (Sundstol Eriksen 2004), lymphocytes (Schatzmayr et al. 2006) and brine shrimp (Swanson et al. 1987). *In vivo* supplementation of DON-contaminated feed, with bacteria and/or an enzyme able to de-epoxidize DON, induced a reduction of the toxicity as shown by measurement of zootechnical or immune parameters (Li et al. 2011; He et al. 1993; Grenier et al. 2013).

In the present study, we observed reduced intestinal toxicity of 3-epi-DON and deepoxy-DON when compared to DON. The toxicity of DON and its bacterial metabolites, was first investigated on proliferating and differentiated Caco-2 cells. As already demonstrated, DON induced a significant decrease in Caco-2 cell proliferation, reduced their barrier function and altered their respiratory capacities (Alassane-Kempbi et al. 2013, Akbari et al. 2014; Bin-Umer et al. 2014). This is the first investigation of the toxicity of deepoxy-DON on human intestinal epithelial cells, although the absence of toxicity of 3-epi-DON on Caco-2 cells has been recently demonstrated (He et al. 2015b).

The toxicity of DON and its bacterial metabolites on intestinal tissues was further evaluated. Because of the difficulties accessing human intestinal samples, the study was performed on porcine intestinal explants. Indeed pigs are very sensitive to DON and can be

399 considered good models for extrapolation to humans, with a digestive physiology very similar
400 to that of humans (Nejdfors et al. 2000; Pinton and Oswald 2014). Histological assessment
401 showed normal villi lined with columnar enterocytes, mild interstitial edema and cell debris
402 on the apical surface for the control, 3-epi-DON and deepoxy-DON treated explants. This is
403 in accordance with the absence of histopathological lesions observed in mice after a 14 day
404 oral exposure to 25 or 100 mg 3-epi-DON /Kg bw (body weight) (He et al. 2015). Effects of
405 purified deepoxy-DON on the intestine have never been tested, however nutritional strategies
406 including bacteria/enzyme transforming DON to deepoxy-DON have reduced the occurrence
407 and extent of intestinal lesions (Grenier et al. 2013) and showed the same zootechnical
408 performance as in control animals (He et al. 1993*;* Li et al. 2011). By contrast, as already
409 shown, treatment with 10µM of DON induces intestinal damage indicated by villi atrophy and
410 villi fusion (Lucioli et al. 2013). To confirm that the two microbial transformation products of
411 DON were not toxic, a pan-genomic analysis using a DNA array containing 62,976 probes
412 was performed on jejunal explants. It revealed that no probes were differentially expressed
413 between control explants and the ones treated with either deepoxy-DON or 3-epi-DON. To
414 the best of our knowledge this is the first genome wide analysis performed for deepoxy-DON
415 and 3-epi-DON.

416 The global transcriptomic analysis of the effect of DON on the intestine indicated that
417 DON does not only interfere with genes involved in the immune response. As already
418 described for human and murine thymus cells (Van Kol et al. 2011; Katika et al. 2012; Mishra
419 2014), DON exposure also targets ER (endoplasmatic reticulum) stress, protein synthesis,
420 oxidative stress, cell cycle regulation and apoptosis in intestinal tissues. The strong alteration
421 of the gene *MLEC* implicated in misfolded glycoprotein quality control observed herein is
422 likely due to the arrest of translation induced by ribotoxic stress. This leads to less protein
423 entering the ER to temper the unfolded protein response and therefore protein synthesis
424 (Katika et al. 2012). The increased gene expression of the ER heat shock protein HSP70 could
425 also reduce the accumulation of unfolded protein in ER lumen. An increased expression of
426 some genes involved in the ubiquitination pathway was observed in the presence of DON.
427 This result could indicate that the presence of DON may induce the increase in proteins
428 involved in protein degradation (Shen et al. 2007; Osman et al. 2010; Katika et al. 2012). Our
429 data also underline the decrease of the unfolded protein response pro-apoptotic gene CHAC1.
430 The CHAC1 protein seems to play a role in glutathione degradation (Kumar et al. 2012). ER
431 stress could also induce leakage of calcium from the reticulum leading to activation of NFkB,
432 NRF2-mediated oxidative stress response and apoptosis (Katika et al. 2012). The present

work emphasizes the effect of DON on metallothioneins MT1A, MT1M and MT2B. A relationship between metallothionein protein levels, used as a marker of oxidative stress, and mycotoxins in the liver of rats fed on naturally contaminated wheat has been reported (Vasatkova et al. 2009). Therefore, it could be assumed that MTs are associated with pathways protecting the intestine against DON toxicity. The present study underlines the effect of DON on the genes of intestinal transporters. DON decreases the expression of the solute carrier *SLC15A1* and *SLC9A2* involved in proton-coupled oligopeptides transporter PepT1 and a Na+/H+ exchanger, respectively (Bookstein et al. 1997; Smith et al. 2013). Similar effects on other mRNA expression transporters as sugars transporters were described in the jejunum and to a lesser extent in the liver of broilers exposed to DON (Dietrich et al. 2012). Accordingly, it has been experimentally shown that DON decreases the intestinal uptake of various nutrients in human epithelial intestinal cell line HT-29-D4 (Maresca et al. 2002). This effect is likely due to a specific modulation of intestinal transporters expression, rather than a consequence of cell damage. The transcriptomic analysis demonstrates that DON down-regulates the expression of *ABCC2* gene that encodes for MRP2, a protein involved in efflux of DON and other mycotoxins and also in the transport of a wide range of organic anions including bile salt flow (Videmann et al. 2007). An action of DON on mitochondrial dysfunction, attested to by the down-regulation of CPT1A mRNA was also observed in this study. CPT1A encodes for a key regulatory enzyme of β-oxidation and is required for transport of long chain fatty acids into mitochondria (Nakamura et al. 2014). The modulation of β-oxidation in addition to the modulation of intestinal transporters could explain the energy failure reported after DON exposure (Maresca et al.2002). It is now necessary to investigate these changes at the protein level.

The use of bacteria is a promising approach to DON decontamination. In the present study we observed that deepoxy-DON and 3-epi-DON were devoid of intestinal toxicity. The underlying mechanism was further investigated. DON is known to develop its toxic potential by interacting with the peptidyl transferase at the 60S ribosomal subunit level, blocking the protein synthesis at the elongation step, inducing a ribotoxic stress and activating MAPKinases (Maresca 2013; Pestka et al. 2004; Garreau de Loubresse et al. 2014). In accordance with literature, we observed that DON induced phosphorylation of JNK and p38 proteins (Sergent et al. 2006; Lucioli et al 2013). By contrast deepoxy-DON and 3-epi-DON did not active these signaling pathways, suggesting an absence of ribotoxic stress. To further the analysis, a modeling of deepoxy-DON and 3-epi-DON in the ribosome peptidyl transferase center was performed. DON and its bacterial metabolites fit into the A-site pocket,

however whereas DON binds to the peptidyl transferase center with three hydrogen bonds, only two hydrogen bonds were identified between deepoxy-DON or 3-epi-DON and the peptidyl transferase center. This suggests that the absence of the epoxy group or the isomeric transformation decreases the affinity of these latter metabolites for the active site pocket A of the ribosome and prevents the induction of ribotoxic stress. *In silico* modeling revealed that a third hydrogen bond (the one between the oxygen of the C15 group CH2OH and the hydrogen of the guanine base G2403) could be involved in the interaction of DON with the ribosome. It would be of interest to establish whether this H-bond is necessary for the toxicity of DON. Unfortunately we were not able to identify a proper DON metabolite or another fusariotoxin metabolite to confirm the involvement of this H-bound in the structure-toxicity relationship.

In conclusion, the present study confirms that the toxicity of DON is not only linked to the epoxy group but is also influenced by the C3 group (Sato and Ueno 1977; Sundstol Eriksen 2004, Karlovsky 2011). It demonstrates that microbial biotransformation of DON into deepoxy-DON or 3-epi-DON decreases the intestinal toxicity of this mycotoxin. The underlying metabolism causes decreased affinity of the metabolites to the ribosome and the lack of MAPKinases activation. These data significantly increase the current knowledge of intestinal toxicity of DON, deepoxy-DON and 3-epi-DON and contribute to the evaluation of the effectiveness of the microbial biotransformation strategies in the fight against mycotoxins.

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640 **Table 1. Primer sequences used for RT-qPCR analysis (F: Forward; R: Reverse)**

641

Gene symbol	Gene name	Primer sequence	References
CycloA	Cyclophilin A	F: CCCACCGTCTTCTTCGACAT	NM_214353
		R: TCTGCTGTCTTTGGAACTTTGTCT	Gourbeyre et al. 2015
RPL32	Ribosomal Protein L32	F: AGTTCATCCGGCACCAGTCA	NM_001001636
		R: GAACCTTCTCCGCACCCTGT	Gourbeyre et al. 2015
IL1A	Interleukin 1- alpha	F: TCAGCCGCCCATCCA	NM_214029,1
		R: AGCCCCCGGTGCCATGT	Cano et al. 2013
IL1B	Interleukin 1 - beta	F: ATGCTGAAGGCTCTCCACCTC	NM_214055
		R: TTGTTGCTATCATCTCCTTGCAC	Gourbeyre et al. 2015
IL8	Interleukin - 8	F: GCTCTCTGTGAGGCTGCAGTTC	NM_213867
		R: AAGGTGTGGAATGCGTATTTATGC	Grenier et al. 2011
TNFA	Tumor necrosis factor -alpha	F: ACTGCACTTCGAGGTTATCGG	NM_214022
		R: GGCGACGGGCTTATCTGA	Gourbeyre et al. 2015
IL12p40	Interleukin 12 - p 40	F: GGTTTCAGACCCGACGAACTCT	NM_214013
		R : CATATGGCCACAATGGGAGATG	Cano et al., 2013
IL17A	Interleukin 17 - alpha	F: CCAGACGGCCCTCAGATTAC	AB102693
		R: CACTTGGCCTCCCAGATCAC	Cano et al. 2013
IL22	Interleukin - 22	F: AAGCAGGTCCTGAACTTCAC	AY937228
		R: CACCCTTAATACGGCATTGG	Cano et al. 2013

642

643

644 **Table 2. DON but not deepoxy-DON & 3-epi-DON up regulated mRNA relative expression levels of pro-**
645 **inflammatory cytokines and chemokines in pig jejunal explants**
646

Cytokines	Explant treatments			
	Control	DON	Deepoxy-DON	3-epi-DON
IL1B	1.00 ± 0.40[a]	17.4 ± 5.1[b]	0.7 ± 0.2[a]	0.8 ± 0.3[a]
IL1A	1.00 ± 0.30[a]	3.9 ± 1.4[b]	0.9 ± 0.2[a]	0.9 ± 0.2[a]
IL8	1.00 ± 0.20[a]	4.5 ± 1.2[b]	1 ± 0.1a	0.9 ± 0.2[a]
IL12p40	1.00 ± 0.31[a]	2.3 ± 0.4[b]	1.2 ± 0.2[a]	0.9 ± 0.2[a]
IL17A	1.00 ± 0.50[a]	15.8 ± 5.6[b]	0.8 ± 0.1[a]	1.3 ± 0.4[a]
IL22	1.00 ± 0.30[a]	7.9 ± 1.3[b]	1.3 ± 0.5[a]	1.4 ± 0.5[a]
TNFA	1.00 ± 0.30[a]	3.5 ± 0.5[b]	1.1 ± 0.4[a]	1.1 ± 0.3[a]

647
648 *Notes:* results are expressed in arbitrary units relative to control group. Results are mean ±SEM of 6 animals.
649 Means in a row without a common letter differ (Newman-Keuls test, $P<0.05$).
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652 **Table 3**: Top scored differentially expressed genes in DON treated porcine jejunal explants

Gene symbol	Gene name	-log (p-value)	Ratio
a. Genes up-regulated			
IL1B	interleukin 1 beta	4.428	1.29E-11
CCL20	chemokine (C-C motif) ligand 20	3.481	1.79E-06
IL1A	interleukin 1. alpha	3.207	6.46E-09
CXCL2	chemokine (C-X-C motif) ligand 2	3.129	1.87E-04
IL22	interleukin 22	2.955	1.13E-07
PRDM1	PR domain containing 1 with ZNF domain	2.793	4.76E-06
AREG/AREGB	amphiregulin	2.662	1.94E-11
CSF2	colony stimulating factor 2 (granulocyte-macrophage)	2.593	1.86E-05
IL8	interleukin 8	2.585	1.25E-06
FOSL1	FOS-like antigen 1	2.447	4.22E-04
IER3	immediate early response 3	2.446	1.95E-04
CCR7	chemokine (C-C motif) receptor 7	2.325	1.79E-08
CALCB	calcitonin-related polypeptide beta	2.313	9.03E-11
GADD45A	growth arrest and DNA-damage-inducible alpha	2.270	5.61E-08
TNFAIP3	tumor necrosis factor alpha-induced protein 3	2.260	1.36E-08
RND1	Rho family GTPase 1	2.255	3.24E-06
IER2	immediate early response 2	2.227	3.44E-06
CD83	CD83 molecule	2.207	1.10E-05
PLAUR	plasminogen activator. urokinase receptor	2.085	9.86E-04
BTG2	BTG family member 2	2.073	1.25E-06
IFRD1	interferon-related developmental regulator 1	2.025	1.14E-08
RGS1	regulator of G-protein signaling 1	2.020	3.24E-06
GEM	GTP binding protein overexpressed in skeletal muscle	2.013	4.52E-05
CCL4	chemokine (C-C motif) ligand 4	2.004	6.44E-04
STX11	syntaxin 11	1.989	4.27E-05
GADD45G	growth arrest and DNA-damage-inducible gamma	1.881	2.26E-06
GADD45B	growth arrest and DNA-damage-inducible beta	1.873	9.01E-04
NEDD9	neural precursor cell expressed developmentally down-regulated 9	1.870	1.15E-10
LAMA3	laminin. alpha 3	1.858	2.07E-05
CD274	CD274 molecule	1.846	8.75E-11
IL17A	interleukin 17A	1.844	2.11E-11
b. Genes down-regulated			
CHAC1	cation transport regulator homolog 1 (*E. coli)*	-1.696	9.18E-04
ABCC2	ATP-binding cassette sub-family C (CFTR/MRP) member 2	-1.015	2.45E-06

SLC15A1	solute carrier family 15 (oligopeptide transporter) member 1	-0.851	3.56E-05
SLC9A2	solute carrier family 9 subfamily A (NHE2 cation proton antiporter 2) member 2	-0.804	9.09E-06
CCL24	chemokine (C-C motif) ligand 24	-0.784	8.75E-04
MTTP	microsomal triglyceride transfer protein	-0.755	3.26E-05
DMBT1	deleted in malignant brain tumors 1	-0.666	2.67E-04
MLEC	Malectin	-0.654	9.50E-04
SSH1	slingshot protein phosphatase 1	-0.628	1.06E-03
VPS26B	vacuolar protein sorting 26 homolog B (*S. pombe*)	-0.610	1.04E-03
ACE2	angiotensin I converting enzyme 2	-0.607	7.35E-04
SCGB2A1	secretoglobin. family 2A member 1	-0.594	2.74E-04
MYEOV	myeloma overexpressed	-0.592	1.58E-04
NPR3	natriuretic peptide receptor 3	-0.582	8.74E-04
CBL	Cbl proto-oncogene. E3 ubiquitin protein ligase	-0.574	3.70E-04
PLOD2	procollagen-lysine 2-oxoglutarate 5-dioxygenase 2	-0.547	3.98E-05
C4BPA	complement component 4 binding protein Alpha	-0.525	1.04E-03
ARHGEF37	Rho guanine nucleotide exchange factor (GEF) 37	-0.521	9.19E-04
DESI2	desumoylating isopeptidase 2	-0.501	3.04E-04
STOML3	stomatin (EPB72)-like 3	-0.487	8.33E-04
UNC119B	unc-119 homolog B (*C elegans*)	-0.467	2.42E-04
ZER1	zyg-11 related. cell cycle regulator	-0.455	4.92E-04
EGLN1	egl-9 family hypoxia-inducible factor 1	-0.443	2.26E-04
TCAP	titin-cap	-0.441	8.90E-04
PECAM1	platelet/endothelial cell adhesion molecule 1	-0.431	1.43E-04
ZCCHC14	zinc finger CCHC domain containing 14	-0.430	5.16E-04
GALNT4	polypeptide N-acetylgalactosaminyltransferase 4	-0.395	6.91E-04
ANKRD13A	ankyrin repeat domain 13A	-0.388	8.85E-04
UNC45A	unc-45 homolog A (C. elegans)	-0.377	7.64E-04
TPP1	tripeptidyl peptidase I	-0.375	5.37E-04
OSBPL7	oxysterol binding protein-like 7	-0.349	1.05E-03

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Table 4. Ten top scored canonical pathways differentially regulated in 10µM DON treated porcine jejuna explants and list of genes in each pathway

a. Up-regulated pathways

Ingenuity Canonical Pathways	-log (p-value)	Ratio	Molecules
Granulocyte Adhesion and Diapedesis	1.18E01	1.1E-01	*CCL3,IL1B,MMP12,EZR,CCL20,CLDN4,CCL3L1/CCL3L3,SELE,MMP13, CXCL2,VCAM1,CXCL8, CXCR4, IL18, IL1RN,TNF,CXCR2,ICCL4,XCL1*
Agranulocyte Adhesion and Diapedesis	1.13E01	1.04E-01	*CCL3,IL1B,MMP12,EZR,CCL20,CLDN4,CCL3L1/CCL3L3,SELE,MMP13, CXCL2,VCAM1,CXCL8, CXCR4,IL18,IL1RN,TNF,CXCR2,IL1A,CCL 4,XCL1*
Glucocorticoid Receptor Signaling	1.11E01	7.69E-02	*HSPA2,NFKB1,CCL3,CSF2,JAK2,IL1B,PLAU,SELE,NFKBIE,NFATC1,NF KBIA,VCAM1,CXCL8, FOS,IL1RN,DUSP1,SGK1,NR3C1,TNF,SMAD3,CDKN1A,IL10,F OXO3*
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	1.07E01	3.91E-01	*CCL3,CSF2,IL1B,TNF,IL1A,IL17A,IL10,CC L4,IL17F*
Communication between Innate and Adaptive Immune Cells	1.01E01	1.25E-01	*CCR7,CCL3,CSF2,IL1B,CCL3L1/CCL3L3,CD40,CXCL8,CD83,IL18,IL1RN ,TNF,IL1A,IL10,CCL4*
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	1.01E01	4.44E-01	*CCL3,CSF2,IL1B,TNF,IL17A,IL10,CCL4,IL 17F*
Hepatic Fibrosis / Hepatic Stellate Cell Activation	9.55E00	1.03E-01	*CCR7,NFKB1,IL1B,EDNRB,MMP13,CD40,VCAM1,CXCL8,IFNGR1,TNF,S MAD3,IL1A,EDN1,IL4R, IL10,TIMP*
IL-10 Signaling	9.48E00	1.54E-01	*FOS,IL18,NFKB1,IL1RN,IL1B,SOCS3,TNF,IL1A,IL4R,IL10,NFK BIE,NFKBIA*
T Helper Cell Differentiation	9.41E00	1.67E-01	*GATA3,IFNGR1,BCL6,IL18,STAT4,TNF,ICOSLG/LOC102723996,IL17A,IL 4R,IL10,CD40,IL17F*
Atherosclerosis Signaling	9.4E00	1.08E-01	*NFKB1,IL1B,SELE,MMP13,CD40,VCAM1,CXCL8,CXCR4,IL18,IL1RN,TN F,IL1A,F3,TNFRSF12A, PLA2G4A*

b. down-regulated pathways

Ingenuity Canonical Pathways	-log (p-value)	Ratio	Molecules
PXR/RXR Activation	2.28E00	2.17E-02	*ABCC2, CPT1A*

FXR/RXR Activation	2.07E00	1.82E-02	*ABCC2, MTTP*
Mitochondrial L-carnitine Shuttle Pathway	1.57E00	4.55E-02	*CPT1A*
Granulocyte Adhesion and Diapedesis	1.49E00	1.1E-02	*CCL24, PECAM1*
Agranulocyte Adhesion and Diapedesis	1.44E00	1.04E-02	*CCL24, PECAM1*
LPS/IL-1 Mediated Inhibition of RXR Function	1.31E00	8.16E-03	*ABCC2, CPT1A*
Complement System	1.28E00	2.86E-02	*C4BPA*
Erythropoietin Signaling	9.87E-01	1.27E-02	*CBL*
Chemokine Signaling	9.75E-01	1.33E-02	*CCL24*
Ephrin B Signaling	9.46E-01	1.22E-02	*CBL*

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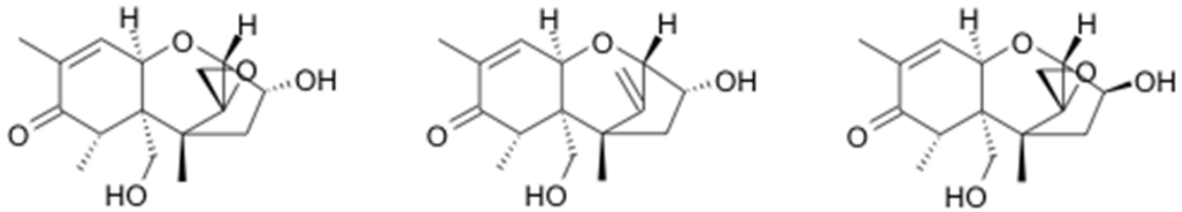


Figure 1. Molecular structures of DON, deepoxy-DON and 3-epi-DON.

