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Bertrand Grenier

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Université
de Toulouse

THÈSE

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Titre :

Effets chez le porcelet d'une exposition à un régime co-contaminé en
mycotoxines, et appréciation des stratégies de lutte

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TITRE : Effet chez le porcelet d'une exposition à un régime co-contaminé en mycotoxines, et appréciation des stratégies de lutte

DIRECTEURS DE THESE : Dr. Isabelle OSWALD et Pr. Martine KOLF-CLAUW

RESUME :

Les mycotoxines sont des métabolites secondaires des moisissures qui peuvent naturellement contaminer de nombreuses denrées alimentaires, notamment les céréales. Dans les travaux de thèse, nous nous sommes intéressés à deux mycotoxines majeures produites par des champignons du genre *Fusarium*, le Déoxynivalénole (DON) et la Fumonisine (FB). Les objectifs de la thèse ont été de déterminer les effets individuels et combinés d'une contamination en DON et FB chez le porc, une espèce cible et sensible aux mycotoxines. Les effets sur les fonctions immunitaires lors d'un challenge antigénique ainsi que sur les fonctions intestinales ont été évalués. Par ailleurs, dans le cadre d'un partenariat avec un industriel, nous avons évalué *in vivo* les effets de méthodes de détoxification par biotransformation et ciblant spécifiquement ces deux toxines. Chez le porc, l'ingestion d'aliments contaminés avec de faibles doses de mycotoxines (DON, 3 mg/kg ; FB, 6 mg/kg) a provoqué des lésions tissulaires (foie, reins et poumons) et a fortement altéré la mise en place d'une réponse immunitaire spécifique de l'antigène (expression des cytokines, prolifération des lymphocytes et anticorps spécifiques). Les animaux ont été significativement plus affectés après la consommation du régime co-contaminé, et l'interaction a pu être considérée comme additive. De plus, les paramètres intestinaux examinés ont révélé des changements dans la morphologie, dans le profil de sécrétion des cytokines et dans l'adhésion cellulaire. L'interaction des deux toxines a pu ici être caractérisée comme moins qu'additive. Les approches de détoxification biologique proposées par l'industriel étaient basées sur la transformation par voie enzymatique du DON et des FB, à partir d'un microorganisme entier et d'une enzyme respectivement. La stratégie d'élimination des FB a suscité un intérêt plus important étant donné que cette méthode est non commercialisée et en cours de développement. Ainsi, la toxicité du produit d'hydrolyse de la FB1 (mycotoxine principale de la famille des FB) obtenu initialement par traitement enzymatique, a été comparée *in vivo* à celle de la molécule mère la FB1. Les résultats ont montré que l'hydrolyse de la FB1 réduisait fortement la toxicité hépatique et intestinale chez les porcelets. L'expérimentation animale avec le DON et la FB, seuls ou en combinaison a ensuite été reproduite afin de déterminer l'efficacité d'hydrolyse de ce procédé chez le porc après incorporation de l'enzyme dans les aliments contaminés. Dans ces aliments, le microorganisme entier ciblant le DON avait également été inclus. La nette diminution du marqueur d'exposition des FB et la neutralisation partielle ou totale des effets ont suggéré que le procédé avait fortement réduit la biodisponibilité des FB dans le tractus gastro-intestinal. Cette observation a aussi été en partie confirmée pour l'approche de dégradation du DON. La biotransformation par voie enzymatique des mycotoxines représente ainsi une stratégie biotechnologique prometteuse dans la lutte contre ces contaminants.

MOT-CLES : Déoxynivalénole, Fumonisine, porc, co-contamination, réponse immunitaire, réponse intestinale, marqueur d'exposition, biotransformation enzymatique

DISCIPLINE : Toxicologie alimentaire

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TITLE : Effect in pigs of the exposure to a mycotoxins co-contaminated diet, and evaluation of control strategies

THESIS SUPERVISORS : Dr. Isabelle OSWALD and Pr. Martine KOLF-CLAUW

ABSTRACT :