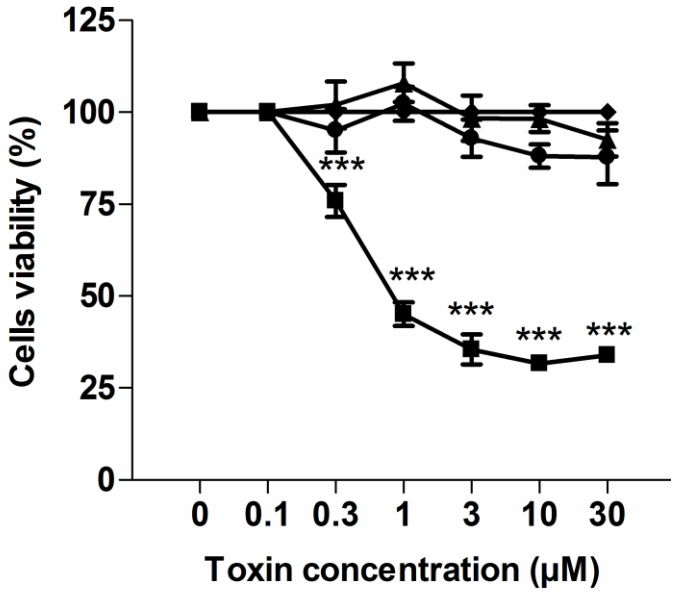
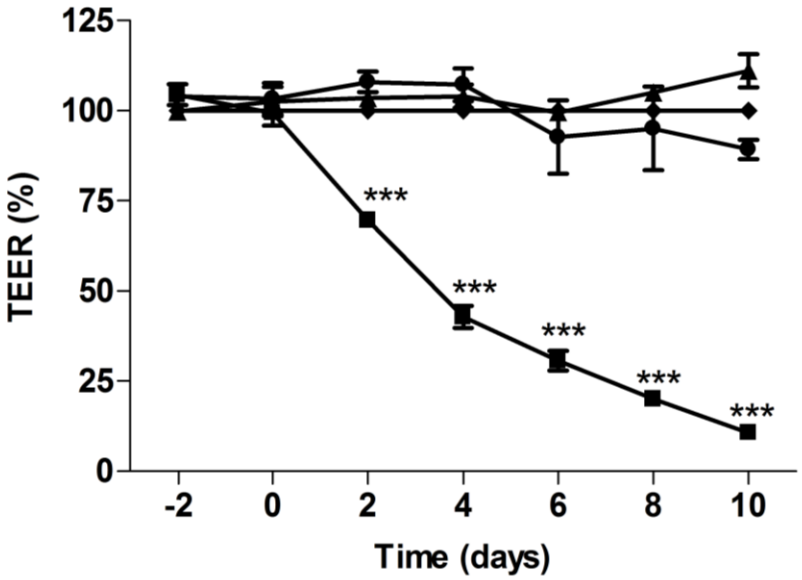


Figure 2. Effects of deepoxy-DON or 3-epi-DON on cytotoxicity of human intestinal epithelial cells. Proliferative Caco-2 cells were incubated with increasing concentrations of diluent (♦), DON (■), DEEPOXY-DON (▲) or 3-epi-DON (●) or for 48 hours. Cell viability evaluated by measurement of ATP, is expressed as % of control cells. Results are expressed as mean ± SEM of 3-4 independent experiments, ***p<0.001.



673

674 **Figure 3. Effects of deepoxy-DON and 3-epi-DON on trans-epithelial electrical resistance**
675 **(TEER) of human intestinal epithelial cells.** Differentiated caco-2 cells, were treated with 10µM of
676 diluent (♦), DON (■), DEEPOXY-DON (▲) or 3-epi-DON (●) and TEER was measured. Results are
677 expressed as mean ± SEM of 3-4 independent experiments, ***p<0.001.

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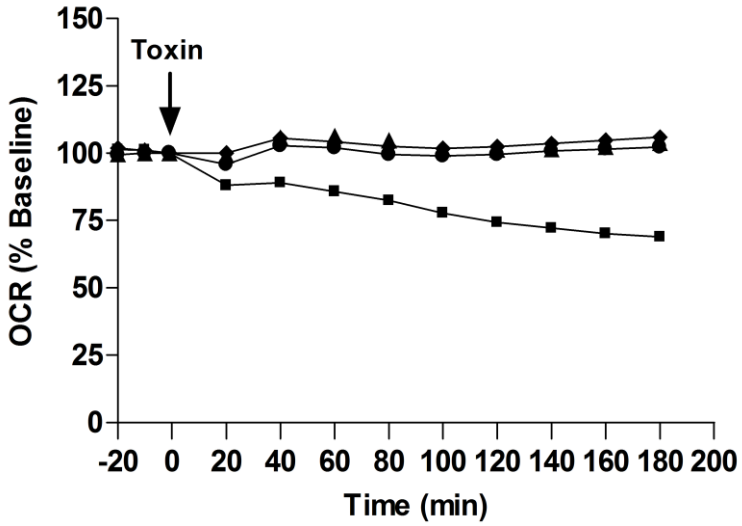

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680 **Figure 4. The acute effect of toxins on oxygen consumption rate (OCR) of cultured Caco-2 cells.**
681 After establishment of baseline oxygen consumption rate in proliferated Caco-2 cells seeded to
682 $1.5x10^4$ cells/well, diluent (♦), DON (■), deepoxy-DON (▲) or 3-epi-DON (●), was injected at final
683 concentration of 10µM as indicated by the arrow. The rate of oxygen consumption was then measured
684 for the indicated time. For visual clarity, statistical indicators were omitted from the graph. The OCR
685 values are shown as the percent of baseline for each group. DON treatment is significantly different
686 from others treatments, ***p<0.001, n=5.

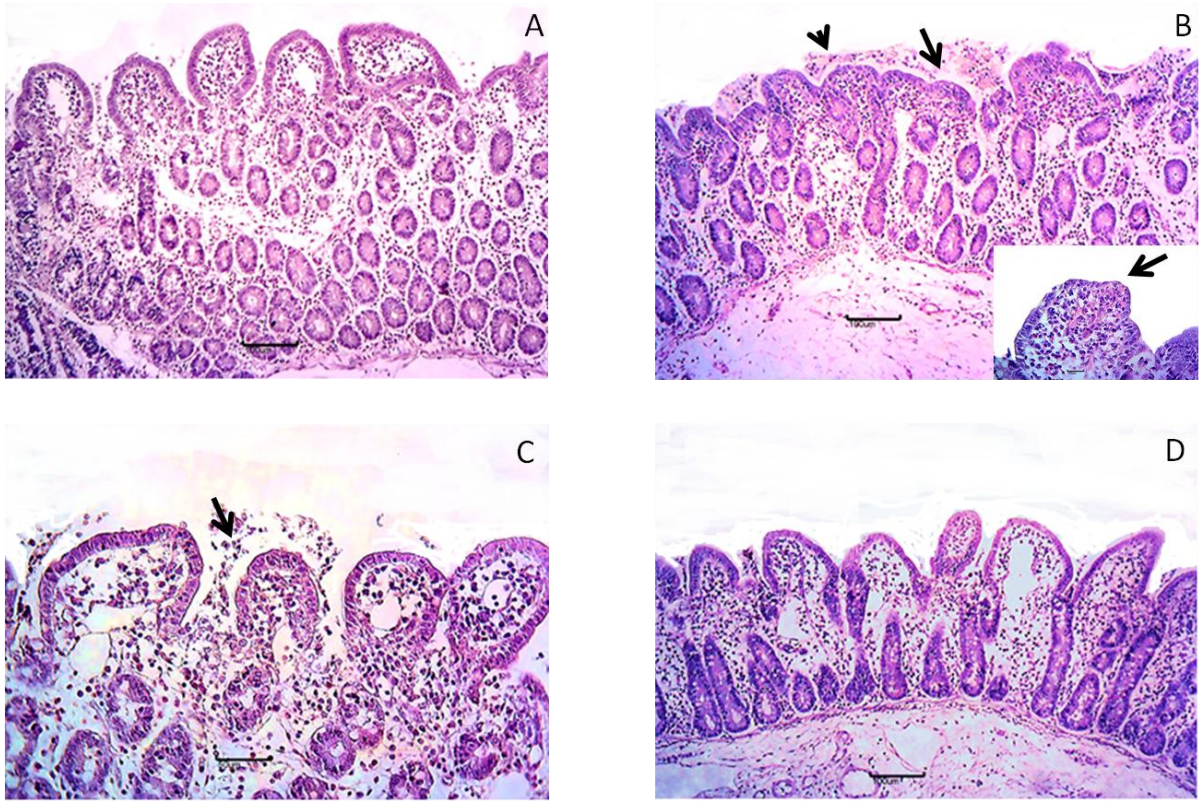

Figure 5. Comparative effect of deepoxy-DON and 3-epi-DON and DON on morphology of intestinal explants.

Jejunal explants from 4 different animals were exposed for 4 hours, to diluent or 10µM toxins and stained with HE for histological analysis. Normal villi lined with columnar enterocytes were observed on control explants (A), multifocal villi atrophy (arrow) and cell debris (arrowhead), apical necrosis (insert) on DON explants (B), histological aspects similar to control group on deepoxy-DON (C) or 3-epi-DON (D) explants. Bar 100 µm; insert bar 20 µm.

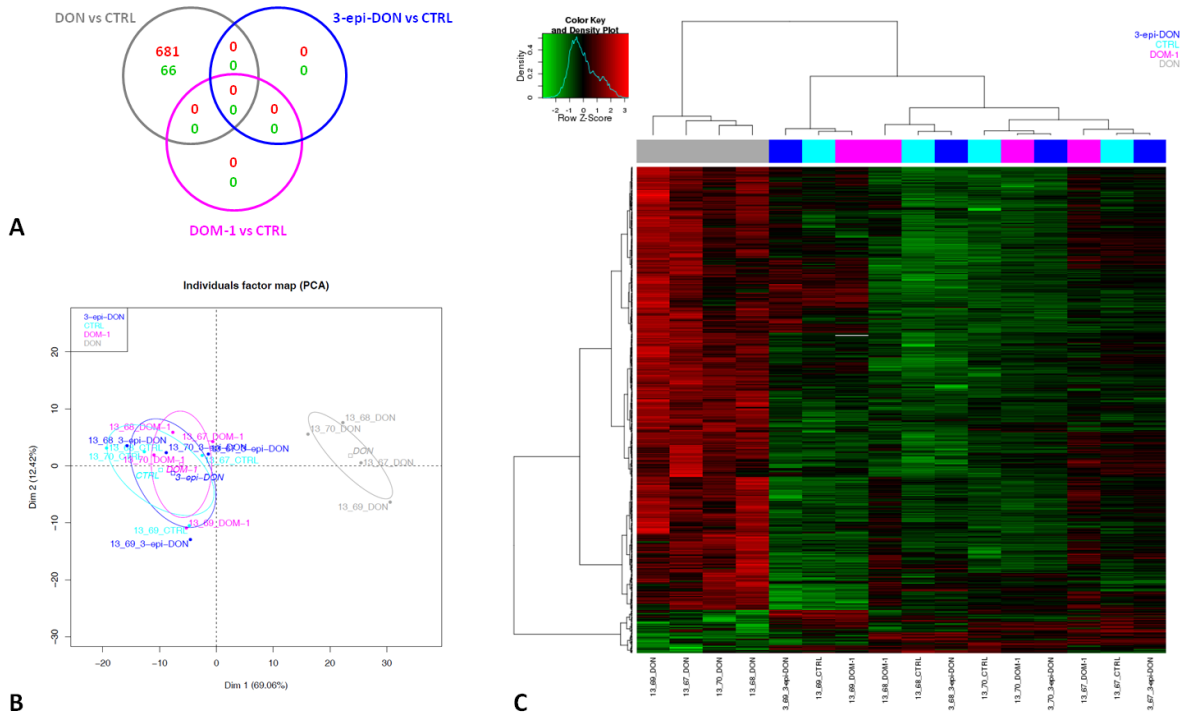

Figure 6. **Gene expression profile of intestinal explants exposed to deepoxy-DON, 3-epi-DON or DON**

Jejunal explants from 4 different animals were exposed for 4 hours, to diluent or 10µM toxins and gene expression was analyzed with a 60K microarray. Groups are represented by different colors: DON in grey, Control in cyan, deepoxy-DON (or DOM-1) in pink and 3-epi-DON in dark blue.

Panel A: Venn diagram illustrating the overlaps between the probes significantly up- or down-regulated in response to DON, deepoxy-DON and 3-epi-DON treatment.

Panel B: Principal Component Analysis of differentially expressed probes between DON/D3G and control (747 with BH adjusted p-*value* < 0.05).

Panel C: Heat map representing differentially expressed probes between DON, deepoxy-DON, 3-epi-DON and control explant. Red and green colors indicate values above and below the mean (average Z-score) respectively. Black color indicates values close to the mean.

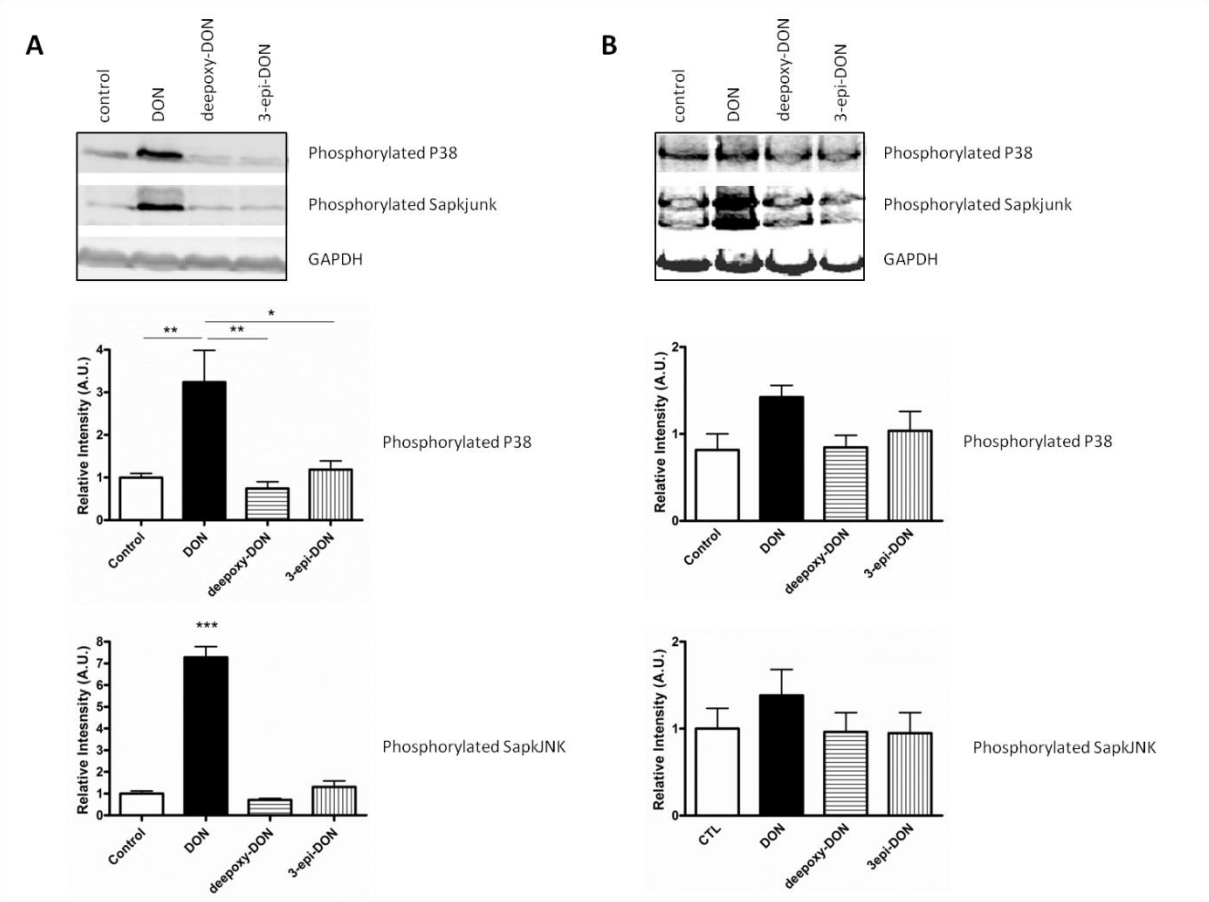

Figure 7. Effects of deepoxy-DON or 3-epi-DON on activation of MAPK on human intestinal epithelial cells.

Panel A: Caco-2 cells, differentiated on inserts. **Panel B:** Jejunal explants.

Samples were treated for 1h with 10µM toxins and analyzed by western blot for expression of phosphorylated P38, phosphorylated JNK and GAPDH, used as a protein loading control. Representative immunoblots and normalized expression graph.

Results are expressed as mean ± SEM of 3-4 independent experiments, *p<0.05, **p<0.01, ***p<0.001.

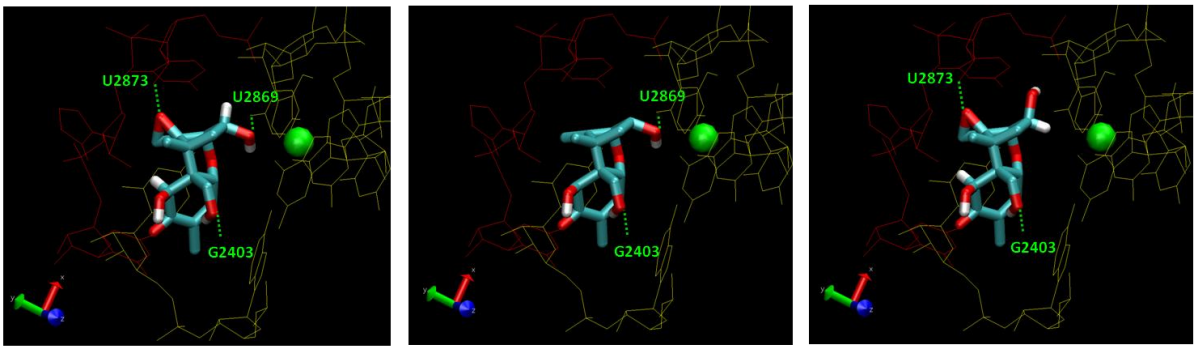

Figure 8. Interaction between the ribosome 60S subunit binding site and DON, deepoxy-DON and 3-epi-DON.

Both sides of the A site of the yeast ribosome 60S subunit are colored in red and yellow respectively. Hydrogen and oxygen atoms are represented in white and red respectively.
Panels A: detailed views of the co-crystal (4U53) of DON inside the A-site.
Panels B: detailed views of deepoxy-DON modeling inside the A-site.
Panels C: detailed views of 3-epi-DON modeling inside the A-site.
The magnesium atom inside the A-site pocket has been highlighted in green.
DON forms 3 hydrogen bonds with the A-site: between the oxygen of the DON epoxy group on C12 and one hydrogen of the sugar of the uracil U2873; between the oxygen of the C15 group CH2OH and one hydrogen of the guanine basis G2403; between the hydrogen of the C3 group and one oxygen of the uracil U2869.

738 Supplementary materials

739 **Supplementary Table1**: Complete list of differentially expressed genes in DON treated intestinal explants

Gene symbol	**Gene name**	**-log (p-value)**	**Ratio**
Up- regulated genes			
IL1B	interleukin 1. beta	4.428	1.29E-11
CCL20	chemokine (C-C motif) ligand 20	3.481	1.79E-06
IL1A	interleukin 1. alpha	3.207	6.46E-09
CXCL2	chemokine (C-X-C motif) ligand 2	3.129	1.87E-04
IL22	interleukin 22	2.955	1.13E-07
PRDM1	PR domain containing 1. with ZNF domain	2.793	4.76E-06
AREG/AREGB	amphiregulin	2.662	1.94E-11
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IER3	immediate early response 3	2.446	1.95E-04
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GADD45A	growth arrest and DNA-damage-inducible. alpha	2.270	5.61E-08
TNFAIP3	tumor necrosis factor. alpha-induced protein 3	2.260	1.36E-08
RND1	Rho family GTPase 1	2.255	3.24E-06
IER2	immediate early response 2	2.227	3.44E-06
CD83	CD83 molecule	2.207	1.10E-05
PLAUR	plasminogen activator. urokinase receptor	2.085	9.86E-04
BTG2	BTG family. member 2	2.073	1.25E-06
IFRD1	interferon-related developmental regulator 1	2.025	1.14E-08
RGS1	regulator of G-protein signaling 1	2.020	3.24E-06
GEM	GTP binding protein overexpressed in skeletal muscle	2.013	4.52E-05
CCL4	chemokine (C-C motif) ligand 4	2.004	6.44E-04
STX11	syntaxin 11	1.989	4.27E-05
GADD45G	growth arrest and DNA-damage-inducible. gamma	1.881	2.26E-06
GADD45B	growth arrest and DNA-damage-inducible. beta	1.873	9.01E-04
NEDD9	neural precursor cell expressed. developmentally down-regulated 9	1.870	1.15E-10
LAMA3	laminin. alpha 3	1.858	2.07E-05
CD274	CD274 molecule	1.846	8.75E-11
IL17A	interleukin 17A	1.844	2.11E-11
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in	1.779	3.53E-05

	B-cells inhibitor. alpha		
SOCS3	suppressor of cytokine signaling 3	1.771	2.54E-06
F3	coagulation factor III (thromboplastin. tissue factor)	1.754	4.29E-05
IDO1	indoleamine 2.3-dioxygenase 1	1.748	2.92E-07
HAMP	hepcidin antimicrobial peptide	1.724	7.77E-07
SPRY2	sprouty homolog 2 (Drosophila)	1.723	1.83E-07
PHLDA1	pleckstrin homology-like domain. family A. member 1	1.708	1.57E-06
NABP1	nucleic acid binding protein 1	1.681	3.78E-07
BCL2A1	BCL2-related protein A1	1.681	5.18E-08
GPR65	G protein-coupled receptor 65	1.677	5.79E-10
TBC1D4	TBC1 domain family. member 4	1.652	2.82E-08
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor. zeta	1.652	6.58E-06
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif. 1	1.652	5.54E-06
TNF	tumor necrosis factor	1.647	4.65E-06
GPR183	G protein-coupled receptor 183	1.609	6.59E-07
ENC1	ectodermal-neural cortex 1 (with BTB domain)	1.602	3.23E-05
BIRC3	baculoviral IAP repeat containing 3	1.596	2.03E-07
IL17F	interleukin 17F	1.527	5.39E-09
RCAN1	regulator of calcineurin 1	1.524	2.18E-04
SELE	selectin E	1.521	2.09E-04
ZFAND5	zinc finger. AN1-type domain 5	1.517	8.21E-09
EPHA2	EPH receptor A2	1.512	1.39E-06
ABTB2	ankyrin repeat and BTB (POZ) domain containing 2	1.504	3.43E-08
ADM	adrenomedullin	1.481	7.47E-08
TXNIP	thioredoxin interacting protein	1.465	1.04E-05
PMAIP1	phorbol-12-myristate-13-acetate-induced protein 1	1.426	1.10E-06
NDEL1	nudE neurodevelopment protein 1-like 1	1.422	4.95E-07
TRIB1	tribbles homolog 1 (Drosophila)	1.394	7.51E-04
CREM	cAMP responsive element modulator	1.390	1.16E-07
TXNIP	thioredoxin interacting protein	1.374	1.31E-05
PDE4B	phosphodiesterase 4B. cAMP-specific	1.373	8.64E-08
MT1M	metallothionein 1M	1.370	1.61E-06
NR1D1	nuclear receptor subfamily 1. group D. member 1	1.364	1.72E-05
BTG1	B-cell translocation gene 1. anti-proliferative	1.361	1.69E-07
BIRC3	baculoviral IAP repeat containing 3	1.349	8.81E-06
PLK2	polo-like kinase 2	1.343	1.23E-08
FEM1C	fem-1 homolog c (C. elegans)	1.334	9.72E-05

FADD	Fas (TNFRSF6)-associated via death domain	1.331	4.02E-07
MCL1	myeloid cell leukemia sequence 1 (BCL2-related)	1.330	1.77E-07
LIF	leukemia inhibitory factor	1.326	3.63E-04
NFIL3	nuclear factor. interleukin 3 regulated	1.322	6.64E-05
LPAR6	lysophosphatidic acid receptor 6	1.319	3.49E-08
IL10	interleukin 10	1.313	3.29E-06
CLDN4	claudin 4	1.309	4.82E-05
MMP12	matrix metallopeptidase 12 (macrophage elastase)	1.308	1.42E-06
TNFRSF12A	tumor necrosis factor receptor superfamily. member 12A	1.299	1.01E-04
STK17B	serine/threonine kinase 17b	1.295	1.57E-05
SOX11	SRY (sex determining region Y)-box 11	1.293	3.16E-06
OTUD1	OTU domain containing 1	1.281	1.02E-04
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21. Cip1)	1.277	6.13E-04
IL1RN	interleukin 1 receptor antagonist	1.270	8.55E-08
BTG2	BTG family. member 2	1.267	7.49E-05
ZBTB10	zinc finger and BTB domain containing 10	1.264	1.54E-06
NR4A3	nuclear receptor subfamily 4. group A. member 3	1.245	2.90E-05
ETV3	ets variant 3	1.239	8.30E-05
NR0B2	nuclear receptor subfamily 0. group B. member 2	1.229	4.76E-05
C1orf116	chromosome 1 open reading frame 116	1.229	2.75E-05
TSC22D2	TSC22 domain family. member 2	1.228	1.90E-05
PLAU	plasminogen activator. urokinase	1.203	1.49E-04
EDN1	endothelin 1	1.203	4.72E-04
NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor. epsilon	1.198	2.47E-07
CD40	CD40 molecule. TNF receptor superfamily member 5	1.173	3.59E-04
THBD	thrombomodulin	1.171	3.11E-06
NR4A2	nuclear receptor subfamily 4. group A. member 2	1.165	8.92E-06
FOS	FBJ murine osteosarcoma viral oncogene homolog	1.165	6.78E-05
TBX3	T-box 3	1.156	9.31E-05
MMP13	matrix metallopeptidase 13 (collagenase 3)	1.151	2.66E-04
HIVEP2	human immunodeficiency virus type I enhancer binding protein 2	1.150	1.81E-04
VCAM1	vascular cell adhesion molecule 1	1.147	7.92E-06
BCL10	B-cell CLL/lymphoma 10	1.142	1.33E-05
EEPD1	endonuclease/exonuclease/phosphatase family domain containing 1	1.133	1.66E-05
PHLDA2	pleckstrin homology-like domain. family A. member 2	1.128	1.79E-04
RHPN2	rhophilin. Rho GTPase binding protein 2	1.127	1.51E-05

DUSP6	dual specificity phosphatase 6	1.127	5.53E-04
JMJD1C	jumonji domain containing 1C	1.121	1.36E-05
BCL6	B-cell CLL/lymphoma 6	1.119	5.51E-05
EVI2A	ecotropic viral integration site 2A	1.116	4.09E-07
XCL1	chemokine (C motif) ligand 1	1.113	1.07E-04
EFNB2	ephrin-B2	1.109	1.86E-05
RASGEF1A	RasGEF domain family. member 1A	1.103	1.04E-04
COQ10B	coenzyme Q10 homolog B (S. cerevisiae)	1.083	7.05E-05
SPRY1	sprouty homolog 1. antagonist of FGF signaling (Drosophila)	1.082	9.08E-04
TIMP1	TIMP metallopeptidase inhibitor 1	1.076	1.68E-04
TRAFD1	TRAF-type zinc finger domain containing 1	1.070	6.59E-06
CXCR4	chemokine (C-X-C motif) receptor 4	1.065	3.32E-05
BTG3	BTG family. member 3	1.063	9.92E-04
KCNK5	potassium channel. subfamily K. member 5	1.048	1.05E-04
CEBPD	CCAAT/enhancer binding protein (C/EBP). delta	1.046	8.18E-05
RHOH	ras homolog family member H	1.033	6.14E-04
REL	v-rel avian reticuloendotheliosis viral oncogene homolog	1.031	2.86E-06
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	1.027	3.48E-04
SKIL	SKI-like oncogene	1.021	2.04E-04
TNFAIP2	tumor necrosis factor. alpha-induced protein 2	1.014	4.77E-04
EGR2	early growth response 2	1.013	5.02E-05
ZFP36L1	ZFP36 ring finger protein-like 1	1.012	4.75E-06
Down-regulated genes			
CHAC1	ChaC. cation transport regulator homolog 1 (E. coli)	-1.696	9.18E-04
ABCC2	ATP-binding cassette. sub-family C (CFTR/MRP). member 2	-1.015	2.45E-06
SLC15A1	solute carrier family 15 (oligopeptide transporter). member 1	-0.851	3.56E-05
SLC9A2	solute carrier family 9. subfamily A (NHE2. cation proton antiporter 2). member 2	-0.804	9.09E-06
CCL24	chemokine (C-C motif) ligand 24	-0.784	8.75E-04
MTTP	microsomal triglyceride transfer protein	-0.755	3.26E-05
DMBT1	deleted in malignant brain tumors 1	-0.666	2.67E-04
MLEC	malectin	-0.654	9.50E-04
SSH1	slingshot protein phosphatase 1	-0.628	1.06E-03
VPS26B	vacuolar protein sorting 26 homolog B (S. pombe)	-0.610	1.04E-03
ACE2	angiotensin I converting enzyme 2	-0.607	7.35E-04
SCGB2A1	secretoglobin. family 2A. member 1	-0.594	2.74E-04

MYEOV	myeloma overexpressed	-0.592	1.58E-04
NPR3	natriuretic peptide receptor 3	-0.582	8.74E-04
CBL	Cbl proto-oncogene. E3 ubiquitin protein ligase	-0.574	3.70E-04
PLOD2	procollagen-lysine. 2-oxoglutarate 5-dioxygenase 2	-0.547	3.98E-05
C4BPA	complement component 4 binding protein. alpha	-0.525	1.04E-03
ARHGEF37	Rho guanine nucleotide exchange factor (GEF) 37	-0.521	9.19E-04
DESI2	desumoylating isopeptidase 2	-0.501	3.04E-04
STOML3	stomatin (EPB72)-like 3	-0.487	8.33E-04
UNC119B	unc-119 homolog B (C. elegans)	-0.467	2.42E-04
ZER1	zyg-11 related. cell cycle regulator	-0.455	4.92E-04
EGLN1	egl-9 family hypoxia-inducible factor 1	-0.443	2.26E-04
TCAP	titin-cap	-0.441	8.90E-04
PECAM1	platelet/endothelial cell adhesion molecule 1	-0.431	1.43E-04
ZCCHC14	zinc finger. CCHC domain containing 14	-0.430	5.16E-04
GALNT4	polypeptide N-acetylgalactosaminyltransferase 4	-0.395	6.91E-04
ANKRD13A	ankyrin repeat domain 13A	-0.388	8.85E-04
UNC45A	unc-45 homolog A (C. elegans)	-0.377	7.64E-04
TPP1	tripeptidyl peptidase I	-0.375	5.37E-04
OSBPL7	oxysterol binding protein-like 7	-0.349	1.05E-03
CPT1A	carnitine palmitoyltransferase 1A (liver)	-0.339	6.12E-04
FOXK2	forkhead box K2	-0.339	9.82E-04

740 **Supplementary Table 2**: Complete list of canonical pathways affected by DON in intestinal explants pathway

Part a: Pathways up-regulated

Canonical Pathways	-log (p-value)	Ratio	Molecules
Granulocyte Adhesion and Diapedesis	1.18E01	1.1E-01	CCL3,IL1B,MMP12,EZR,CCL20,CLDN4,CCL3L1/CCL3L3,SELE,MMP13,CXCL2,VCAM1,CXCL8, CXCR4,IL18,IL1RN,TNF,CXCR2,IL1A,CCL4,XCL1
Agranulocyte Adhesion and Diapedesis	1.13E01	1.04E-01	CCL3,IL1B,MMP12,EZR,CCL20,CLDN4,CCL3L1/CCL3L3,SELE,MMP13,CXCL2,VCAM1,CXCL8, CXCR4,IL18,IL1RN,TNF,CXCR2,IL1A,CCL4,XCL1
Glucocorticoid Receptor Signaling	1.11E01	7.69E-02	HSPA2,NFKB1,CCL3,CSF2,JAK2,IL1B,PLAU,SELE,NFKBIE,NFATC1,NFKBIA,VCAM1,CXCL8, FOS,IL1RN,DUSP1,SGK1,NR3C1,TNF,SMAD3,CDKN1A,IL10,FOXO3
Differential Regulation of Cytokine Production in Intestinal Epithelial Cells by IL-17A and IL-17F	1.07E01	3.91E-01	CCL3,CSF2,IL1B,TNF,IL1A,IL17A,IL10,CCL4,IL17F
Communication between Innate and Adaptive Immune Cells	1.01E01	1.25E-01	CCR7,CCL3,CSF2,IL1B,CCL3L1/CCL3L3,CD40,CXCL8,CD83,IL18,IL1RN,TNF,IL1A,IL10,CCL4
Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F	1.01E01	4.44E-01	CCL3,CSF2,IL1B,TNF,IL17A,IL10,CCL4,IL17F
Hepatic Fibrosis / Hepatic Stellate Cell Activation	9.55E00	1.03E-01	CCR7,NFKB1,IL1B,EDNRB,MMP13,CD40,VCAM1,CXCL8,IFNGR1,TNF,SMAD3,IL1A,EDN1,IL4I IL10,TIMP1
IL-10 Signaling	9.48E00	1.54E-01	FOS,IL18,NFKB1,IL1RN,IL1B,SOCS3,TNF,IL1A,IL4R,IL10,NFKBIE,NFKBIA
T Helper Cell Differentiation	9.41E00	1.67E-01	GATA3,IFNGR1,BCL6,IL18,STAT4,TNF,ICOSLG/LOC102723996,IL17A,IL4R,IL10,CD40,IL17F
Atherosclerosis Signaling	9.4E00	1.08E-01	NFKB1,IL1B,SELE,MMP13,CD40,VCAM1,CXCL8,CXCR4,IL18,IL1RN,TNF,IL1A,F3,TNFRSF12A, PLA2G4A
Role of Cytokines in Mediating Communication between Immune Cells	9.36E00	2E-01	IL18,IL1RN,CSF2,IL1B,TNF,IL22,IL1A,IL17A,IL10,IL17F,CXCL8
TREM1 Signaling	9.01E00	1.47E-01	CD83,IL18,NFKB1,CCL3,CSF2,IL1B,JAK2,TNF,IL10,CD40,CXCL8
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	8.92E00	6.43E-02	NFKB1,CEBPD,CSF2,JAK2,IL1B,SOCS3,SELE,NFKBIE,MMP13,NFATC1,NFKBIA,VCAM1, CXCL8,FOS,IL18,IL1RN,CREB5,TNF,IL1A,IL17A,IL10,IRAK2
TNFR1 Signaling	8.83E00	1.85E-01	FOS,BIRC2,NFKB1,CYCS,TNFAIP3,TNF,FADD,NFKBIE,NFKBIA,BIRC3
TNFR2 Signaling	8.28E00	2.35E-01	FOS,BIRC2,NFKB1,TNFAIP3,TNF,NFKBIE,NFKBIA,BIRC3
Dendritic Cell Maturation	7.97E00	7.58E-02	CCR7,NFKB1,STAT4,CSF2,JAK2,IL1B,NFKBIE,CD40,NFKBIA,CD83,IL18,IL1RN,CREB5,TNF,

			IL1A,IL10
HMGB1 Signaling	7.87E00	1.1E-01	FOS,IFNGR1,RHOH,NFKB1,TNF,RHOB,IL1A,PLAT,SELE,RND3,VCAM1,CXCL8
Role of Hypercytokinemia/hyperchemokinemia in the Pathogenesis of Influenza	7.84E00	1.96E-01	IL18,CCL3,IL1RN,IL1B,TNF,IL1A,IL17A,CCL4,CXCL8
PPAR Signaling	7.82E00	1.12E-01	FOS,IL18,NFKB1,IL1RN,IL1B,TNF,IL1A,NFKBIE,PPARG,NFKBIA,CITED2,NR0B2
IL-6 Signaling	7.72E00	1.05E-01	NFKB1,JAK2,IL1B,MCL1,SOCS3,NFKBIE,NFKBIA,CXCL8,FOS,IL18,IL1RN,TNF,IL1A
TWEAK Signaling	7.66E00	2.05E-01	BIRC2,NFKB1,CYCS,TNFRSF12A,FADD,NFKBIE,NFKBIA,BIRC3
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	7.45E00	6.8E-02	BIRC2,NFKB1,CSF2,IL1B,NFKBIE,BMP2,MMP13,NFATC1,NFKBIA,BIRC3,FOS,IL18,IL1RN, TNF,IL1A,IL17A,IL10
IL-17A Signaling in Fibroblasts	7.44E00	2E-01	FOS,NFKB1,CEBPD,IL17A,NFKBIE,NFKBIZ,NFKBIA,NFKBID
Altered T Cell and B Cell Signaling in Rheumatoid Arthritis	7.22E00	1.1E-01	IL18,NFKB1,IL1RN,CSF2,IL1B,TNF,IL22,IL1A,IL17A,IL10,CD40
p38 MAPK Signaling	6.7E00	1E-01	DUSP10,IL18,IL1RN,DUSP1,IL1B,CREB5,TNF,IL1A,H3F3A/H3F3B,FADD,IRAK2,PLA2G4A
Induction of Apoptosis by HIV1	6.68E00	1.34E-01	BIRC2,CXCR4,NFKB1,CYCS,TNF,FADD,NFKBIE,NFKBIA,BIRC3
iNOS Signaling	6.61E00	1.51E-01	FOS,IRF1,IFNGR1,NFKB1,JAK2,NFKBIE,IRAK2,NFKBIA
Role of IL-17F in Allergic Inflammatory Airway Diseases	6.53E00	1.67E-01	NFKB1,CSF2,IL1B,CREB5,CCL4,MMP13,IL17F,CXCL8
NF-κB Signaling	6.48E00	7.73E-02	NFKB1,IL1B,BMP2,NFKBIE,FADD,CD40,NFKBIA,PELI1,IL18,IL1RN,TNFAIP3,BCL10,TNF,IL1A
Type I Diabetes Mellitus Signaling	6.2E00	9.09E-02	IRF1,IFNGR1,NFKB1,CYCS,IL1B,JAK2,SOCS3,TNF,FADD,NFKBIE,NFKBIA
IL-17A Signaling in Gastric Cells	5.84E00	2.14E-01	FOS,NFKB1,TNF,CCL20,IL17A,CXCL8
Acute Phase Response Signaling	5.81E00	7.18E-02	NFKB1,JAK2,IL1B,SOCS3,NFKBIE,NFKBIA,FOS,IL18,IL1RN,NR3C1,TNF,IL1A,HAMP
Role of PKR in Interferon Induction and Antiviral Response	5.73E00	1.43E-01	IRF1,NFKB1,CYCS,TNF,FADD,NFKBIE,NFKBIA
Death Receptor Signaling	5.66E00	1.18E-01	BIRC2,NFKB1,CYCS,TNF,FADD,NFKBIE,NFKBIA,BIRC3
Cholecystokinin/Gastrin-mediated Signaling	5.57E00	9.43E-02	FOS,RHOH,IL18,CREM,IL1RN,IL1B,TNF,RHOB,IL1A,RND3
Activation of IRF by Cytosolic Pattern Recognition Receptors	5.38E00	1.08E-01	NFKB1,TNF,IFIH1,IL10,FADD,CD40,NFKBIE,NFKBIA
IL-12 Signaling and Production in Macrophages	5.27E00	7.01E-02	FOS,REL,IRF1,IFNGR1,IL18,NFKB1,STAT4,TNF,IL10,CD40,PPARG
Apoptosis Signaling	5.19E00	9E-02	BIRC2,NFKB1,CYCS,MCL1,BCL2A1,TNF,NFKBIE,NFKBIA,BIRC3
Hepatic Cholestasis	5.12E00	6.01E-02	IL18,NFKB1,IL1RN,IL1B,TNF,IL1A,NFKBIE,IRAK2,NFKBIA,CXCL8,NR0B2
Role of IL-17A in Arthritis	4.83E00	1.09E-01	NFKB1,CCL20,IL17A,NFKBIE,MMP13,NFKBIA,CXCL8
PI3K Signaling in B Lymphocytes	4.65E00	6.99E-02	FOS,NFKB1,BCL10,IL4R,CD40,NFKBIE,NFKBIA,NFATC1,FOXO3,ATF3
Glioma Invasiveness Signaling	4.62E00	1.06E-01	RHOH,ITGAV,PLAU,RHOB,PLAUR,TIMP1,RND3
IL-8 Signaling	4.6E00	5.33E-02	FOS,HBEGF,RHOH,NFKB1,ANGPT2,ITGAV,RHOB,CXCR2,IRAK2,RND3,VCAM1,CXCL8
Role of Tissue Factor in Cancer	4.37E00	6.92E-02	HBEGF,CSF2,ITGAV,IL1B,JAK2,PLAUR,F3,MMP13,CXCL8

ERK5 Signaling	4.34E00	1.03E-01	FOS,CREB5,SGK1,LIF,FOXO3,FOSL1,SH2D2A
Molecular Mechanisms of Cancer	4.23E00	4.12E-02	BIRC2,RHOH,NFKB1,JAK2,BMP2,NFKBIE,FADD,NFKBIA,BIRC3,FOS,CYCS,SMAD3,RHOB, CDKN1A,RND3,PMAIP1
Crosstalk between Dendritic Cells and Natural Killer Cells	4.23E00	7.55E-02	CCR7,CD83,IL18,NFKB1,CSF2,TNF,CD40,CD69
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	4.1E00	5.19E-02	FOS,IRF1,IFNGR1,RHOH,NFKB1,JAK2,TNF,RHOB,NFKBIE,NFKBIA,RND3
MIF-mediated Glucocorticoid Regulation	3.92E00	1.19E-01	NFKB1,NR3C1,NFKBIE,NFKBIA,PLA2G4A
ERK/MAPK Signaling	3.91E00	5.21E-02	FOS,DUSP1,CREB5,H3F3A/H3F3B,DUSP6,ETS1,PPARG,ELF3,NFATC1,PLA2G4A,ETS2
Lymphotoxin β Receptor Signaling	3.83E00	9.68E-02	BIRC2,NFKB1,CYCS,NFKBIA,NFKBID,VCAM1
Coagulation System	3.8E00	1.32E-01	PLAU,THBD,PLAUR,F3,PLAT
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	3.76E00	7.87E-02	FOS,NFKB1,BCL10,SMAD3,NFKBIE,NFKBIA,NFATC1
GADD45 Signaling	3.71E00	1.67E-01	CDKN1A,GADD45B,GADD45A,GADD45G
April Mediated Signaling	3.62E00	1.14E-01	FOS,NFKB1,NFKBIE,NFKBIA,NFATC1
ATM Signaling	3.58E00	9.09E-02	CREB5,CDKN1A,GADD45B,GADD45A,NFKBIA,GADD45G
FXR/RXR Activation	3.53E00	6.36E-02	IL18,IL1RN,IL1B,TNF,IL1A,PPARG,NR0B2
B Cell Activating Factor Signaling	3.52E00	1.09E-01	FOS,NFKB1,NFKBIE,NFKBIA,NFATC1
RANK Signaling in Osteoclasts	3.49E00	7.22E-02	FOS,BIRC2,NFKB1,NFKBIE,NFKBIA,NFATC1,BIRC3
MIF Regulation of Innate Immunity	3.47E00	9.62E-02	FOS,NFKB1,NFKBIE,NFKBIA,PLA2G4A
CD40 Signaling	3.46E00	8.45E-02	FOS,NFKB1,TNFAIP3,CD40,NFKBIE,NFKBIA
IL-17A Signaling in Airway Cells	3.42E00	7.89E-02	NFKB1,JAK2,CCL20,IL17A,NFKBIE,NFKBIA
Role of RIG1-like Receptors in Antiviral Innate Immunity	3.32E00	1.02E-01	NFKB1,IFIH1,FADD,NFKBIE,NFKBIA
Erythropoietin Signaling	3.32E00	7.59E-02	FOS,NFKB1,JAK2,SOCS3,NFKBIE,NFKBIA
PI3K/AKT Signaling	3.29E00	5.26E-02	NFKB1,JAK2,MCL1,CDKN1A,NOS3,NFKBIE,NFKBIA,FOXO3
Tec Kinase Signaling	3.25E00	4.89E-02	FOS,RHOH,NFKB1,STAT4,JAK2,TNF,RHOB,FADD,RND3
Graft-versus-Host Disease Signaling	3.23E00	9.8E-02	IL18,IL1RN,IL1B,TNF,IL1A
Antioxidant Action of Vitamin C	3.21E00	6.31E-02	NFKB1,CSF2,JAK2,TNF,NFKBIE,NFKBIA,PLA2G4A
B Cell Receptor Signaling	3.19E00	5.14E-02	BCL6,NFKB1,BCL10,CREB5,BCL2A1,ETS1,NFKBIE,NFKBIA,NFATC1
IL-17 Signaling	3.15E00	8E-02	NFKB1,JAK2,IL17A,TIMP1,IL17F,CXCL8
Role of IL-17A in Psoriasis	3.08E00	2.14E-01	CCL20,IL17A,CXCL8
CD27 Signaling in Lymphocytes	3.02E00	8.47E-02	FOS,NFKB1,CYCS,NFKBIE,NFKBIA
Hematopoiesis from Pluripotent Stem Cells	3.02E00	7.94E-02	CSF2,IL1A,LIF,IL10,CXCL8
Colorectal Cancer Metastasis Signaling	2.98E00	4.1E-02	FOS,IFNGR1,RHOH,NFKB1,JAK2,TNF,RHOB,SMAD3,MMP12,MMP13,RND3
VDR/RXR Activation	2.96E00	6.82E-02	CSF2,THBD,CDKN1A,MXD1,GADD45A,KLF4