Mycotoxins are secondary metabolites of fungi that are natural contaminants of several commodities, in particular cereals. In the present work, we focused on two major mycotoxins produced by the *Fusarium* genus, Deoxynivalenol (DON) and Fumonisin (FB). The main objectives of the thesis were to determine the toxic effects of individual and combined DON and FB contamination in pig, a target species highly sensitive to mycotoxins. The effects on the immune functions following an antigenic challenge and also on the intestinal functions were evaluated. Besides, within the framework of an industrial partnership, we evaluated *in vivo* the effects of detoxifying methods by biotransformation and targeting specifically these two toxins. In pigs, ingestion of contaminated feeds with low doses of mycotoxins (DON, 3 mg/kg ; FB, 6 mg/kg) triggered tissular lesions (liver, kidneys and lungs) and strongly impaired the establishment of the antigenic immune response (cytokines expression, lymphocytes proliferation and specific antibodies). Animals consuming the co-contaminated diet were more affected and the interaction could be considered as additive. In addition, changes in morphology, in profile of cytokines secretion and in cell adhesion were observed at intestinal level. The interaction here could be characterized as less than additive. The biological detoxification approaches proposed by the industrial were based on the transformation by enzymatic way of DON and FB, from intact microorganism and enzyme respectively. We paid a particular attention to the strategy of FB removal as this method is not marketed and still in development. Therefore, the toxicity of the hydrolysis product of FB1 (major mycotoxin in the FB group) initially obtained by enzymatic way, was compared *in vivo* to the toxicity of the parent compound FB1. Results showed that the hydrolysis of FB1 strongly reduced the toxicity in piglets at intestinal and hepatic levels. The animal experiment with DON and FB, alone or in combination was then repeated in order to determine in pigs the hydrolysis efficiency of this process when enzyme was incorporated in contaminated feeds. In these feeds, the intact microorganism toward DON was also included. The marked decrease of the biomarker of exposure to FB and the partial or total counteraction of the effects suggested that the process had greatly reduced the FB bioavailability in the gastrointestinal tract. This observation was also in part confirmed for the method degrading DON. The biotransformation method of mycotoxins by enzymatic way represents therefore a promising biotechnological strategy in the control of these contaminants.

KEY WORDS : Deoxynivalenol, Fumonisin, pig, co-contamination, immune response, intestinal response, biomarker of exposure, enzymatic biotransformation

DISCIPLINE : Food Toxicology

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LISTE DES ABREVIATIONS

AF	Aflatoxine	DOM-1	De-époxy-déoxynivalénol
AFB1	Aflatoxine B1	DON	Déoxynivalénol
AFM1	Aflatoxine M1	EFSA	European Food Safety Authority
AJ	Adherens Junction	e.g.	exempli gratia (par exemple)
ANOVA	Analysis of variance	ELISA	Enzyme Linked Immuno Sorbent Assay
ANRT	Agence Nationale de la Recherche Technique	ERK	Extracellular signal-Regulated Kinases
APC	Antigen Presenting Cells	FAO	Food and Agriculture Organization of the United Nations
A.U.	Arbitrary Units	FB	Fumonisine
b.w.	body weight	FB1	Fumonisine B1
BGY	Bright Greenish Yellow	FLD	Fluorescent detection
CAST	Council for Agricultural and Sciences Technology	GALT	Gut-Associated Lymphoid Tissue
CIFRE	Convention Industrielle de Formation par la Recherche	GGT	Gamma-Glutamyl Transferase
CIT	Citrinine	HACCP	Hazard Analysis Critical Control Point
CMH	Complexe Majeur d'Histocompatibilité	HE	Hématoxyline-Eosine
ConA	Concanavoline A	HFB	Hydrolyzed Fumonisin
CPA	Cyclopiazonic acid	HFB1	Hydrolyzed Fumonisin B1
cpm	counts per minute	HPLC	High Performance Liquid Chromatography
DA	Deactivating Agents	HRP	Horseradish peroxydase
DART	Data Analysis for Real Time	HSCAS	Hydrated Sodium Calcium Aluminosilicate
DAS	Diacetoxyscirpenol	H ₂ O ₂	Hydrogen peroxyde
DC	Dendritic Cells	IARC	International Agency for Research on Cancer
DDGS	Dried Distillers' Grains and Solubles		