4-1BB Signaling in T Lymphocytes	2.96E00	1.11E-01	NFKB1,TNFRSF9,NFKBIE,NFKBIA
Toll-like Receptor Signaling	2.84E00	7.81E-02	FOS,NFKB1,TNFAIP3,IRAK2,NFKBIA
Gαq Signaling	2.76E00	4.68E-02	RHOH,NFKB1,RHOB,RGS2,NFKBIE,NFKBIA,NFATC1,RND3
Type II Diabetes Mellitus Signaling	2.75E00	4.09E-02	ACSL4,NFKB1,SOCS3,TNF,NFKBIE,PPARG,NFKBIA
Protein Kinase A Signaling	2.73E00	3.42E-02	DUSP10,NFKB1,H3F3A/H3F3B,NFKBIE,PDE4B,H1F0,NFATC1,NFKBIA,CREM,CREB5,DUSP1, SMAD3,DUSP6,NOS3
Systemic Lupus Erythematosus Signaling	2.71E00	3.91E-02	FOS,IL18,CREM,IL1RN,IL1B,TNF,IL1A,IL10,CD40,NFATC1
ILK Signaling	2.7E00	4.39E-02	FOS,RHOH,NFKB1,CREB5,SNAI2,TNF,RHOB,BMP2,RND3
IL-1 Signaling	2.57E00	5.5E-02	FOS,NFKB1,IL1A,NFKBIE,IRAK2,NFKBIA
Angiopoietin Signaling	2.55E00	6.67E-02	NFKB1,ANGPT2,NOS3,NFKBIE,NFKBIA
Hypoxia Signaling in the Cardiovascular System	2.52E00	7.35E-02	CREB5,EDN1,NOS3,NFKBIE,NFKBIA
JAK/Stat Signaling	2.49E00	7.04E-02	FOS,STAT4,JAK2,SOCS3,CDKN1A
Neurotrophin/TRK Signaling	2.46E00	6.58E-02	FOS,CREB5,SPRY2,SPRY1,BDNF
p53 Signaling	2.46E00	5.31E-02	SNAI2,CDKN1A,GADD45B,GADD45A,PMAIP1,GADD45G
Small Cell Lung Cancer Signaling	2.38E00	5.32E-02	BIRC2,NFKB1,CYCS,NFKBIE,NFKBIA
PEDF Signaling	2.38E00	6.33E-02	NFKB1,NFKBIE,PPARG,NFKBIA,BDNF
Prolactin Signaling	2.33E00	5.95E-02	FOS,IRF1,JAK2,SOCS3,NR3C1
Aryl Hydrocarbon Receptor Signaling	2.3E00	4.09E-02	FOS,NFKB1,IL1B,TNF,IL1A,CDKN1A,NR0B2
RAR Activation	2.29E00	4.1E-02	FOS,REL,NFKB1,DUSP1,JAK2,SMAD3,BMP2,CITED2
Role of JAK1, JAK2 and TYK2 in Interferon Signaling	2.28E00	1.07E-01	IFNGR1,NFKB1,JAK2
iCOS-iCOSL Signaling in T Helper Cells	2.27E00	4.76E-02	NFKB1,ICOSLG/LOC102723996,CD40,NFKBIE,NFKBIA,NFATC1
G-Protein Coupled Receptor Signaling	2.26E00	3.62E-02	NFKB1,DUSP1,CREB5,CXCR2,RGS2,ADORA3,DUSP6,NFKBIE,PDE4B,NFKBIA
Airway Pathology in Chronic Obstructive Pulmonary Disease	2.23E00	1.82E-01	TNF,CXCL8
IL-15 Production	2.13E00	9.68E-02	IRF1,NFKB1,JAK2
Pathogenesis of Multiple Sclerosis	2.13E00	2E-01	CCL3,CCL4
Semaphorin Signaling in Neurons	2.13E00	7.41E-02	RHOH,RHOB,RND3,RND1
CD28 Signaling in T Helper Cells	2.1E00	4.41E-02	FOS,NFKB1,BCL10,NFKBIE,NFKBIA,NFATC1
PKCθ Signaling in T Lymphocytes	2.1E00	4.17E-02	FOS,NFKB1,BCL10,NFKBIE,NFKBIA,NFATC1
Prostate Cancer Signaling	2.1E00	4.85E-02	NFKB1,CREB5,CDKN1A,NFKBIE,NFKBIA
LXR/RXR Activation	2.02E00	4.32E-02	IL18,NFKB1,IL1RN,IL1B,TNF,IL1A
TGF-β Signaling	2.01E00	5.32E-02	FOS,INHBA,SMAD3,PMEPA1,BMP2
Leukocyte Extravasation Signaling	2.01E00	3.81E-02	RHOH,CXCR4,MMP12,EZR,CLDN4,TIMP1,MMP13,VCAM1
IL-15 Signaling	1.89E00	5.56E-02	NFKB1,CSF2,JAK2,IL17A

SAPK/JNK Signaling	1.86E00	4.76E-02	DUSP10,GADD45A,FADD,NFATC1,SH2D2A
Circadian Rhythm Signaling	1.86E00	7.89E-02	BHLHE40,CREB5,NR1D1
IL-9 Signaling	1.86E00	7.5E-02	NFKB1,SOCS3,TNF
Oncostatin M Signaling	1.86E00	8.57E-02	JAK2,PLAU,MMP13
Interferon Signaling	1.86E00	8.33E-02	IRF1,IFNGR1,JAK2
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	1.84E00	4.59E-02	NFKB1,IL1B,TNF,IFIH1,IL10
GM-CSF Signaling	1.84E00	5.88E-02	CSF2,JAK2,BCL2A1,ETS1
T Cell Receptor Signaling	1.82E00	4.59E-02	FOS,NFKB1,BCL10,NFKBIA,NFATC1
Relaxin Signaling	1.77E00	3.66E-02	FOS,NFKB1,NOS3,NFKBIE,PDE4B,NFKBIA
HGF Signaling	1.77E00	4.5E-02	FOS,CDKN1A,ETS1,ELF3,ETS2
PPARα/RXRα Activation	1.77E00	3.5E-02	NFKB1,IL1B,JAK2,SMAD3,NFKBIE,NFKBIA,NR0B2
cAMP-mediated signaling	1.75E00	3.54E-02	CREM,DUSP1,CREB5,CXCR2,RGS2,ADORA3,DUSP6,PDE4B
PXR/RXR Activation	1.75E00	4.35E-02	NR3C1,TNF,FOXO3,NR0B2
Retinoic acid Mediated Apoptosis Signaling	1.75E00	5.48E-02	TIPARP,IRF1,CYCS,FADD
LPS/IL-1 Mediated Inhibition of RXR Function	1.74E00	3.27E-02	ACSL4,IL18,IL1RN,IL1B,TNF,IL1A,SMOX,NR0B2
Docosahexaenoic Acid (DHA) Signaling	1.7E00	6E-02	CYCS,IL1B,BCL2A1
Inhibition of Matrix Metalloproteases	1.7E00	7.5E-02	MMP12,TIMP1,MMP13
Pancreatic Adenocarcinoma Signaling	1.66E00	3.91E-02	HBEGF,NFKB1,JAK2,SMAD3,CDKN1A
Extrinsic Prothrombin Activation Pathway	1.64E00	9.09E-02	THBD,F3
LPS-stimulated MAPK Signaling	1.63E00	4.82E-02	FOS,NFKB1,NFKBIE,NFKBIA
NF-κB Activation by Viruses	1.61E00	4.82E-02	NFKB1,ITGAV,NFKBIE,NFKBIA
IL-4 Signaling	1.61E00	5E-02	JAK2,NR3C1,IL4R,NFATC1
Corticotropin Releasing Hormone Signaling	1.57E00	3.45E-02	FOS,JUND,CREB5,NOS3,BDNF
Integrin Signaling	1.54E00	3.37E-02	RHOH,ITGAV,RHOB,NEDD9,BCAR3,ARF6,RND3
Thiosulfate Disproportionation III (Rhodanese)	1.53E00	1.67E-01	MOCS3
Ceramide Signaling	1.5E00	4.4E-02	FOS,NFKB1,CYCS,TNF
Polyamine Regulation in Colon Cancer	1.38E00	6.67E-02	MXD1,PPARG
Role of NFAT in Regulation of the Immune Response	1.36E00	3E-02	FOS,NFKB1,RCAN1,NFKBIE,NFKBIA,NFATC1
Bladder Cancer Signaling	1.36E00	4.12E-02	MMP12,CDKN1A,MMP13,CXCL8
IL-22 Signaling	1.31E00	8E-02	SOCS3,IL22
Tumoricidal Function of Hepatic Natural Killer Cells	1.31E00	7.41E-02	CYCS,FADD
Actin Nucleation by ARP-WASP Complex	1.29E00	4.48E-02	RHOH,RHOB,RND3
Role of JAK family kinases in IL-6-type Cytokine	1.28E00	7.14E-02	JAK2,SOCS3

Signaling			
Regulation of the Epithelial-Mesenchymal Transition Pathway	1.27E00	3.06E-02	NFKB1,JAK2,ID2,SNAI2,SMAD3,ETS1
IGF-1 Signaling	1.24E00	3.74E-02	FOS,JAK2,SOCS3,FOXO3
Airway Inflammation in Asthma	1.23E00	1.67E-01	TNF
Spermine and Spermidine Degradation I	1.23E00	7.14E-02	SMOX
Melatonin Degradation II	1.23E00	8.33E-02	SMOX
Molybdenum Cofactor Biosynthesis	1.23E00	6.25E-02	MOCS3
Phospholipase C Signaling	1.21E00	2.64E-02	RHOH,NFKB1,CREB5,RHOB,NFATC1,PLA2G4A,RND3
Telomerase Signaling	1.19E00	3.77E-02	CDKN1A,ETS1,ELF3,ETS2
Role of JAK1 and JAK3 in γc Cytokine Signaling	1.19E00	4.41E-02	JAK2,SOCS3,IL4R
Estrogen-Dependent Breast Cancer Signaling	1.17E00	4.11E-02	FOS,NFKB1,CREB5
HIF1α Signaling	1.17E00	3.57E-02	MMP12,EDN1,NOS3,MMP13
Tetrahydrofolate Salvage from 5,10-methenyltetrahydrofolate	1.14E00	1E-01	MTHFD1L
Citrulline-Nitric Oxide Cycle	1.14E00	6.25E-02	NOS3
Role of PI3K/AKT Signaling in the Pathogenesis of Influenza	1.14E00	3.95E-02	NFKB1,NFKBIE,NFKBIA
Tight Junction Signaling	1.1E00	2.99E-02	FOS,NFKB1,MPP5,TNF,CLDN4
CXCR4 Signaling	1.1E00	2.87E-02	FOS,RHOH,CXCR4,RHOB,RND3
fMLP Signaling in Neutrophils	1.1E00	3.03E-02	NFKB1,NFKBIE,NFKBIA,NFATC1
Renin-Angiotensin Signaling	1.1E00	3.17E-02	FOS,NFKB1,JAK2,TNF
Growth Hormone Signaling	1.08E00	3.85E-02	FOS,JAK2,SOCS3
Chemokine Signaling	1.08E00	4E-02	FOS,CXCR4,CCL4
CCR5 Signaling in Macrophages	1.06E00	3.09E-02	FOS,CCL3,CCL4
Role of JAK2 in Hormone-like Cytokine Signaling	1.04E00	5.41E-02	JAK2,SOCS3
STAT3 Pathway	1.02E00	3.75E-02	JAK2,SOCS3,CDKN1A
Hereditary Breast Cancer Signaling	1.02E00	2.99E-02	CDKN1A,GADD45B,GADD45A,GADD45G
Cell Cycle Regulation by BTG Family Proteins	1.02E00	5.13E-02	BTG1,BTG2
Folate Polyglutamylation	1E00	5E-02	MTHFD1L
Gα12/13 Signaling	1E00	3.15E-02	LPAR6,NFKB1,NFKBIE,NFKBIA
RhoA Signaling	1E00	3.25E-02	LPAR6,EZR,RHPN2,RND3
VEGF Family Ligand-Receptor Interactions	9.81E-01	3.41E-02	FOS,NOS3,PLA2G4A
BMP signaling pathway	9.81E-01	3.49E-02	NFKB1,FST,BMP2
Histidine Degradation III	9.46E-01	5.56E-02	MTHFD1L
Tryptophan Degradation to 2-amino-3-	9.46E-01	5.56E-02	IDO1

carboxymuconate Semialdehyde			
Endothelin-1 Signaling	9.39E-01	2.6E-02	FOS,EDN1,EDNRB,NOS3,PLA2G4A
Thyroid Cancer Signaling	9.23E-01	4.55E-02	PPARG,BDNF
Ephrin Receptor Signaling	9.16E-01	2.38E-02	EFNB2,CXCR4,JAK2,CREB5,EPHA2
Regulation of Actin-based Motility by Rho	8.96E-01	3.26E-02	RHOH,RHOB,RND3
Allograft Rejection Signaling	8.96E-01	3.09E-02	TNF,IL10,CD40
Signaling by Rho Family GTPases	8.79E-01	2.28E-02	FOS,RHOH,NFKB1,RHOB,EZR,RND3
ErbB Signaling	8.74E-01	3.33E-02	FOS,HBEGF,AREG
Insulin Receptor Signaling	8.7E-01	2.68E-02	JAK2,SOCS3,SGK1,FOXO3
OX40 Signaling Pathway	8.63E-01	3.09E-02	NFKB1,NFKBIE,NFKBIA
Folate Transformations I	8.55E-01	3.03E-02	MTHFD1L
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	8.55E-01	4.08E-02	CDKN1A,GADD45A
Human Embryonic Stem Cell Pluripotency	8.44E-01	2.47E-02	INHBA,SMAD3,BMP2,BDNF
MSP-RON Signaling Pathway	8.39E-01	3.92E-02	JAK2,TNF
UVA-Induced MAPK Signaling	8.11E-01	3.06E-02	FOS,TIPARP,CYCS
Autoimmune Thyroid Disease Signaling	8.09E-01	3.23E-02	IL10,CD40
Thrombin Signaling	8.03E-01	2.37E-02	GATA3,RHOH,NFKB1,RHOB,RND3
VEGF Signaling	8.01E-01	2.75E-02	NOS3,FOXO3,SH2D2A
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	7.91E-01	2.83E-02	CSF2,EZR,ARF6
Hematopoiesis from Multipotent Stem Cells	7.83E-01	8.33E-02	CSF2
Chronic Myeloid Leukemia Signaling	7.82E-01	2.83E-02	NFKB1,SMAD3,CDKN1A
Mouse Embryonic Stem Cell Pluripotency	7.72E-01	3.03E-02	JAK2,ID2,LIF
Glioblastoma Multiforme Signaling	7.52E-01	2.38E-02	RHOH,RHOB,CDKN1A,RND3
Fatty Acid Activation	7.51E-01	5.26E-02	ACSL4
Amyotrophic Lateral Sclerosis Signaling	7.36E-01	2.38E-02	BIRC2,CYCS,BIRC3
UVB-Induced MAPK Signaling	7.28E-01	3.45E-02	FOS,H3F3A/H3F3B
Superpathway of Citrulline Metabolism	7.22E-01	2.63E-02	NOS3
Phenylalanine Degradation IV (Mammalian, via Side Chain)	7.22E-01	2.56E-02	SMOX
Thrombopoietin Signaling	7.04E-01	3.12E-02	FOS,JAK2
Nur77 Signaling in T Lymphocytes	7.04E-01	3.12E-02	CYCS,NFATC1
Axonal Guidance Signaling	7E-01	1.85E-02	EFNB2,CXCR4,BMP2,MMP13,EPHA2,NFATC1,BDNF,RND1,ADAMTS1
NAD biosynthesis II (from tryptophan)	6.95E-01	2.86E-02	IDO1

Germ Cell-Sertoli Cell Junction Signaling	6.9E-01	2.37E-02	RHOH,TNF,RHOB,RND3
Granzyme B Signaling	6.7E-01	5.56E-02	CYCS
Parkinson's Signaling	6.7E-01	5.26E-02	CYCS
Myc Mediated Apoptosis Signaling	6.69E-01	3.17E-02	CYCS,FADD
Xenobiotic Metabolism Signaling	6.6E-01	2.08E-02	NFKB1,IL1B,TNF,IL1A,SMOX,CITED2
Fc Epsilon RI Signaling	6.54E-01	2.56E-02	CSF2,TNF,PLA2G4A
Role of BRCA1 in DNA Damage Response	6.48E-01	2.82E-02	CDKN1A,GADD45A
γ-linolenate Biosynthesis II (Animals)	6.47E-01	4.17E-02	ACSL4
Mitochondrial L-carnitine Shuttle Pathway	6.47E-01	4.55E-02	ACSL4
Putrescine Degradation III	6.47E-01	3.33E-02	SMOX
Sphingosine-1-phosphate Signaling	6.46E-01	2.44E-02	RHOH,RHOB,RND3
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	6.31E-01	2.52E-02	JAK2,LIF,BMP2
Tryptophan Degradation X (Mammalian, via Tryptamine)	6.25E-01	3.45E-02	SMOX
Huntington's Disease Signaling	6.2E-01	1.98E-02	HSPA2,CYCS,CREB5,SGK1,BDNF
Cardiomyocyte Differentiation via BMP Receptors	6.05E-01	4.55E-02	BMP2
Granzyme A Signaling	6.05E-01	5E-02	H1F0
1D-myo-inositol Hexakisphosphate Biosynthesis II (Mammalian)	6.05E-01	3.57E-02	ITPKC
D-myo-inositol (1,3,4)-trisphosphate Biosynthesis	6.05E-01	4E-02	ITPKC
PTEN Signaling	5.96E-01	2.17E-02	NFKB1,CDKN1A,FOXO3
Cell Cycle: G1/S Checkpoint Regulation	5.88E-01	2.78E-02	SMAD3,CDKN1A
DNA Methylation and Transcriptional Repression Signaling	5.86E-01	4.35E-02	ARID4B
RhoGDI Signaling	5.82E-01	1.98E-02	RHOH,RHOB,EZR,RND3
Role of MAPK Signaling in the Pathogenesis of Influenza	5.7E-01	2.78E-02	TNF,PLA2G4A
Tryptophan Degradation III (Eukaryotic)	5.68E-01	2.08E-02	IDO1
P2Y Purigenic Receptor Signaling Pathway	5.63E-01	2.08E-02	FOS,NFKB1,CREB5
D-myo-inositol (1,4,5,6)-Tetrakisphosphate Biosynthesis	5.56E-01	2.08E-02	DUSP10,DUSP1,SOCS3
D-myo-inositol (3,4,5,6)-tetrakisphosphate Biosynthesis	5.56E-01	2.08E-02	DUSP10,DUSP1,SOCS3
Renal Cell Carcinoma Signaling	5.52E-01	2.53E-02	FOS,ETS1
NRF2-mediated Oxidative Stress Response	5.51E-01	2.05E-02	FOS,JUND,FOSL1,ENC1

IL-3 Signaling	5.44E-01	2.67E-02	FOS,JAK2
Estrogen Receptor Signaling	5.32E-01	2.21E-02	NR3C1,H3F3A/H3F3B,NR0B2
Ephrin B Signaling	5.19E-01	2.44E-02	EFNB2,CXCR4
Estrogen-mediated S-phase Entry	5.19E-01	3.57E-02	CDKN1A
Dopamine Degradation	5.19E-01	2.63E-02	SMOX
Superpathway of D-myo-inositol (1,4,5)-trisphosphate Metabolism	5.19E-01	3.03E-02	ITPKC
GNRH Signaling	5.14E-01	1.96E-02	FOS,NFKB1,CREB5
FLT3 Signaling in Hematopoietic Progenitor Cells	5.12E-01	2.53E-02	STAT4,CREB5
Leptin Signaling in Obesity	5.12E-01	2.35E-02	JAK2,SOCS3
eNOS Signaling	5.08E-01	1.94E-02	HSPA2,LPAR6,NOS3
HER-2 Signaling in Breast Cancer	5.04E-01	2.44E-02	AREG,CDKN1A
Superpathway of Inositol Phosphate Compounds	5.02E-01	1.71E-02	DUSP10,DUSP1,SOCS3,ITPKC
PDGF Signaling	4.96E-01	2.33E-02	FOS,JAK2
Lipid Antigen Presentation by CD1	4.9E-01	3.33E-02	ARF6
Antiproliferative Role of TOB in T Cell Signaling	4.9E-01	3.85E-02	SMAD3
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	4.68E-01	2.27E-02	CYCS,FADD
Intrinsic Prothrombin Activation Pathway	4.64E-01	2.7E-02	THBD
Pyrimidine Ribonucleotides Interconversion	4.64E-01	2.5E-02	ENTPD7
D-myo-inositol-5-phosphate Metabolism	4.6E-01	1.85E-02	DUSP10,DUSP1,SOCS3
3-phosphoinositide Degradation	4.6E-01	1.85E-02	DUSP10,DUSP1,SOCS3
Pyrimidine Ribonucleotides De Novo Biosynthesis	4.4E-01	1.85E-02	ENTPD7
Fatty Acid β-oxidation I	4.4E-01	2.22E-02	ACSL4
Neuregulin Signaling	4.22E-01	1.92E-02	HBEGF,AREG
B Cell Development	4.18E-01	2.78E-02	CD40
Retinoate Biosynthesis I	4.18E-01	2.7E-02	BMP2
Serotonin Receptor Signaling	4.08E-01	2.04E-02	SMOX
3-phosphoinositide Biosynthesis	4.03E-01	1.66E-02	DUSP10,DUSP1,SOCS3
Aldosterone Signaling in Epithelial Cells	3.98E-01	1.78E-02	HSPA2,DUSP1,SGK1
G Protein Signaling Mediated by Tubby	3.98E-01	2.27E-02	JAK2
Inhibition of Angiogenesis by TSP1	3.98E-01	2.38E-02	NOS3
tRNA Splicing	3.88E-01	2.17E-02	PDE4B
Stearate Biosynthesis I (Animals)	3.88E-01	2.04E-02	ACSL4
Noradrenaline and Adrenaline Degradation	3.79E-01	1.89E-02	SMOX

Neuropathic Pain Signaling In Dorsal Horn Neurons	3.56E-01	1.83E-02	FOS,BDNF
Netrin Signaling	3.53E-01	1.72E-02	NFATC1
Paxillin Signaling	3.51E-01	1.71E-02	ITGAV,ARF6
Transcriptional Regulatory Network in Embryonic Stem Cells	3.45E-01	2.33E-02	SKIL
Melanoma Signaling	3.3E-01	2E-02	CDKN1A
UVC-Induced MAPK Signaling	3.3E-01	2.27E-02	FOS
NGF Signaling	3.23E-01	1.64E-02	NFKB1,CREB5
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	3.08E-01	1.92E-02	JARID2
Sertoli Cell-Sertoli Cell Junction Signaling	3.07E-01	1.52E-02	TNF,CLDN4,NOS3
Androgen Signaling	3.02E-01	1.38E-02	NFKB1,SMAD3
Gαs Signaling	3.02E-01	1.6E-02	CREB5,RGS2
Calcium Signaling	2.97E-01	1.38E-02	CREB5,RCAN1,NFATC1
Ephrin A Signaling	2.89E-01	1.85E-02	EPHA2
14-3-3-mediated Signaling	2.82E-01	1.65E-02	FOS,TNF
Protein Ubiquitination Pathway	2.76E-01	1.48E-02	HSPA2,BIRC2,NEDD4L,BIRC3
Sperm Motility	2.74E-01	1.39E-02	PDE4B,PLA2G4A
CNTF Signaling	2.66E-01	1.75E-02	JAK2
Endometrial Cancer Signaling	2.66E-01	1.67E-02	FOXO3
Primary Immunodeficiency Signaling	2.66E-01	1.56E-02	CD40
mTOR Signaling	2.64E-01	1.41E-02	RHOH,RHOB,RND3
Gαi Signaling	2.63E-01	1.48E-02	CXCR2,ADORA3
IL-2 Signaling	2.6E-01	1.64E-02	FOS
Role of CHK Proteins in Cell Cycle Checkpoint Control	2.5E-01	1.69E-02	CDKN1A
EGF Signaling	2.45E-01	1.56E-02	FOS
Cellular Effects of Sildenafil (Viagra)	2.4E-01	1.29E-02	NOS3, PDE4B
Regulation of Cellular Mechanics by Calpain Protease	2.4E-01	1.37E-02	EZR
Phospholipases	2.35E-01	1.47E-02	PLA2G4A
Superpathway of Melatonin Degradation	2.26E-01	1.23E-02	SMOX
Serotonin Degradation	2.21E-01	1.28E-02	SMOX
Eicosanoid Signaling	2.13E-01	1.16E-02	PLA2G4A
Antiproliferative Role of Somatostatin Receptor 2	2.09E-01	1.39E-02	CDKN1A

Non-Small Cell Lung Cancer Signaling	2.05E-01	1.2E-02	FOXO3
Pyridoxal 5'-phosphate Salvage Pathway	2.01E-01	1.32E-02	SGK1
Mitotic Roles of Polo-Like Kinase	1.97E-01	1.35E-02	PLK2

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Part b: Pathways down-regulated

Ingenuity Canonical Pathways	**-log (p-value)**	**Ratio**	**Molecules**
PXR/RXR Activation	2.28E00	2.17E-02	ABCC2,CPT1A
FXR/RXR Activation	2.07E00	1.82E-02	ABCC2,MTTP
Mitochondrial L-carnitine Shuttle Pathway	1.57E00	4.55E-02	CPT1A
Granulocyte Adhesion and Diapedesis	1.49E00	1.1E-02	CCL24,PECAM1
Agranulocyte Adhesion and Diapedesis	1.44E00	1.04E-02	CCL24,PECAM1
LPS/IL-1 Mediated Inhibition of RXR Function	1.31E00	8.16E-03	ABCC2,CPT1A
Complement System	1.28E00	2.86E-02	C4BPA
Erythropoietin Signaling	9.87E-01	1.27E-02	CBL
Chemokine Signaling	9.75E-01	1.33E-02	CCL24
Ephrin B Signaling	9.46E-01	1.22E-02	CBL
FLT3 Signaling in Hematopoietic Progenitor Cells	9.41E-01	1.27E-02	CBL
RANK Signaling in Osteoclasts	8.81E-01	1.03E-02	CBL
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	8.54E-01	9.43E-03	CBL
T Cell Receptor Signaling	8.37E-01	9.17E-03	CBL
Telomerase Signaling	8.2E-01	9.43E-03	TPP1
HIF1α Signaling	8.13E-01	8.93E-03	EGLN1
CCR3 Signaling in Eosinophils	7.69E-01	7.46E-03	CCL24
14-3-3-mediated Signaling	7.62E-01	8.26E-03	CBL
PTEN Signaling	7.58E-01	7.19E-03	CBL
Gαi Signaling	7.45E-01	7.41E-03	NPR3
PI3K Signaling in B Lymphocytes	7.26E-01	6.99E-03	CBL
Insulin Receptor Signaling	7.14E-01	6.71E-03	CBL
AMPK Signaling	7.06E-01	5.52E-03	CPT1A
Hepatic Cholestasis	6.94E-01	5.46E-03	ABCC2

Mitochondrial Dysfunction	6.26E-01	4.65E-03	CPT1A
Acute Phase Response Signaling	6.19E-01	5.52E-03	C4BPA
NRF2-mediated Oxidative Stress Response	5.95E-01	5.13E-03	ABCC2
Clathrin-mediated Endocytosis Signaling	5.85E-01	5.05E-03	CBL
Leukocyte Extravasation Signaling	5.6E-01	4.76E-03	PECAM1
Actin Cytoskeleton Signaling	5.31E-01	4.13E-03	SSH1
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	5.21E-01	4E-03	CBL
cAMP-mediated signaling	5.21E-01	4.42E-03	NPR3
Systemic Lupus Erythematosus Signaling	5.18E-01	3.92E-03	CBL
Protein Ubiquitination Pathway	4.68E-01	3.7E-03	CBL
G-Protein Coupled Receptor Signaling	4.64E-01	3.62E-03	NPR3
Xenobiotic Metabolism Signaling	4.47E-01	3.47E-03	ABCC2
Molecular Mechanisms of Cancer	3.68E-01	2.58E-03	CBL

Chapter II

In vivo toxicity of purified deepoxy-deoxynivalenol (DOM-1) in piglets

I. Context of the study

In the previous chapter, it was shown that DOM-1, in comparison to DON, has a reduced toxicity on intestinal cells and intestinal explants. The aim of this study was to assess the toxicity of purified DOM-1 on animal model. Actually, the toxicity of purified DOM-1 has never been assessed. Previous experiments were conducted on pigs receiving DON and the bacteria (bacillus sp. Ls100 or contents of the large intestine of chickens (CLIC)), able to deepoxidize DON into DOM-1 into the gut of the animal. In addition, in these experiments that are more focused on the zootechnical parameters such as feed intake and weight gain, they described no effect on treated animals. This prove that microbial detoxification of DON in contaminated feed can eliminate the negative effects of the mycotoxin, and the pre-feeding detoxification approach may be applied in the livestock industry (He et al. 1993; Li et al. 2011).

The aim of the present study was to evaluate the toxic effect of purified DOM-1 based on several parameters, including zootechnical parameters but also on the intestinal and immune responses. In fact, the effects of DON on the intestine and immunity are well characterized and represent a serious risk for animal health. Among animal species, pig constituted a relevant model to evaluate the toxicity of DOM-1, due to its high sensibility to mycotoxin, especially DON and its cereals rich diet.

II. Material and Methods

A. Experimental design

1. Animals

All animal experimentation procedures were carried out in accordance with the European Directive on the protection of animals used for scientific purposes (Directive 2010/63/EU). All the experimental design is summary in the Figure 1.

For the experiment, twenty-two 4-wk-old weaned castrated male pigs (PIC 410) were obtained locally (GAEC Calvignac, St Vincent d'Autejac, France). As previously described a greater effect of DON occurs on male pigs compared to female pigs (Cote et al. 1985; Marin et al. 2006). Animals were acclimatized for 2 weeks in the animal facility of the Toxalim Laboratory (INRA, Toulouse, France), prior to being used in experimental protocols. Eight pigs were allocated per box to each treatment on the basis of body weight for (1) control group and (2) DON group, and six pigs for (3) DOM-1 group. At the start of the experiment, there were no differences in body weights among all treatment groups including the control; piglets weighted 10.9±0.08Kg, $p>0.05$. Then they were weighed and observed daily, and the animal behaviour was noted. During the three weeks of experimental period, each group was given free access to water and feed. They were fed with a commercial feed adapted to their age, complete food for the first piglet age Belecla (Annex 1) and complete food for the second piglet age Stimiouti (Annex 2). A period of transition of 3 days was respected between the two foods. After the experimental period the animals were submitted to electrical stunning, and euthanized by exsanguination.

2. Toxins

Purified DON was purchased from Sigma-Aldrich (St Louis, MO, USA). DOM-1 was obtained by transforming the crystalline DON (Romer Labs, Tulln, Austria), dissolved in medium 10 (Caldwell and Bryant, 1966) at a concentration of 2 mg/ml, and then by inoculating with BBSH 797, Gen. nov. sp. nov. of family Coriobacteriaceae in sterile medium, at 37°C for six days. Biotransformation of DON to deepoxy-DON was confirmed by LC-MS/MS, and deepoxy-DON was purified by solid phase extraction and preparative HPLC (Schwartz-Zimmermann et al., 2014). The purity of the deepoxy-DON preparation was 99%, based on chromatograms recorded at 220nm.

3. Gavage

During the experiment, animals were exposed to DON and DOM-1 as powder at 100% and 63% of purity respectively (provides by Austria BIOMIN GmgH, Tulln). The two molecules were administered by gavage for 21 days, at the same molar concentration (0.15 mg/kg BW /day for DON and 0.14 mg/Kg BW/day for DOM-1). Toxins were diluted in water and black current syrup, to increase the palatability.

4. Sample collection

The experimental design used in this study was randomized with eight or six repetitions (each animal represented one repetition). To evaluate the vaccine response, all

piglets were immunized by subcutaneous inoculation with 1 mg of ovalbumin (OVA) for the primary injection and 2 mg for the booster vaccine, respectively (Sigma, St Quentin Fallavier, France), dissolved in sterile PBS and mixed with incomplete Freund's adjuvant (Sigma). At weekly time intervals, blood samples were aseptically collected from the left jugular vein. Blood was collected in tubes containing sodium heparin or EDTA (Vacutainer®, becton-Dickinson, USA) for blood culture or blood formula, respectively. Serum samples were obtained after centrifugation of blood and stored at -20°C for later analysis. After 21 days of exposure pigs were killed by electrical stunning and exsanguination.

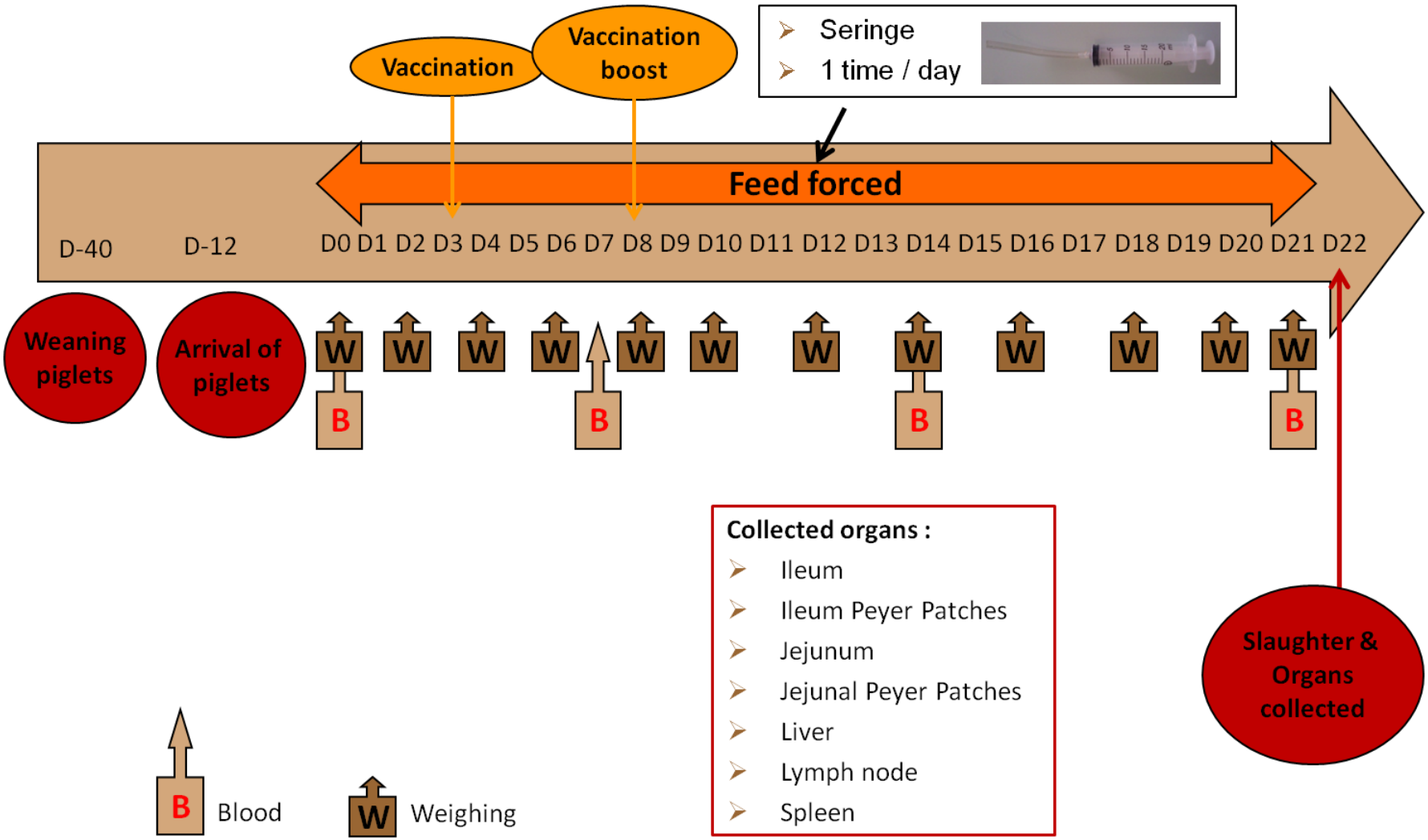
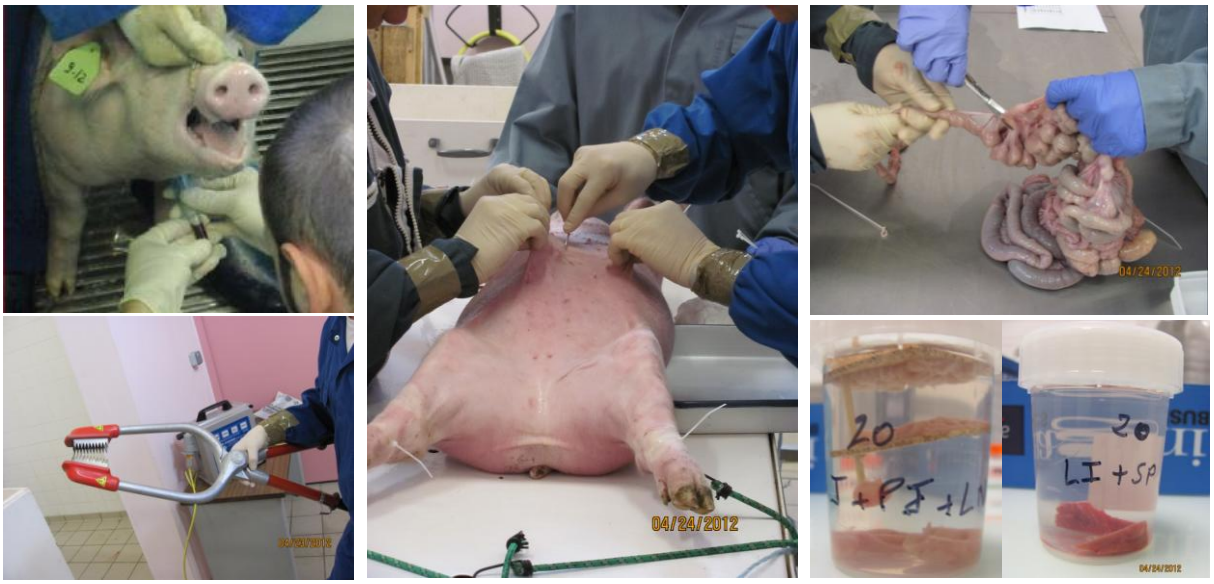


Figure 1 - Experimental design of the *in vivo* experiment



B. Samples analysis

1. Plasma analysis

Total concentration of the immunoglobulin subsets (IgG) was measured by ELISA as already described (Taranu et al. 2005). Briefly, plasma samples diluted 1:15.000 to detect IgG, in Tris–buffered saline and added to plates coated with immunoglobulin class specific pig antibody (Bethyl, Interchim, Montluçon, France). The different subsets were detected with the appropriate peroxidase anti-pig IgG (Bethyl) and were quantified by reference to standard curves constructed with known amounts of pig immunoglobulin subsets. Antibody titles against ovalbumin were also measured by ELISA (Meissonnier et al. 2008). Briefly, ELISA plates were coated with 2µg/mL ovalbumin diluted in 0.05 M NaHCO3 (pH 9.6). Diluted plasma, were then added to the plates and the anti-ovalbumin antibodies were detected with peroxidase-labeled anti-pig IgG (Bethyl). Absorbance was read at 450 nm using an ELISA plate reader (Spectra thermo scan, Tecan, NC, USA).

2. Histology

Fragments of liver, spleen, lymph node and intestine (jejunum with and without Peyer patches, ileum with and without Peyer patches) were fixed in 10% buffered formalin for 24 h,

fixed in ethanol 70%, embedded in paraffin and cut into sections of 3µm. Paraffin sections of all the fragments were stained with the hematoxylin-eosin method.

Histological findings were graduated in scores. The score is based in histological changes and severity of lesions as previously reported (Bracarense et al. 2012; Grenier et al. 2011). Morphometry of intestinal villous and crypt was performed using a MOTIC Image Plus 2.0 ML® image analysis system. Thirty measurements from each fragment of intestine (duodenum, jejunum, ileum and colon) were done to determine villous height and crypts length.

3. mRNA expression for cytokines by real-time PCR

RNA on tissue was extracted with lysing matrix D tubes (MP Biomedicals, Illkirch, France) containing guanidine thiocyanate-acid phenol (Extract-All ; Eurobio, les Ulis, France) for use with the FastPrep-24 (MP Biomedicals, Illkirch, France). Concentration and quality of samples were analysed using Nanodrop ND1000 (Labtech International, Paris France) and using Bioanalyser (Agilent RNA 6000 Nano Kit Quick Start Guide). Then, reverse transcription and real-time qPCR steps were performed as previously described (Meissonnier, 2008). Specificity of qPCR products was assessed at the end of the reactions by analyzing dissociation curves. Primers were purchased from Invitrogen (Invitrogen, Life Technologies Corporation, Paisley, UK). Specific sequences were specified in Table 1. Amplification efficiency and initial fluorescence were determined by the ΔCt method, then obtained values were normalized using a reference gene, the cyclophillin A (Bruel et al. 2010). Stability of these genes was demonstrated previously in intestinal tissues (Delgado-Ortega et al. 2011). mRNA expression levels were expressed relative to the mean of the control group.

Table 1- Primer sequences used for RT-qPCR analysis (F: Forward; R: Reverse)

Gene symbol	Gene name	Primer sequence	References
CycloA	Cyclophilin A	F: CCCACCGTCTTCTTCGACAT	NM_214353
		R: TCTGCTGTCTTTGGAACTTTGTCT	Curtis MM 2009
IL1beta	Interleukin 1 beta	F: ATGCTGAAGGCTCTCCACCTC	NM_214055
		R: TTGTTGCTATCATCTCCTTGCAC	Von der Hardt et al.2004
IL6	Interleukin 6	F: TTCACCTCTCCGGACAAAACTG	NM_214399
		R: TCTGCCAGTACCTCCTTGCTGT	Grenier et al. 2011
IL8	Interleukin 8	F: GCTCTCTGTGAGGCTGCAGTTC	NM_213867
		R: AAGGTGTGGAATGCGTATTTATGC	Grenier et al. 2011
IL1alpha	Interleukin 1 alpha	F: TCAGCCGCCCATCCA	NM_214029,1
		R: AGCCCCCGGTGCCATGT	Cano et al. 2013
IL10	Interleukin 10	F: GGCCCAGTGAAGAGTTTCTTTC	NM_214041
		R: CAACAAGTCGCCCATCTGGT	Bracarense 2012
IL17alpha	Interleukin 17 alpha	F: CCAGACGGCCCTCAGATTAC	AB102693
		R: CACTTGGCCTCCCAGATCAC	Cano et al. 2013
IFNgamma	Interferon gamma	F: TGGTAGCTCTGGGAAACTGAATG	NM_213948
		R: GGCTTTGCGCTGGATCTG	Royaee et al. 2004
TNFalpha	Tumor necrosis factor alpha	F: ACTGCACTTCGAGGTTATCGG	NM_214022
		R: GGCGACGGGCTTATCTGA	Meissonnier et al. 2008
TGFbeta	Transforming growth factor beta	F: GAAGCGCATCGAGGCCATTC	NM_214015
		R: GGCTCCGGTTCGACACTTTC	Meurens et al. 2009
CCL20	Chemokine (C-C motif) ligand 20	F: GCTCCTGGCTGCTTTGATGTC	NM_001024589
		R: CATTGGCGAGCTGCTGTGTG	Meurens et al. 2009
TLR2	Toll like receptor 2	F: TCACTTGTCTAACTTATCATCCTCTTG	AB085935
		R: TCAGCGAAGGTGTCATTATTGC	Arce et al. 2010
TLR4	Toll like receptor 4	F: GCCATCGCTGCTAACATCATC	AB188301
		R: CTCATACTCAAAGATACACCATCGG	Arce et al. 2010

4. Statistical analysis

For statistical analysis, a One-way or Two-way ANOVA were realized with a Bonferroni test as Post-hoc, $p<0.05$.

III. Results

A. Comparative effect of DOM-1 and DON on animal performance

During the experiment, the mean body weight gained per animal was no significantly different between groups (Fig. 2), excepted on week 2, where DOM-1 group had a weight gain higher in comparison to other groups (Fig. 2).

In another study with similar design but higher dose of toxins (0.3 mg DON or DOM-1 /Kg BW for 2 weeks) we obtained similar results. Animals in the DOM-1 group had a significant higher weight gain on the second week compared to than animals in the control and DON groups, while animals in the DON group had a significant lower weight gain on the first week (Fig. 3). Morover 4 of 6 pigs receiving DON vomitted during the experiment.

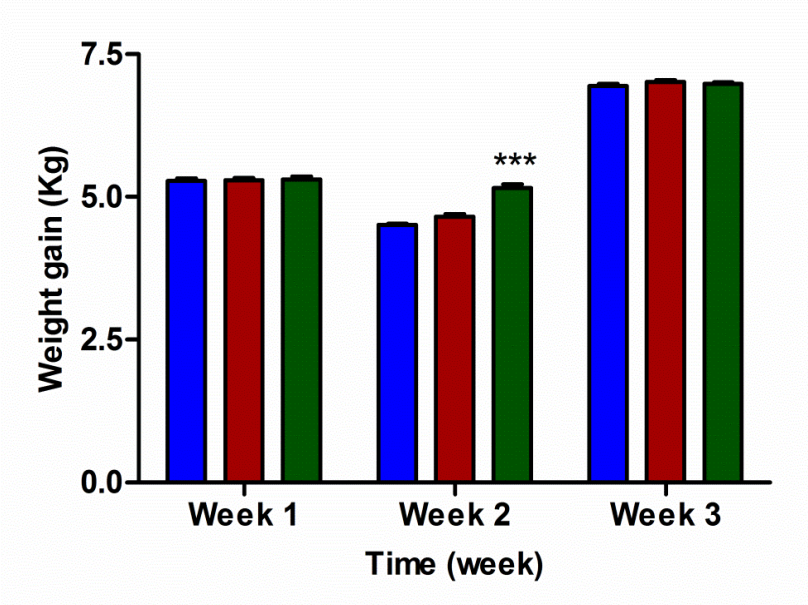


Figure 2 - Mean weight gain per piglets measured on animals submitted to the gavage to control treatment (blue), DON (0.15mg/Kg BW, red) or DOM-1 (0.14mg/Kg BW, green) during 21 days. Two-way ANOVA, Bonferroni test as Post-hoc, $p<0.05$, n=6 to 8.

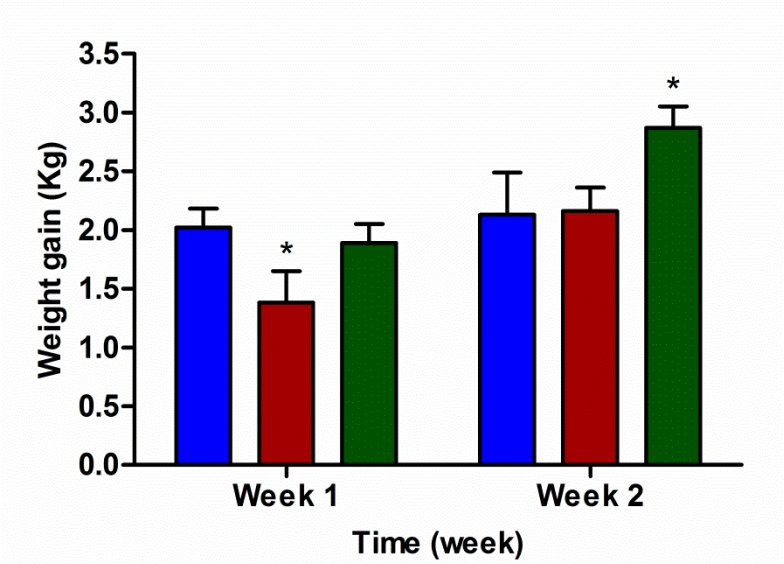


Figure 3 - Mean weight gain per piglets measured on animals submitted by gavages to control treatment (blue), DON (0.3 mg/Kg BW, red) and DOM-1 (0.28 mg/Kg BW, green), during 14 days at 0.3mg/Kg BW. Two-way ANOVA, Bonferroni test as Post-hoc, $p<0.05$, n=6.