IFN	Interféron	ppm	partie par million (e.g. mg/kg)
IBD	Inflammatory Bowel Disease	pv	poids vif
Ig	Immunoglobuline	RPL32	Ribosomal Protein L32
IL	Interleukine	RT	Reverse Transcription
INRA	Institut National de la Recherche Agronomique	Sa	Sphinganine
IPEC-1	Intestinal Porcine Epithelial Cells-1	SDS	Sodium Dodecyl Sulfate
kGy	kilogray	SEM	Standard Error Mean
LD	Limite de Détection	So	Sphingosine
MAPK	Mitogen-Activated Protein Kinase	TEER	Résistance électrique transépithéliale
MIP	Macrophage Inflammatory Protein	TCA	Tricarballic acids side chains
MLN	Mesenteric Lymph Nodes	TCT	Trichothécènes
MON	Moniliformine	Th	T helper cells
MS	Mass Spectrometry	TJ	Tight Junction
NIV	Nivalénol	TNF	Tumor Necrosis Factor
NTC	Non Template Control	T-2	Toxine T-2
OTA	Ochratoxine A	UE/EU	Union Européenne/European Union
OVA	Ovalbumine	USFDA	United State Food and Drug Administration
PAGE	Polyacrylamide gel electrophoresis	ZEA	Zéaralénone
PAS	Period Acid-Schiff	μCi	μCurie
PCR	Polymerase Chain Reaction	3-aDON	3-acétyldéoxynivalénol
ppb	partie par milliard (e.g. μg/kg)		

FIGURES ET TABLEAUX

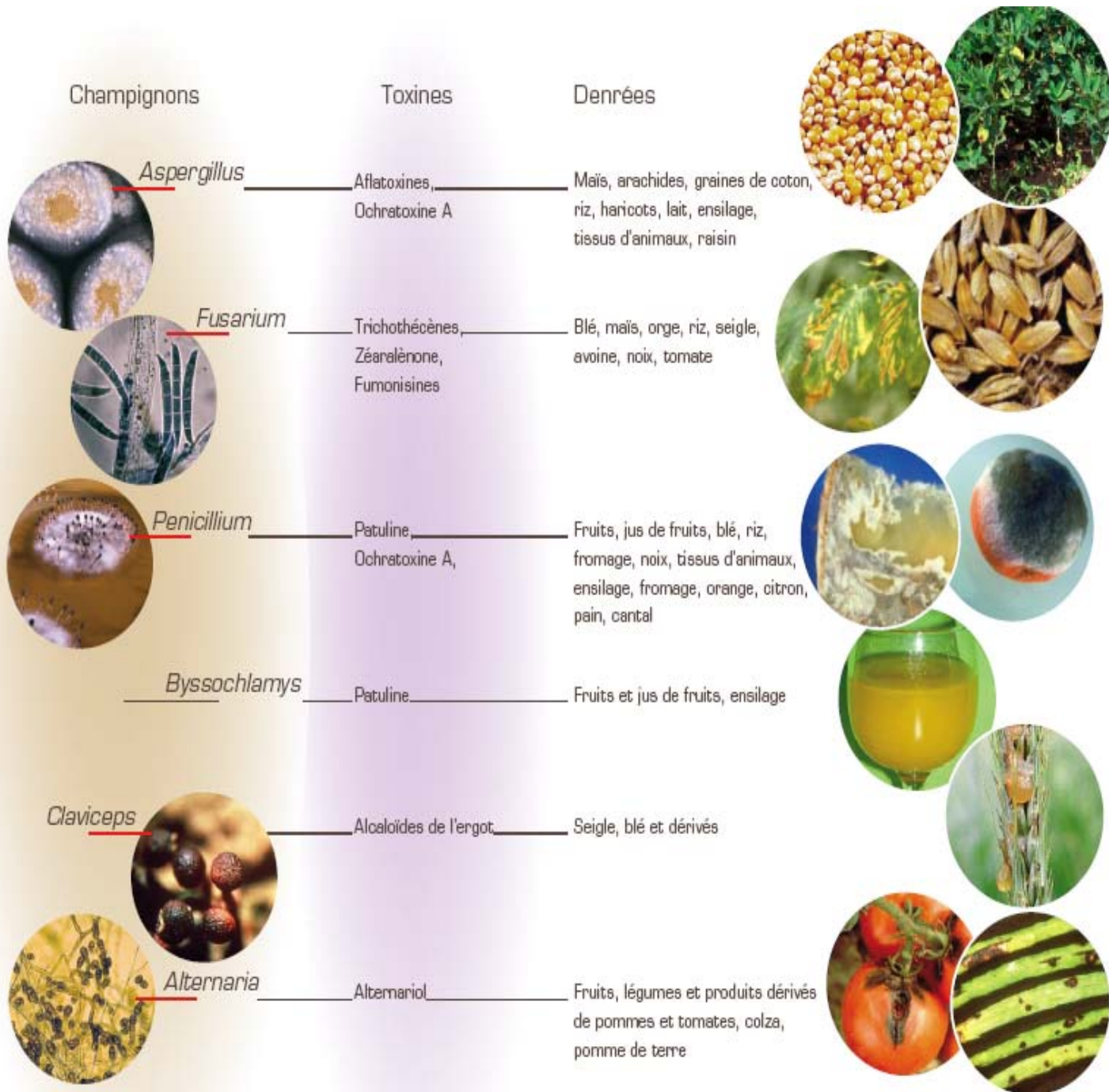
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INTRODUCTION