B. Comparative intestinal toxicity of DOM-1 and DON

Ingestion of DOM-1 induced no significant change on intestinal morphology when compared to control. By contrast, animal fed with DON showed a significant increase in the lesional score of the jejunum (4.5 fold higher than control) and the ileum (3 fold higher than control). Villi atrophy and fusion with flattening of enterocytes and denuded villi were the main histological changes observed on the intestine (Figure 4) in animals receiving DON.

The effects of DON and DOM-1 on the expression of cytokines were assessed, on the jejunum without (Fig. 5, A.) and with Peyer patches (Fig. 5, B.). Whatever the intestinal part and the cytokine considered, no significant increase in mRNA level were observed in DOM-1 treated animals when compared to control ones. In DON treated animals, IL-8, IL-10 and IL-17A mRNA levels were significantly increased in the jejunum when compared to control and DOM-1- treated animals. In jejunum with Peyer's patches, IL1 beta and IL17A significantly increased in animals from the DON group when compared to control and DOM-1 groups (Fig. 5, B.). In this organ, a slight but non significant increase of IL-6, IL8, IL1alpha, IL10 and IFNgamma mRNA levels was also observed in DON treated animals.

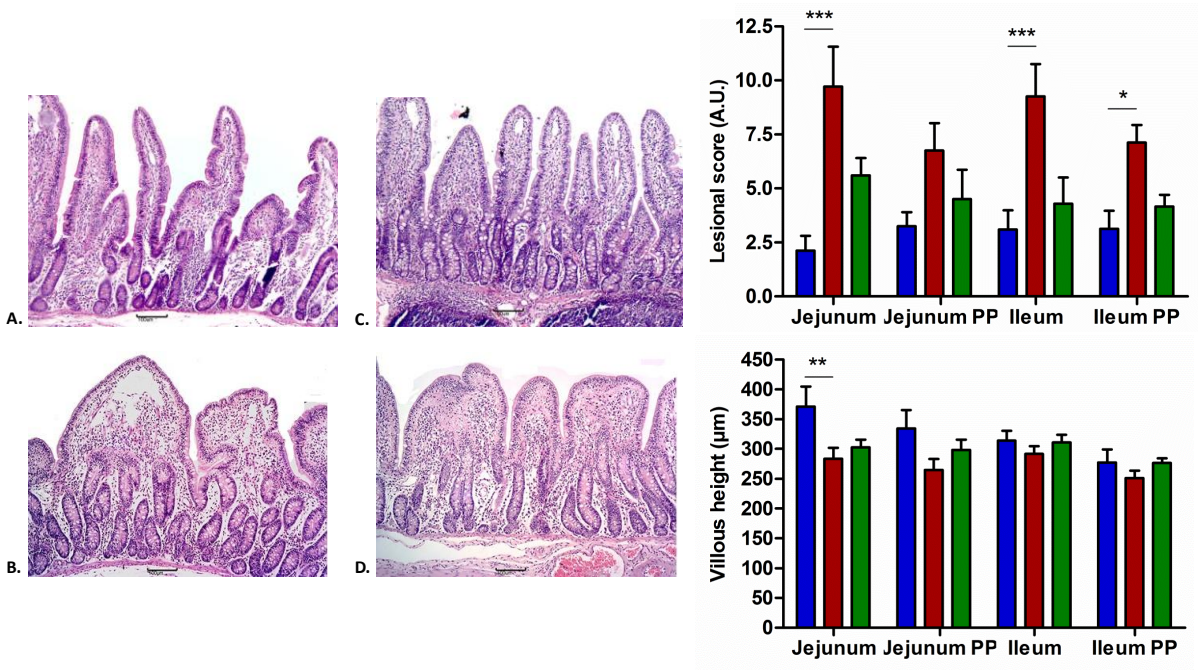


Figure 4 - Histological intestinal samples from piglets submitted by gavage to control treatment (blue), DON (0.15 mg/Kg BW, red) or DOM-1 (0.14 mg/Kg BW, green) during 21 days. Two-way ANOVA, Bonferroni test as Post-hoc, $p<0.05$, n=6 to 8.

A- Jejunum, control group. Villi with normal morphology. B- Jejunum, DON group. Villi fusion and lymphatic vessel expansion. C- Jejunum with Peyer patches, DOM-1 group. Villi with normal morpholy. D- Ileum, DON group. Villi atrophy, villi fusion and edema of lamina propria. Hematoxilin-eosin. Bar 100 µm.

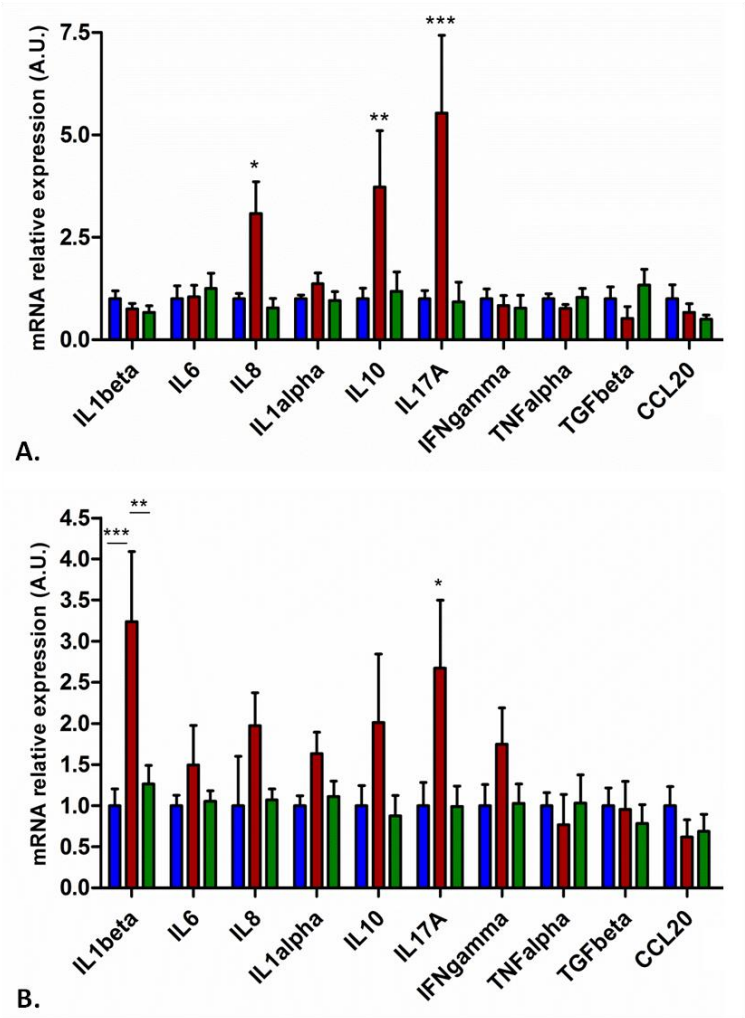


Figure 5 - The mRNA expression levels of markers of inflammation are affected by gavage to control treatment (blue), DON (0.15 mg/Kg BW, red) or DOM-1 (0.14 mg/Kg BW, green) during 21 days. At the end of the experiment, samples from different organs (A.jejunum, B.jejunal Peyer patches) were collected, and mRNA levels of inflammatory markers (IL1beta, IL6, IL8, IL1alpha, IL10, IL17A, IFNgamma, TNFalpha, TGFbeta, CCL20) were measured by RT-PCR. Results are expressed as the relative mRNA expression as means ± SEM; n=6 to 8 animals/group.*$p<0.05$, **$p<0.01$ and ***$p<0.001$, Two Away ANOVA with Bonferroni Post-hoc test.

C. Comparative effects of DOM-1 and DON on liver

Animals receiving DOM-1 were similar to the control ones. By contrasts, a significant increase on liver lesional score was observed in the DON-treated group (3 fold higher than control). In these animals the liver, showed a disorganization of hepatocytes, a vacuolization and a megalocytosis of these cells (Figure 6).

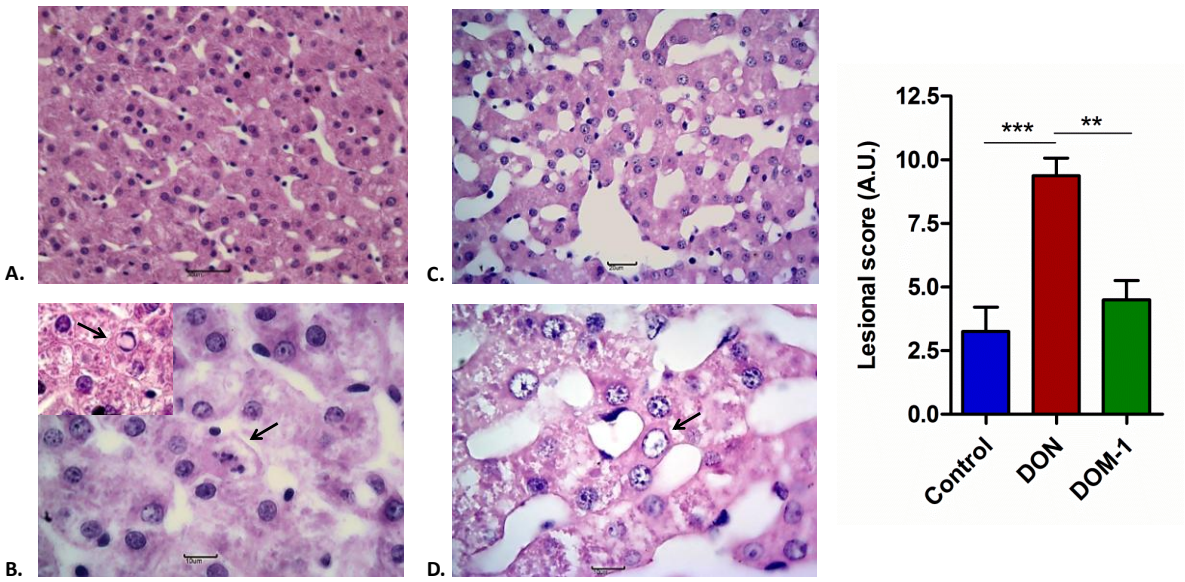


Figure 6 - Histological liver samples from piglets submitted by gavage to control treatment (blue), DON (0.15 mg/Kg BW, red) or DOM-1 (0.14 mg/Kg BW, green) during 21 days. One-way ANOVA, Bonferroni test as Post-hoc, $p<0.05$, n=6 to 8.
A- Control group. Hepatocytes' trabeculae with normal arrangement. B- DON group. Hepatocyte apoptosis (arrows) and mild vacuolation of hepatocytes cytoplasm. C- DOM-1 group. Disorganization of hepatocytes' trabeculae and mild vacuolation of cytoplasm. D- DON group. Nuclear vacuolation of hepatocyte (arrow) and megalocytosis. Hematoxilin-eosin. Bar 30 µm (A), 20 µm (C), 10 µm (B,D).

D. Comparative effect of DOM-1 and DON on immune parameters

The last part of the experiment was to evaluate the effect of DOM-1 and DON on immunity related parameters such as the histology of the spleen and lymph node, total and specific antibody response.

A significant increase in histological changes was observed in animals treated with DOM-1 in spleen (2.4 fold), and a non-significant increase in mesenteric lymph nodes in comparison to control group was also seen (1.4 fold). A similar increase in histological changes was also observed in animals treated with DON (1.5 fold and 2.5 fold for lymph node and spleen, respectively). In these organs, lymphoid depletion and apoptosis of lymphocytes were the most frequent changes observed in DOM-1 or DON treated animals. Reactive germinal center with macrophages containing tangible bodies was also observed in spleen of DON treated group (Figure 7).

The effect of DON and DOM-1 were also measured on the total and specific IgG. The plasmatic concentration of IgG was not significantly modified by the presence of toxins (Fig. 8). The immunization protocol allowed investigating the effects of mycotoxins on antigen-specific immunity. Ingestion of either DOM-1 or DON significantly increased the production of OVA-specific IgG at day

21 when compared to control (15.2 ± 2.9; 86.3 ± 25.9 and 82.3 ± 37.4 Arbitrary Units in DOM-1, DON and control animals respectively) (Fig. 9).

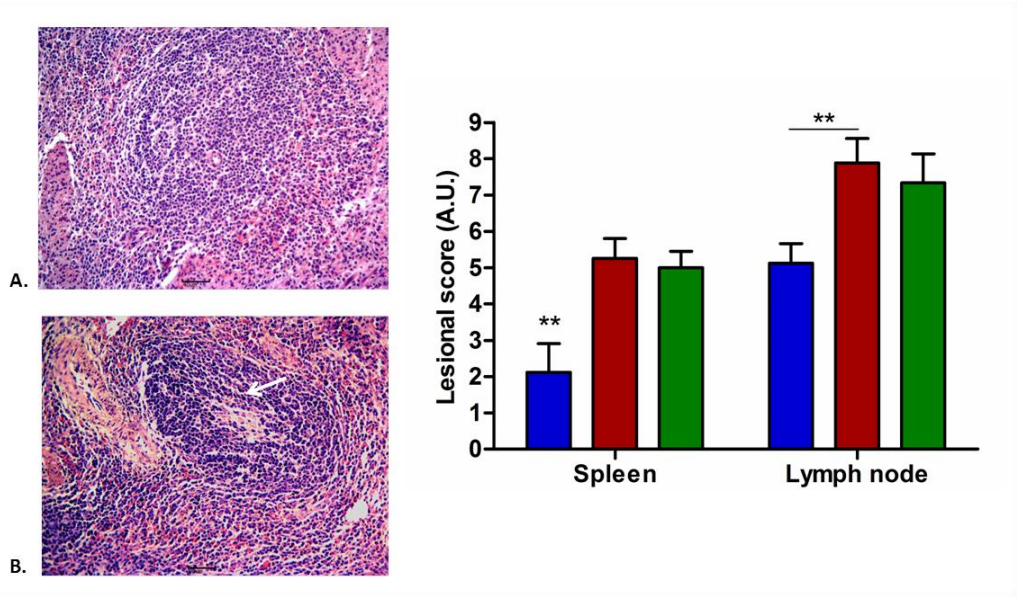


Figure 7 - Histological spleen from piglets submitted by gavage to control treatment (blue), DON (0.15 mg/Kg BW, red) or DOM-1 (0.14 mg/Kg BW, green) during 21 days. Two-way ANOVA, Bonferroni test as Post-hoc, $p<0.05$, n=6 to 8.
A- Spleen, control group. Lymphoid follicle with normal arrangement. B- Spleen, DON group. Reactive germinal center with macrophages containing tangible bodies (arrow). Hematoxilin-eosin. Bar 40 µm (A), 50 µm (B).

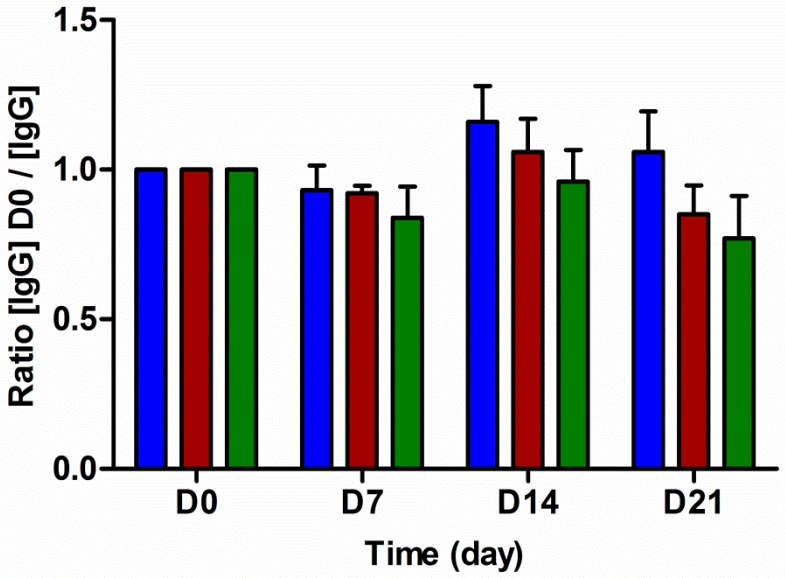


Figure 8 - Total Immune response for each treatment control (blue), DON (0.15 mg/Kg BW, red) or DOM-1 (0.14 mg/Kg BW, green) during the experiment.
Ratio of Total IgG/Total IgG at DO of piglets for each treatment at D7, D14 and D21. N=8 for control and DON groups and 6 for DOM-1 group, Two-way ANOVA, $p<0.05$

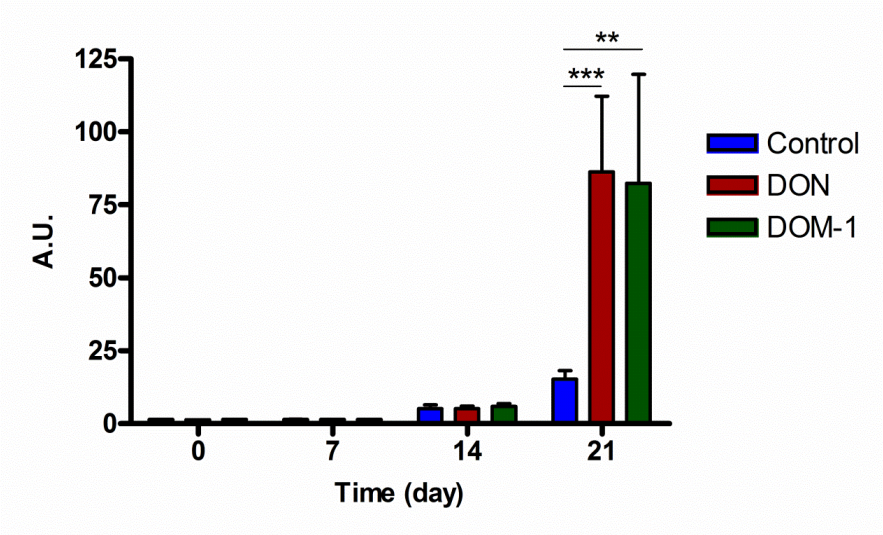


Figure 9 - IgG-antiOVA during the experiment per treatment control (blue), DON (0.15 mg/Kg BW, red) or DOM-1 (0.14 mg/Kg BW, green) during the experiment, following one vaccine injection against OVA at day 3 and one booster at day 8. Two-way ANOVA, Bonferroni test as Post-hoc, $p<0.05$, n=6 to 8.

IV. Discussion

In this present 3-week study, piglets were exposed to low doses of one major mycotoxin, the DON, and one of its derivatives, the DOM-1. Previous studies on the toxicity of DOM-1 toxicity were done with feed contaminated with DON in presence of the bacteria that is able to deepoxydise the DON and focused on zootechnical parameters (He et al. 1993; Li et al. 2011). By contrast, this study assessed the effect of purified DOM-1 on animals, and assessed major markers of toxicity, zootechnical parameters, intestine, systemic and immunologic parameters.

The use of purified toxin by gavages allowed to precisely control the dose used and to make sure that each animal received the same amount. Purified DOM-1 were given to pig at a dose of 0.14mg/Kg BW, molecular equivalent of 0.15mg/Kg BW of DON corresponding approximately to 3-4 ppm (Sergent et al. 2006), at levels commonly found in crops (SCOOP 2003).

From an agronomic point of view, pigs are exposed to mycotoxins due to the proportion of cereals in their diet. In addition, pigs are very sensitive to mycotoxins, due to the absence of ruminal tank, known to contain microorganisms capable of degrading toxins before their

intestinal absorption (Rotter et al. 2006). Finally, considering the similarity in the immune and digestive systems of pigs and humans, the use of pig model allow to extrapolate data to man (Heinritz et al. 2013; Nedjfors et al. 2000; Helm et al. 2003; Kimber et al. 2003; Rothkotter et al. 2002). For the choice of doses and time of exposure, this work fits in the current dynamic of a toxicological study, for chronic exposure going from low doses to moderate ones, not inducing major clinical manifestations (Forsyth 1977).

In summary, results showed that DOM-1 does not have toxic effects on zootechnical parameters, intestinal histology, intestinal and inflammatory response and liver histology. For these parameters we found toxic effects of DON as already described by others (Grenier et al. 2011; Pestka 2010; Rotter et al. 1996). The zootechnical parameters were not impacted by DOM-1 ingestion, with no decrease of weight gain and no emesis observed. Concerning the impact of DOM-1 on the intestine, which is the first organ targeted by feed contaminant (Prelusky et al. 1996; Oswald 2006), no tissue damage were induced. Moreover, DOM-1 didn't induce a pro-inflammatory response on intestinal tissue like it was observed with DON. One organ of the systemic response was analyzed, the liver, to complete the information about the action of DOM-1 once absorbed in the intestine. Histomorphological analysis on liver showed no damage induced by DOM-1 in contrary to DON. Finally, concerning the global immune response, DOM-1 didn't elicit total immune response.

On secondary lymphoid organs and on total and specific immune response, DOM-1 elicited the same increased immune response as DON. Indeed, concerning the specific immune response, and after a vaccine injection DOM-1 seems to act like an adjuvant, in increasing the immune response against the vaccine. Moreover, lymphoid depletion and apoptosis of lymphocytes were observed on lymph node and spleen like in DON group, may be correlated to the important specific immune response. In a previous study (see results of paper Chapter I, Part 2), we found that DOM-1 didn't induce MAPKs activation and a pro-inflammatory response like DON. The results obtained in the present study indicate that DOM-1, not activating MAPKinase have the same effect on the immune response than DON that activates the MAPKinase. This suggests that all effects induced by DON, especially the ones on the immune response are not mediated by the way MAPKs works. This was already proposed by Wu et al. (2014) when investigating the effect on the immune response induced by D3G, another DON derivative that do not activate MAPKinase.

In conclusion these results are in accordance with previous results found in others *in vivo* study. DOM-1, deepoxidated from DON by a strain BBSH 797, *Gen. nov. sp. nov. of family Coriobacteriaceae,* sold as a food additive, is globally not toxic for animals as DON. Taking into account that degrading product of DON (DOM-1) formed by this bacteria is much less toxic than DON, associated to the information given by previous *in vivo* studies (He et al. 1993; Li et al. 2011); the use of these detoxifying products seems to be a relevant strategy to protect animals, against the toxic effects of DON found in pig feed. However, this study has also shown that DOM-1 is able to act on the immune response. The underlying mechanism still need to be investigated.

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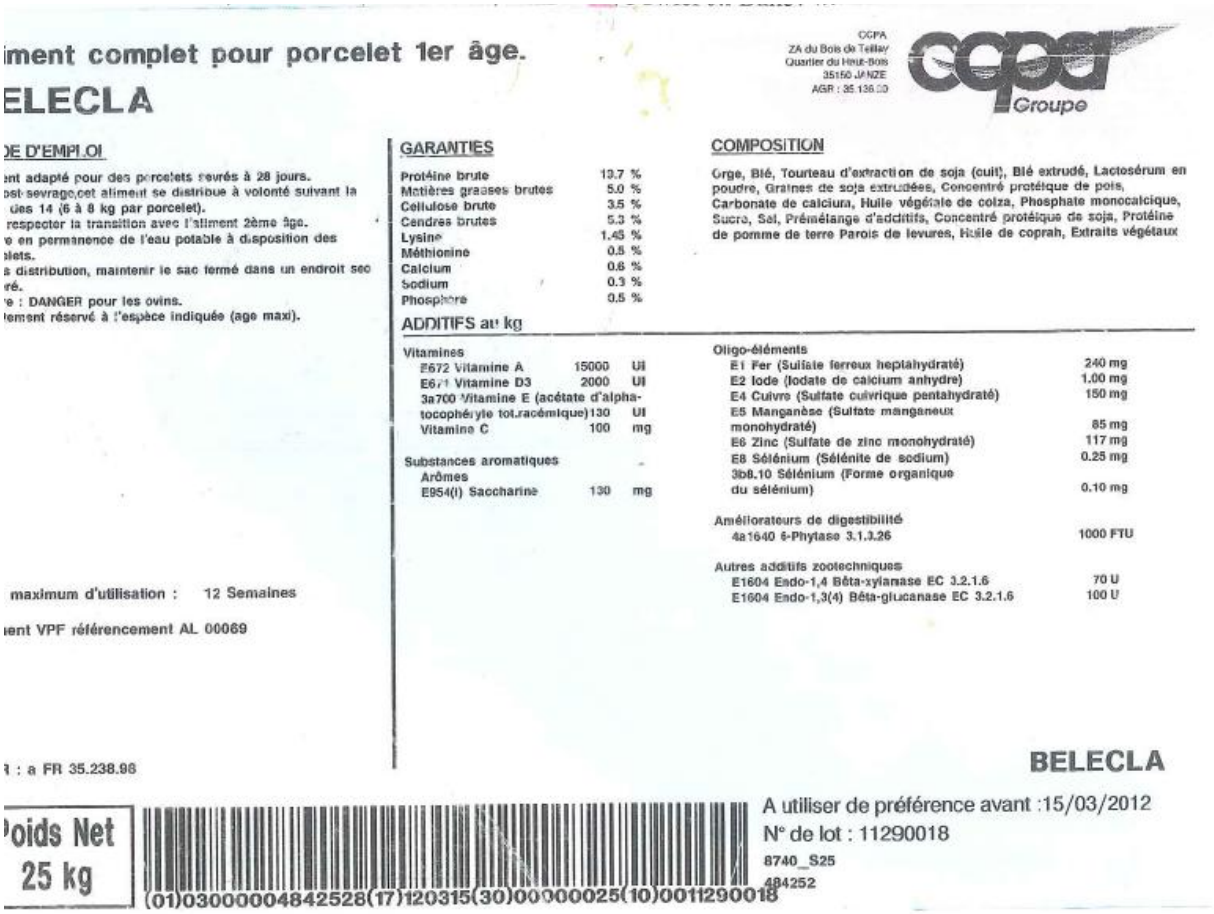
Annex 1

BELECLA: Complete feed for first age piglet



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ELECLA

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Quartier du Haut-Bois
35150 JANZE
AGR : 35.138.00

cgpa Groupe

DE D'EMPLOI

ent adapté pour des porcelets sevrés à 28 jours.
ost-sevrage,cet aliment se distribue à volonté suivant la
des 14 (6 à 8 kg par porcelet).
respecter la transition avec l'aliment 2ème âge.
e en permanence de l'eau potable à disposition des
lets.
distribution, maintenir le sac fermé dans un endroit sec
ré.
e : DANGER pour les ovins.
lement réservé à l'espèce indiquée (age maxi).

maximum d'utilisation : 12 Semaines

ient VPF référencement AL 00069

R : a FR 35.238.98

GARANTIES

Protéine brute	13.7 %
Matières grasses brutes	5.0 %
Cellulose brute	3.5 %
Cendres brutes	5.3 %
Lysine	1.45 %
Méthionine	0.5 %
Calcium	0.6 %
Sodium	0.3 %
Phosphore	0.5 %

COMPOSITION

Orge, Blé, Tourteau d'extraction de soja (cuit), Blé extrudé, Lactosérum en poudre, Graines de soja extrudées, Concentré protéique de pois, Carbonate de calcium, Huile végétale de colza, Phosphate monocalcique, Sucre, Sel, Prémélange d'additifs, Concentré protéique de soja, Protéine de pomme de terre Parois de levures, Huile de coprah, Extraits végétaux

ADDITIFS au kg

Vitamines		
E672 Vitamine A	15000	UI
E671 Vitamine D3	2000	UI
3a700 Vitamine E (acétate d'alpha-tocophéryle tot.racémique)	130	UI
Vitamine C	100	mg
Substances aromatiques		
Arômes		
E954(i) Saccharine	130	mg

Oligo-éléments	
E1 Fer (Sulfate ferreux heptahydraté)	240 mg
E2 Iode (Iodate de calcium anhydre)	1.00 mg
E4 Cuivre (Sulfate cuivrique pentahydraté)	150 mg
E5 Manganèse (Sulfate manganeux monohydraté)	85 mg
E6 Zinc (Sulfate de zinc monohydraté)	117 mg
E8 Sélénium (Sélénite de sodium)	0.25 mg
3b8.10 Sélénium (Forme organique du sélénium)	0.10 mg
Améliorateurs de digestibilité	
4a1640 6-Phytase 3.1.3.26	1000 FTU
Autres additifs zootechniques	
E1604 Endo-1,4 Bêta-xylanase EC 3.2.1.6	70 U
E1604 Endo-1,3(4) Bêta-glucanase EC 3.2.1.6	100 U

BELECLA

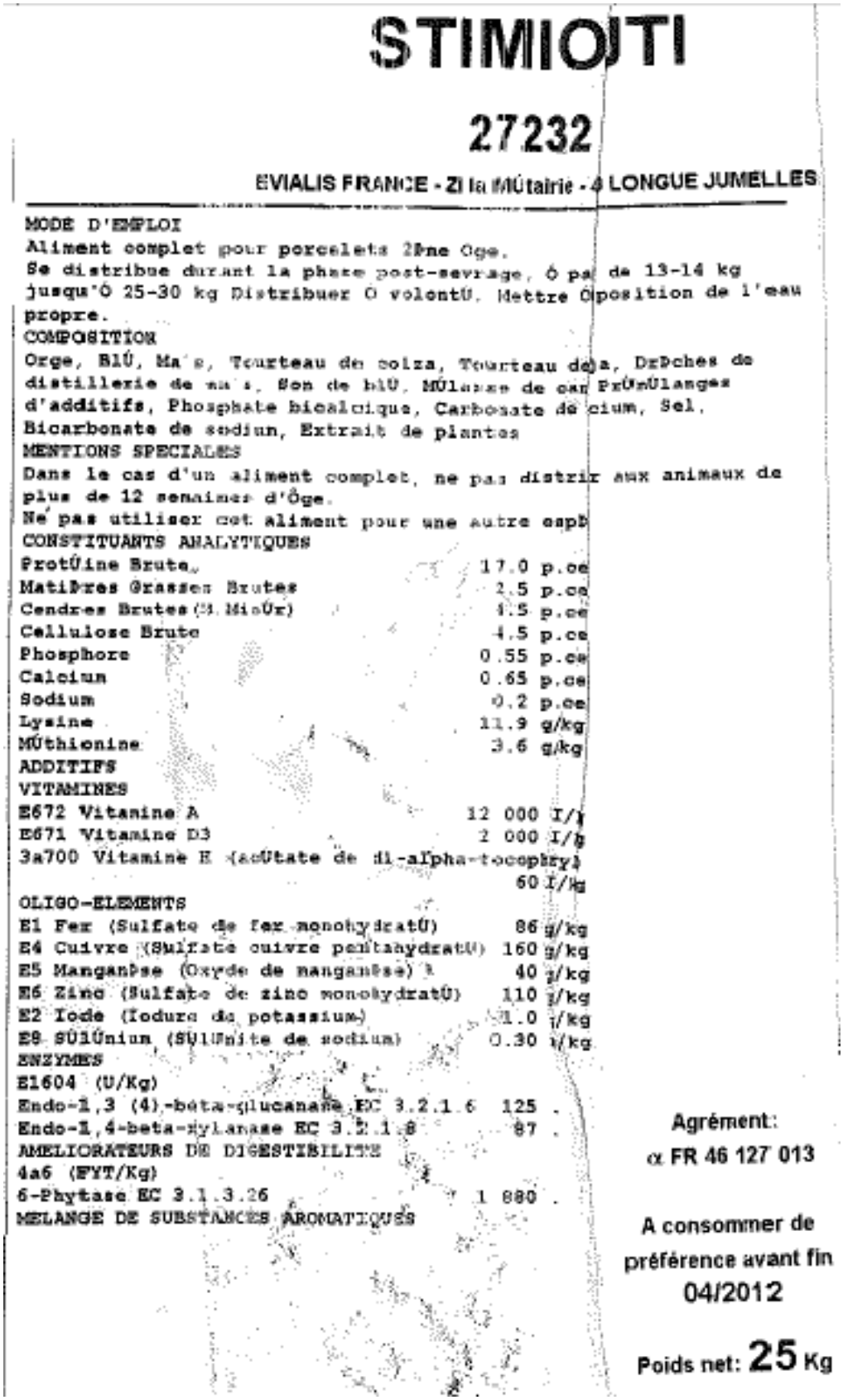
Poids Net 25 kg

(01)03000004842528(17)120315(30)00000025(10)0011290018

A utiliser de préférence avant :15/03/2012
N° de lot : 11290018
8740_S25
484252

Annex 2

STIMIOUTI : Complete feed for second age piglet



STIMIOUTI

27232

EVIALIS FRANCE - ZI la MÚtairie - 4 LONGUE JUMELLES

MODE D'EMPLOI
Aliment complet pour porcelets 2ème Óge.
Se distribue durant la phase post-sevrage, Ó pa de 13-14 kg jusqu'Ó 25-30 kg Distribuer O volontÚ. Mettre Óposition de l'eau propre.
COMPOSITION
Orge, BlÚ, Ma s, Tourteau de colza, Tourteau de a, DrÞches de distillerie de ma s, Son de blÚ, MÚlasse de can PrÚmÚlanges d'additifs, Phosphate bicalcique, Carbonate de cium, Sel, Bicarbonate de sodium, Extrait de plantes
MENTIONS SPECIALES
Dans le cas d'un aliment complet, ne pas distrir aux animaux de plus de 12 semaines d'Óge.
Ne pas utiliser cet aliment pour une autre espÞ
CONSTITUANTS ANALYTIQUES

ProtÚine Brute	17.0 p.ce
MatiÞres Grasses Brutes	2.5 p.ce
Cendres Brutes (3 MinÚr)	1.5 p.ce
Cellulose Brute	4.5 p.ce
Phosphore	0.55 p.ce
Calcium	0.65 p.ce
Sodium	0.2 p.ce
Lysine	11.9 g/kg
MÚthionine	3.6 g/kg

ADDITIFS
VITAMINES

E672 Vitamine A	12 000 I/k
E671 Vitamine D3	2 000 I/k
3a700 Vitamine E (acÚtate de di-alpha-tocophÚryl	60 I/kg

OLIGO-ELEMENTS

E1 Fer (Sulfate de fer monohydratÚ)	86 g/kg
E4 Cuivre (Sulfate cuivre pentahydratÚ)	160 g/kg
E5 Manganèse (Oxyde de manganèse)	40 g/kg
E6 Zinc (Sulfate de zinc monohydratÚ)	110 g/kg
E2 Iode (Iodure de potassium)	1.0 g/kg
E8 SÚlÚnium (SÚlÚnite de sodium)	0.30 g/kg

ENZYMES

E1604 (U/Kg)	
Endo-1,3 (4)-beta-glucanase EC 3.2.1.6	125 .
Endo-1,4-beta-xylanase EC 3.2.1.8	87 .

AMELIORATEURS DE DIGESTIBILITE

4a6 (FYT/Kg)	
6-Phytase EC 3.1.3.26	1 880 .

MELANGE DE SUBSTANCES AROMATIQUES

Agrément:
α FR 46 127 013

A consommer de préférence avant fin 04/2012

Poids net: 25 Kg

GENERAL DISCUSSION

&

PERSPECTIVES

I. Discussion

Once present in cereals or feed materials, the high stability of mycotoxins make them very difficult to eliminate. Several strategies have been developed to manage mycotoxins contamination, such as physical (cleaning, sieving), chemical (ammoniation) and biological (binding agents, feed additives, resistant crops) treatments. In this context, my thesis project deals with problematic of mycotoxin detoxification and was in particular focused on DON.

Indeed, among mycotoxins, DON is the most common in the world and can be present in various cereals and raw materials. As other mycotoxins, it is really hard to manage and eliminate. New strategies of biological detoxification led to the formation of metabolites, such as the deepoxy-deoxynivalenol (DOM-1) and the 3-epi-deoxynivalenol (3-epi-DON). The "masked" mycotoxin, deoxynivalenol-3-glucoside (D3G) is produced in plant resistant to *Fusarium* and is very often present simultaneously with DON. Thereby it becomes important to assess the toxicity of these biological degradation products of detoxification of DON. Limited toxicological information on DON-transformation products are available, this may be due to the lack of suitable methods to purify sufficient amounts of the chemicals for structure identification and toxicological studies.

The main purpose of this work was to assess the toxicity of "modified" and "masked" forms of DON, to evaluate the efficacy of the detoxification. This thesis investigates the toxicity of these DON derivatives on human and animal models, mostly on immune and intestinal parameters, using several approaches. These new molecules issued from a natural way of transformation can be a promising approach to detoxify mycotoxins and protect human and animals from their hazard effects.

Problematic of modified and masked mycotoxin in context of risk assessment, effects of DON on health pig and comparative toxicological effects of DON, DOM-1, 3-epi-DON and D3G have been summarized and discussed in the previous papers and will not be repeated here. This general discussion will focus on the studied models used, the specific results obtained with *in silico* and IPA analysis, and to finish the perspectives on experiments that could be realized to further investigate or complete information of toxicity and mechanisms of toxicity on DON and its derivatives.

A. Discussion on the analysis performed in the thesis

In silico, in vitro, and *ex vivo* models, were used to compare the intestinal and immune toxicity of D3G, DOM-1 and 3-epi-DON to those of DON.

In silico analysis allowed to draw three dimensions molecules and to visualize their interaction with the ribosome, especially with the petptidyl transferase center (PTC) of the ribosome. *In silico* approach is widely used in medical chemistry to decipher the mechanism of binding between protein and ligands, to predict interactions between molecules or to study structure activity relationship. This approach is not so commonly used in food safety, however some studies show that this approach allow in food safety studies to discover and study, quickly and economically, new putative endocrine

disruptors (Amadasi et al., 2009), like ZEN and its masked derivative (Cozzini and Dellafiora 2012). In this thesis, modeling allow the better understanding of the molecular mechanism of DON toxicity.

In vitro model using cell lines is a powerful tool for the exploration of fundamental questions regarding drug delivery dynamics and kinetics (Artursson et al. 2001; Boveri et al. 2004). To study the toxicity of DON and its derivatives on intestinal epithelial cells, we chose a human intestinal cell line. Caco-2 has the advantage to express characteristics markers of adults' intestinal cells, to be a reference line in toxicology studies, and to be largely used to study intestinal absorption (Sambruy et al. 2001).

Explant model allows a direct testing of the toxicity of molecules on intestinal living tissue keeping its polarity and extracellular integrity (Gonzalez-Vallina et al. 1996). Moreover, in the context of 3R, explants limit the use of animals, as well as the variability. Indeed many conditions are tested on the same animal, thus an animal is its own control. This model also allows to investigate in detail the impact of each derivatives on the expression of many genes on the intestinal tissue of pigs. Using a microarray encompassing the genome, associated with a functional analysis allows to go deeper on the investigation on the pathways targeted by these derivatives.

Finally the use of *in vivo* model, allow assessing the toxicity on the entire organism, looking at several organs and parameters that can be targeted by mycotoxins.

1. *In silico* analysis of the interaction between DON, DON derivatives and the ribosome

The *in silico* analysis lets us go deeper in the understanding of the mechanism of toxicity. The molecules binding of DON, DOM-1 and 3epi-DON into the peptidyl transferase center of the ribosome were modeled. The *in silico* analysis demonstrates that DON interacts with the two chains of the A-site of the ribosome through three hydrogen bonds (with G2403, U2873 and U2869). The deepoxydation of the epoxy group (into one hydrogen double bound) leads to a loose of the hydrogen bond U2873 and to the absence of toxicity. We can thus imagine that the three hydrogen bounds are required to maintain the molecule in the peptidyl transferase center; when only two bounds forming the stability is too weak to maintain the molecule in the peptidyl transferase center and to induce a subsequent cellular response. A similar analysis was performed with the 3-epi-DON; due to the isomeric change, the hydrogen bond with the uracile U2869 is lost. The molecule is only maintained in the ribosome pocket by two hydrogen bonds, this reduces the stability of the interaction and prevent cellular activation.

As both DOM-1 and 3-epi DON do not activate MAPKinase, the three hydrogen bonds seem required to maintain stably the molecule of DON into the A-site of the ribosome, to activate the MAPKinase and to induce the toxicity of this mycotoxin. This finding is in line with the hypothesis

that the toxicity of DON is linked to the epoxy group and the C3 group (Karlovsky 2011; Sundstol Eriksen et al. 2004) (Rotter et al. 1996). The loss of one hydrogen bound decreases the toxicity of DON. Most of the A-site inhibitors molecules, such as DON, impair peptide bond formation during translation elongation. They all block protein synthesis by competing with the amino acid side chains of incoming aminoacyl-tRNAs for binding in the A-site cleft in the PTC, which is universally conserved (Garreau de Loubresse et al. 2014). In addition, these structures support the hypothesis that the species specificity exhibited by the A-site cleft inhibitors is determined by the interactions they make, or fail to make, with a single nucleotide, U2504 (*Escherichia coli*) (Gurel et al. 2009). In our case, it confirms that just one change of interaction can change the toxicity of the molecule. However, we did not determine the role of the third hydrogen bond (between the oxygen of C15 group CH2OH and the hydrogen of the guanine basis G2403) on the binding of DON to the PTC of the ribosome and the subsequent toxicity of DON. Indeed, we couldn't identify a DON metabolite or another fusariotoxin metabolite to confirm the involvement of this H-bound in the structure-toxicity relationship. It would be interesting to test a molecule having both the epoxy and the C3 group but not the G2403 hydrogen bound. A second possibility would be to make a double mutations on the A-site of the ribosome, replacing the uracile basis U2869 by a guanine and the guanine G2403 by an adenosine, and to test the interaction of this mutated ribosome with DON.

The molecular basis to explain the lack of toxicity of D3G is easier. Due to its size, D3G can't interact with the A-site. Indeed, glucose molecule, added during the phase II of metabolization to easily eliminate the molecule, importantly increase its size.

2. Pan genomic analysis of the effect of DON and its derivatives

After the microarray analysis of explant treated with DON, or its derivative, a functional analysis using Ingenuity Pathways Analysis (IPA), was performed. IPA is a database gathering information on molecules (genes, proteins, metabolites, xenobiotics) and their interactions between each other or with pathologies, phenotypes, cells or cellular processes. The sources of information of IPA consist in existing databases (GenBank, Ensembl Entrez Gene, Gene Ontology, GEO ...), and knowledge extracted from the literature. This software allows to rapidly obtain information on genes expressed in the tissue and find genes who interferes (in different ways) with these genes and that are differentially expressed in our analysis. The Ingenuity Pathways Analysis demonstrates the absence of toxicity of DOM-1, 3-epi-DON and D3G, with no genes differentially expressed and thus no functions impacted. It also allows identifying key biological functions regulated by DON. This is a powerful tool to assess, on one tissue, all the functions that could be regulated by one molecule. It both confirms

the implication of function and genes that have been already described but also demonstrates the implication of new functions/genes.

IPA highlighted several genes and pathways already known to be impaired by DON in the intestine. For example we observed that in the intestine, DON modulates cytokines involved in the inflammatory pathway, tight junction signaling, p38MAPK Signaling, Protein Ubiquitination pathways, oxidative stress, protein degradation pathway as already described (Katika et al. 2012; Mishra et al. 2014; Osman et al. 2010; Shen et al. 2007; van Kol et al. 2011). Functional analysis pathway also brings new information on DON effects in the intestine. It's the case on metallothionein, shown to be a marker of oxidative stress and mycotoxins contamination in rats (Vasatkova et al. 2009). Results also underline the effect of DON on genes of intestinal transporters (Bookstein et al. 1997; Smith et al. 2013; Videmann et al. 2007) and on the decrease of the unfolded protein response pro-apoptotic gene CHAC1 that seems to play a role in glutathione degradation (Kumar et al. 2012). This study also shows the impact of DON on mitochondrial dysfunction.

3. The *in vivo* experimental protocol

During the experiments, purified mycotoxins were used. In cereals naturally contaminated, there are always co-contamination that doesn't allow to discriminate the effect of each mycotoxin (Schatzmayr and Streit 2013). Moreover, fungi contamination on cereals affects nutritional quality and change the final quality of pig feed. So the use of purified mycotoxins allows to keep the nutritional quality of feed and to attribute observed effect to the tested mycotoxins. In addition, gavage allowed to adjust precisely the dose given and to be sure that all animals received the calculated dose per Kg/BW.

A moderate dose was used in the perspective to assess the toxicity of probable quantity of DON in animals. Many studies performed with DON contaminated feed using moderate amounts corresponding to contamination doses that can be found in natural conditions (Poolman et a., 1985; Harvey et al., 1996; Trenholm et al., 1984; Friend et al., 1992; Rotter et al. 1994).

B. Toxicity assessment of biological strategies to reduce toxic effects of DON

Despite all preventive efforts and improvement of agricultural practices, important concentrations of mycotoxins still occur in agricultural products. Due to the difficulty to eliminate and detoxify DON, alternative effective strategies of detoxification are needed. the use of plants resistant to fungi infection or biological biotransformation to detoxify are new promising strategies (He et al. 2010). Indeed, biological methods are a good way to eliminate toxicity without acting on the grain quality or on the processed feed. There have been several transformation products of DON reported (Table 1), such as DON-sulfonate, DON lactone, norDON A, norDON B and norDON C, 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), diacetyl-DON, triacetyl-DON, iso-DON, 3-keto-deoxynivalenol (3-keto-DON), deepoxy-deoxynivalenol (DOM-1), DON-3-glucuronide,

DON-3-glucoside, and 3-epi-deoxynivalenol (3-epi-DON) (He et al. 2010; Ikunaga et al. 2011; Sato et al. 2012; Zhou and He 2009).

Table 1. Biological transformation of DON by microorganisms (He Jian We thesis)

Condition	Transformation reaction	Transformation product	Microorganism	Origin	Reference
Anaerobic	Acetylation	3-ADON	*Fusarium nivale*	Fungus	Yoshizawa *et al.*, 1975
	Reduction by deepoxidation	DOM-1	Rumen microorganisms (mixture)	(Dairy) Cow	King *et al.*, 1984; Côté *et al.*, 1986; Swanson *et al.* 1987a; 1987b; He *et al.*, 1992
			Chicken gut microorganisms (mixture)	Chicken	He *et al.*, 1992
			Pig gut microorganisms (mixture)	Pig	Kollarczik *et al.*, 1994
			A catfish digesta microbial culture (mixture)	Fish	Guan *et al.*, 2009
			Bacterial strain BBSH 797	Rumen fluid	Fuchs *et al.*, 2002
			Ten isolates belonging to *Clostridiales*, *Anaerofilum*, *Collinsella*, and *Bacillus* genera	Chicken digesta	Young *et al.*, 2007; Yu *et al.*, 2010
Aerobic	Reduction by deepoxidation	DOM-1	A culture from a mixture of 165 agricultural soils, containing six bacterial genera (*Serratia*, *Clostridium*, *Citrobacter*, *Enterococcus*, *Stenotrophomonas* and *Streptomyces*) (mixture)	Soil	Islam *et al.*, 2012
	Oxidation	3-keto-DON	*Agrobacterium-Rhizobium* sp. E3-39	Soil	Shima *et al.*, 1997
			A culture D107 (mixture)	Agricultural commodity	Volkl *et al.*, 2004
			Devosia sp. 17-2-E-8	Soil	Zhou and He, 2009
	Hydroxylation	Unidentified	*Aspergillus tubingensis* NJA-1	Soil	He *et al.*, 2008
	Epimerization	3-*epi*-DON	*Devosia* sp. 17-2-E-8	Soil	Zhou and He, 2009
			Nocardioides sp. WSN05-2	Soil	Ikunaga *et al.*, 2011
			Nine *Nocardioides* spp. & five *Devosia* spp.	Soil	Sato *et al.*, 2012
	Unidentified	Unidentified	*Marmoricola* sp. MIM116	Wheat	Ito *et al.*, 2012

1. Efficiency of detoxifying strategies

a) Bacterial transformation

The use of feed additives is a well-established practice in the animal feed industry. Successful mycotoxin-binding agents act by preventing its intestinal absorption by the animal from the contaminated feed (Bryden 2012). However, adsorption approaches for DON are relatively ineffective and so the use of microorganisms is recommended for trichothecene (Awad et al. 2010). Moreover, due to the chemical diversity of mycotoxins, often present in food in the same time, additional approaches to detoxifying feedstuffs are required, even if adsorbing, binding or trapping agents can be very effective.

Concerning the use of detoxifying agents, the major part of my work was to assess the global *in vivo* toxicity of DOM-1. Indeed, one strategy to protect animal from mycotoxin, is the use of microorganisms/enzymes detoxifying mycotoxins to limit the absorption in the organism (Schatzmayr et al. 2006). These microorganisms realize some transformation not feasible by the plant or by the animal. These transformations on the basic structure will play on the original toxicity of the molecule. The toxicity of trichothecene mycotoxins varies and is determined by their molecular structures, particularly functional groups such as epoxy, ester, and hydroxyl groups (Betina 1989; Nagy et al.

2005; Zhou et al. 2008). The number and the position of hydroxyl groups influence trichothecene toxicity. Currently, information on the toxicity of these transformation products (or DON derivatives) are limited.

BIOMIN group isolated and stabilized *bacterium*, now called BBSH 797, Gen. nov. sp. nov. of family Coriobacteriaceae able to remove the epoxide group of trichothecenes *in vivo*, and to transform DON into DOM-1 (Binder et al. 2001; Fuchs et al. 2002). Since its discovery in 1997, *Eubacterium* specific of trichothecenes, named BBSH797 in honour of the research team who discovered it (BBSH, Binder Binder Schatzmayr Heidler), was the first additive detoxifying mycotoxins formulated from a life microorganism. This derivative is recognized to be less toxic than DON, based on *in vitro* and *in vivo* experiment with animal ingesting the bacteria in presence of DON (He et al. 1993; Li et al. 2011; Schatzmayr et al. 2006; Sundstol Eriksen et al. 2004; Swanson et al. 1987). However, actual DOM-1 cytotoxic assays are not sufficient to correctly evaluate the overall toxicological relevance (Rychlik et al. 2014). And these *in vivo* studies didn't evaluate the global toxicity of purified DOM-1 on the animal.

The 3-epi-DON is also issued from a bacterial transformation. A bacteria, issued from the an alfalfa soil enriched with *F. graminearum*-infested corn, bacterium Devosia mutans 17-2-E-8 that is capable of completely transforming DON into a major product 3-epi-DON and a minor product 3-keto-DON in aerobic conditions (He et al. 2015b; Zhou and He 2009, 2010). 3-epi-DON, is an epimer of DON, the only difference between these two chemicals is the stereochemistry at the 3-OH Group (He et al. 2015b). 3-epi-DON, has received very little attention. Nevertheless, it is known that epimers can have different physicochemical properties, biological activities and toxicities. A study shows that a epimeric form of catechin was less active than the initial form (Mendoza-Wilson and Glossman-Mitnik 2006). A second study showed a five-fold difference in *in vitro* toxicity that has been demonstrated for the marine biotoxins azaspiracid-1 (AZA1) and 37-epi- AZA1 (Kilcoyne et al. 2014). Moreover, it seems that, once it is formed, the molecule is stable and the transformation is irreversible (Karlovsky 2011). This is a very interesting point in the context of the problem of the metabolization into the animal. 3-epi-DON could be a promising new commercial product if is not toxic and if it stay stable into the organism. Today, only one study assesses its toxicity on *in vitro* and *in vivo* models (He et al. 2015a) and there are no information on its metabolization. The above mentioned study demonstrated that 3-epi-DON is far less toxic than DON on Caco-2 cells and mouse models.

Different analysis realized in this thesis complete the information known about DOM-1 and 3-epi-DON toxicity. In conclusion, our results confirm the lack of *in vitro* toxicity of DOM-1 and 3-epi-DON as seen in previous studies performed on Caco-2 cells, a relevant model in toxicology studies on the intestine (He et al. 1993; Li et al. 2011; Sambruy et al. 2001; Schatzmayr et al. 2006; Sundstol

Eriksen et al. 2004; Swanson et al. 1987). Our results also show that these DON metabolites did not impair the integrity of intestinal tissue and the barrier function. They also demonstrate that DOM-1 and 3-epi-DON do not alter gene expression, do not induce a pro-inflammatory response and do not induce the activation of MAPKs. Moreover, results obtained *in vivo* show that DOM-1 did not alter most of the parameters investigated. Finally, our results bring a mechanistic hypothesis on the lack of toxicity of DOM-1 and 3-epi-DON.

b) Plant transformation

In natural conditions, some plants are able with efficacy to manage xenobiotics and mycotoxin, as DON. Plants could accumulate toxic levels of these molecules without efficient detoxification strategy. The natural way of defence includes biosynthesis pathways, phase I transformation (hydrolysis, reduction or oxidation), phase II solubilisation (conjugaison) and phase III compartmentalisation. These different phases (I and II) aim at increasing the polarity of the molecule making it more water soluble and facilitating by that its transport mediation by ATP-dependent glutathione-conjugate transporters to the vacuole or apoplastic space outside cell (phase III) (Coleman et al. 1997).

Mycotoxins issued from this biosynthesis pathway, are “modified” mycotoxins and more specifically called “masked” mycotoxins by ILSI as they are issued from a conjugation transformation into the plant (Berthiller et al. 2013). Thereby, masked derivatives of DON can be found in co-contamination in raw cereals and processed food, such as deoxynivalenol-3-β-D-glucoside (D3G). A study showed that D3G was present in all tested wheat and maize samples (Berthiller et al. 2009).

One strategy of fight against mycotoxin is the use of plant, for instance wheat cultivars, that carry a major QTL for increased *Fusarium* resistance and increased ability to conjugate DON into D3G. Indeed DON enhances the virulence on wheat (Jansen et al. 2005; Lemmens et al. 2005). These plants resistant to fungi over express UDP-glycosyl-transferase, an enzyme able to glycosylate DON in higher proportion (Berthiller et al. 2013; Karlovsky 2011; Poppenberger et al. 2003). So, this increased use of wheat cultivars may lead to a higher D3G/DON ratio in the future. In this case, the contribution of D3G to overall DON toxicity has to be taken into account.

Different analyses realized in this thesis bring new information about D3G toxicity, and show that it is non toxic. These results imply that the increasing use of resistant plant to *Fusarium* could be a promising strategy to reduce mycotoxin production and their occurrence in cereals and food process products. However, studies show that D3G can be hydrolyzed into DON by acid lactic of some bacteria inside the gut (Berthiller et al. 2011). A study realized on pig shows that the major part of D3G ingested, was excreted by urine in DON form and in minority in DON-glucuronidate forms (Nagl et al. 2014). So, even if D3G is not toxic by itself, if hydrolyzed into DON by the animal, it could be reabsorbed and thus the total amount of DON to which pig is exposed would be increased.

II. Perspectives

Results obtained in the present thesis demonstrate the non toxicity of several derivatives of DON on numerous parameters. To completely evaluate the toxicity of each molecule, an *in vivo* experiment on D3G and 3-epi-DON would be necessary. Actually, as we have seen for DOM-1, an *in vivo* experiment can make reveal unexpected properties of the molecule. No studies evaluating the toxicity of D3G and 3-epi-DON on farm animal were done yet. In order to assess in a significant way all the parameters impacted by D3G and 3-epi-DON, a longer exposure could be interesting to asses chronic effect of the toxin. The use of more animals, even if it is difficult to handle, should reduce the important individual variability that we observed in our *in vivo* experiment.

Our results associated with previous studies assessing the effectiveness and toxicity of bacteria transforming DON into DOM-1, as well as the safety for pig with tolerance studies (He et al. 1993; Li et al. 2011; EFSA 2013), seems to show that the use of bacteria to transform DON into a less toxic compound is be an efficient approach. It will be thus interesting to extend this approach to other mycotoxins. Indeed, as described in the introduction, the detoxification methods have not the same efficacy according to the mycotoxin. The use of bacteria efficient to transform other mycotoxins in less toxic compounds is already tested (Grenier et al. 2011); the toxicity of all the transformed products obtained on many different parameters has not been completely investigated.

In this context, the explants model, was shown to be efficient to evaluate the intestinal toxicity. Combined with others tools, such as microarray and functional analysis, it allows to deeply investigate the impact of a molecule on the tissue and to determine all the genes impacted by the exposure in the tested tissue. This model also allows to perform functional tests. For example, the evaluation of the barrier function can be measured by the TEER of jejunal explants placed in Ussing Chamber. Nevertheless, to completely assess the impact of a mycotoxin on the organism, *in vivo* experiments are required. These type of experiment especially allow to assess zootechnical effects, systemic or specific immune responses as well as the metabolism of the ingested molecule. Indeed, as we saw for DOM-1, its effect on the immune response was not observed *in vitro* or *ex vivo*. Concerning the metabolization, as described for D3G, modified mycotoxins could be retransformed into its native form by the host or the microbiota. Thus, *in vivo* experiment cannot be completely substituted by the use of *in vitro* and *ex vivo* models. Livestock feeding studies (e.g. swine, goat and chicken) are recommended to confirm the detoxification of the transformed product and to determine the metabolism/toxicokinetic of this modified mycotoxin. The toxicological results are critical for future industrial application, but they also provide fundamental scientific information for toxicological research.

In order to assess, the metabolization of modified and masked mycotoxin into the organism, the pig constitutes a relevant model. The bacterial profil of pig matches quite well with human, which is important to investigate the metabolization of “modified” DON in pig. Herein, pig is a good model; beside is similar intestinal tract to those of man (Heinritz et al, 2013), it also possess a similar bacterial

profile to human (Maresca 2013). Plus, since it's a monogastric just like human its similar tract is organized in the same way.

Concerning the use of resistant plants to mycotoxins, the toxicity analysis and the metabolization of products transformed by these plants is important. This step is required to correctly estimate the risk of exposure to native mycotoxin. It's essential to determine if D3G or other masked mycotoxin, present in high quantity in selected or genetically modified plants, won't be all re-transformed to DON in the animal. Even if D3G is not toxic by itself (because issued from a detoxification pathway into plant), it could be transformed back to its aglycone into the organism and thus increase the amount of DON present in the organism. So, in this case to correctly assess the risk, it is necessary to take into account both "native" and "masked" mycotoxins in the calculation of exposure and the calculation of NOEL and LOAEL by regulator organism like European Food Safety Authorities (EFSA).

An alternate application of bacteria is to only use for detoxification the enzymes and/or the genes implicated in the detoxification process. The genes may be cloned and expressed in crops to develop varieties that are resistant to the production of mycotoxins or which detoxify mycotoxin and, thus prevent this mycotoxin from entering the human and animal food chains. The detoxification genes may also be cloned and expressed in microorganisms to produce recombinant microorganisms that are suitable in an industrial scale enzyme production and purification (Altalhi and El-Deeb 2009). These detoxification enzymes should have great potential to eliminate DON and other mycotoxins present in the human and animal food chains.

In conclusion, this work fits in context of animal production, where expenses linked to prophylaxis, cares and productivity loss are important points in the agricultural sector. Mycotoxins present a problem all over the world for both human and animals. Therefore, the necessity to continue developing strategies of prevention and detoxification, is encouraged by the global warming, which will have an impact on the development of fungi (Bryden 2009; Paterson and Lima 2009; Tirado et al. 2010). In the long run, these new ways of detoxification, using plants and bacteria, have great potentials and could help the food safety sector, the livestock production and the crop disease management face these mycotoxins.

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CONCLUSION

Conclusion

The thesis brings new data on modified forms issued from strategies to detoxify mycotoxins. The general objective of this research was to evaluate the toxicity of three DON derivatives issued from biological transformation, DOM-1, 3-epi-DON and D3G. This thesis allowed to examine numerous parameters, through *in silico*, *in vitro* and *ex vivo* experiments. Results increased the knowledge on the toxicity and the mechanism of toxicity of DON, using pangenomic array on a jejunal tissue and *in silico* analysis. Finally, *in vivo* experiments allowed assessing the intestinal and immune toxicity of purified DOM-1 in comparison to DON, on piglets.

In silico, *in vitro* and *ex vitro* results showed that the tested DON derivatives were less toxic than DON; they were not able to induce a ribotoxic stress, a “keystone” of DON toxicity. Another important aspect that was not studied during this thesis is the metabolism of these derivatives in the organism. Certainly, each derivative and mycotoxin has its own behavior into organism, and *in vitro* and *ex vivo* assessment are not enough to give use a complete information on the molecule. *In vivo* studies allow investigating the organ toxicity of the tested molecules. In the present work we did not investigate the transformation of the derivatives, to the “native” mycotoxin into the intestine. Such a transformation, especially with "masked" mycotoxin, could lead to an underestimation of the risk.

In perspectives, assessing the *in vivo* toxicity of 3-epi-DON, D3G and the metabolisation of all the molecules on farm animals, will bring us all the information needed regarding the management of risk assessment for these molecules.

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TITLE : Toxicity of 3 biological derivatives of deoxynivalenol : deepoxy-deoxynivalenol, 3-epi-deoxynivalenol and deoxynivalenol-3-glucoside on pig

SUPERVISORS : Dr. Isabelle OSWALD and Dr. Wulf Dieter Moll

SUMMARY :
The *Fusarium sp.* mycotoxin deoxynivalenol (DON) is one of the most frequently widespread mycotoxin worldwide. Due to its high structural stability, the elimination of DON, once present in cereals or feed materials, becomes difficult. Thereby, it is present in many cereals and final feed products, inducing several toxic effects on human and animals, and causing big economic losses.
New strategies of to fight against mycotoxins were developed, as biological transformation, either by the use of bacteria or plants. Indeed, some microorganisms are able to transform DON in new products, by enzymatic reaction, forming the deepoxy-deoxynivalenol (DOM-1) and the 3-epi-deoxynivalenol (3-epi-DON). Moreover, some plants naturally own the capacity to glycosylate DON in the aim to detoxify it, forming the deoxynivalenol-3-β-D-glucoside (D3G).
The aim of this thesis was to assess the toxicity of these DON derivatives, on the intestine and immune response, using several approaches such as *in silico, in vitro, ex vivo* and *in vivo* models.
On the human intestinal Caco-2 cell line, DOM-1, 3-epi-DON and D3G were not cytotoxic; they did not alter its viability and barrier function, as measured by the trans epithelial electrical resistance.
The expression profile of DOM-1, 3-epi-DON and D3G-treated jejunal explants was similar to that of controls and these explants did not show any histomorphology alteration. On the other hand, the treatment of intestinal explants with DON, induced morphological lesions and upregulated the expression of proinflammatory cytokines. The impact of these three derivatives was also studied on intestinal explants with a pan-genomic transcriptomic analysis. Results show that the derivatives of DON did not induce any change on the gene expression in comparison to the control-treated explants. In contrary, DON-treated explants differentially expressed 747 probes, representing 323 genes involved in immune and inflammatory responses, oxidative stress, cell death, molecular transport and mitochondrial function.
In silico analysis revealed that D3G, opposing to DON, was unable to bind to the A site of the ribosome, which is the main target for DON toxicity. Both DOM-1 and 3-epi-DON were able to fit into the pockets of the A site of the ribosome but only by forming two hydrogen bonds, while in this position, DON forms three hydrogen bonds. Moreover, the three derivatives do not elicit a ribotoxic stress, MAPKinase activation, and inflammatory response.
Then, an *in vivo* study was carried out to assess the toxicity of DOM-1 on pig (feed forced during 21 days at 0.14 mg/Kg BW). The results showed that DOM-1 does not have as much toxic effects as DON on zootechnical parameters (no emesis induced, no decrease of food consumption or weight loss observed), on intestine and liver (no tissues damages), or on the immune response (no inflammatory response induced).
Our data demonstrate that bacterial de-epoxidation or epimerization of deepoxy-DON modified its interaction with the ribosome, leading to an absence of MAPKinase activation and toxicity; and that the glycosylation of DON suppresses its ability to bind to the ribosome and decreases its intestinal toxicity. The mycotoxin deoxynivalenol (DON) remains an important challenge in many regions in the world. Thus, these biological detoxifications of DON seem to represent a new promising approach helping manage the problem of its contamination.

KEYWORDS : Deoxynivalenol (DON), Deepoxy-deoxynivalenol (DOM-1), Deoxynivalenol-3-glucoside (D3G), 3-epi-deoxynivalenol (3-epi-DON), mycotoxins, pig, co-contamination, immune response, intestine, enzymatic biotransformation

DISCIPLINE: Food Toxicology

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TITRE : Toxicité de 3 dérivés biologiques du déoxynivalénol : le déepoxy-déoxynivalénol, le 3-épi-déoxynivalénol et le déoxynivalénol-3-glucoside sur le porc

DIRECTEURS DE THESE : Dr. Isabelle OSWALD et Dr. Wulf Dieter Moll

RESUME :
Les mycotoxines sont des métabolites secondaires de moisissures contaminant de façon naturelle de nombreuses denrées alimentaires, notamment les céréales. Le déoxynivalénol (DON), produit par *Fusarium sp.*, est la mycotoxine la plus répandue dans le monde. Du fait de sa grande stabilité chimique, le DON est difficile à éliminer, et se retrouve dans les céréales et les produits finis ou il induit des effets toxiques pour l'homme et l'animal. De nouvelles stratégies de lutte sont mises en places, telle la transformation biologique utilisant des bactéries ou des plantes. En effet certaines bactéries possèdent des enzymes capables de transformer le DON en de nouveaux composés, le déepoxy-déoxynivalénol (DOM-1) et le 3-épi-déoxynivalénol (3-epi-DON). De plus, certaines plantes sont naturellement capables de transformer le DON dans le but de l'éliminer et de le détoxifier, formant ainsi le deoxynivalénol-3-β-D-glucoside (D3G).
L'objectif de cette thèse était d'évaluer la toxicité de ces dérivés du DON au niveau de l'intestin et du système immunitaire par le biais d'analyses *in silico, in vitro, ex vivo* et *in vivo.*
Les tests de toxicité *in vitro* sur la lignée humaine intestinale cellulaire Caco-2 montrent que le DOM-1, le 3-epi-DON et le D3G n'étaient pas cytotoxiques, ils ne modifiaient ni la viabilité, ni la fonction de barrière des cellules, mesurée par la résistance électrique transépithéliale. Les tests de toxicité *ex vivo* sur des explants jéjunum porcin ont montré que le DOM-1, le 3-epi-DON ou le D3G n'induisaient pas de modifications histomorphologiques. En revanche, les explants exposés au DON montraient des lésions morphologiques et une régulation positive de l'expression des cytokines pro-inflammatoires. L'impact de ces trois dérivés a été également analysé sur l'expression de l'ensemble des gènes du tissu, avec une analyse microarray. Ceci a montré que ces dérivés du DON n'induisaient aucun changement dans l'expression des gènes par rapport au groupe contrôle. Le DON quand a lui exprimait différentiellement 747 sondes, correspondantes à 333 gènes impliqués dans l'immunité, la réponse inflammatoire, le stress oxydatif, la mort cellulaire, le transport moléculaire et la fonction mitochondriale.
L'analyse *in silico* a montré que le D3G, contrairement au DON était incapable de se lier au site-A du ribosome, principale cible de la toxicité pour le DON. Les deux dérivés microbiens eux, étaient capables de se fixer au site-A au sein du ribosome, mais contrairement au DON ils ne formaient que deux liaisons hydrogènes au lieu de trois. De plus, ces trois dérivés n'induisaient pas de stress ribotoxique, d'activation des MAPKs (mitogen-activated protein kinases), et de réponse pro-inflammatoire.
Une étude complémentaire a été menée *in vivo* pour évaluer la toxicité du DOM-1 chez le porc (gavage pendant 21 jours avec .0.14mg / kg de poids vif). Les résultats ont montré que le DOM-1, contrairement au DON n'induisait pas les effets toxiques du DON au niveau des paramètres zootechniques (pas de vomissements, aucune diminution de la consommation alimentaire ou de perte de poids), sur l'intestin et le foie (pas de dommages tissulaires), ou sur la réponse immunitaire (pas de réponse inflammatoire induite).
En conclusion, nos résultats montrent l'efficacité de ces transformations enzymatiques. La déepoxydation et l'épimérisation bactérienne, ainsi que la glycosylation par les plantes permettent de sensiblement diminuer la toxicité du DON, passant par une absence de toxicité sur le ribosome avec une absence d'activation des MAPKs et de réponses inflammatoires. Dans ce contexte de contamination par les mycotoxines, ces méthodes de luttes alternatives semblent être des approches prometteuses.

MOT-CLES : Déoxynivalénol, Déepoxy-déoxynivalénol (DOM-1), Déoxynivalénol-3-glucoside (D3G), 3-epi-déoxynivalénol (3-epi-DON), mycotoxines, porc, co-contamination, réponse immunitaire, réponse intestinale, biotransformation enzymatique

DISCIPLINE : Toxicologie alimentaire

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