Figure 1 :

Mycotoxines majeures produites par les champignons et se retrouvant à l'état naturel dans différents produits de consommation



CONTEXTE DE L'ETUDE

Les mycotoxines sont des produits du métabolisme secondaire de moisissures pouvant se développer sur la plante au champ ou en cours de stockage, et douées de potentialités toxiques à l'égard de l'homme et des animaux. Les moisissures toxigènes peuvent se développer sous tous les climats, sur tous les supports solides ou liquides, dès l'instant qu'il y a des éléments nutritifs et de l'humidité (activité en eau A_w supérieure à 0,6).

Les toxines produites se retrouvent ainsi à l'état de contaminants naturels dans de nombreuses denrées d'origine végétale, notamment les céréales (maïs, blé, orge, soja) mais aussi les fruits, noix, amandes, grains, fourrages, et les aliments composés et manufacturés contenant ces matières premières destinés à l'alimentation humaine et animale (**Figure 1**).

Il a été recensé que les mycotoxines contaminent 25 à 40 % des productions céréalières mondiales, à des niveaux mesurables. Leurs effets toxiques sur l'homme et l'animal, leur stabilité lors des processus de transformation et de cuisson des aliments, et leur présence sur de nombreux produits agricoles justifient ainsi l'attention croissante qui leur est portée (nombreuses recherches en cours, renforcement de la législation en Europe).

Historiquement, de nombreuses pathologies liées à des épisodes de contamination en mycotoxines ont été rapportés. L'une des plus connues s'est produite au Moyen-Age, également appelée le « Mal des Ardents » ou « Feu de Saint-Antoine », et provoquée par les toxines de *Claviceps* élaborées par l'ergot de seigle (**Figure 2**).

Figure 2 :

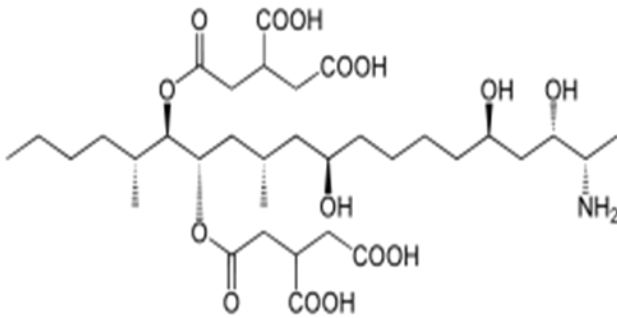
Tentation de Saint Antoine peint entre 1512 et 1516 par Grünewald (photo à droite)

Epis parasités par *Claviceps purpurea*, excroissance (sclérote) qui s'accroche aux épis de seigle (photo ci-dessous)

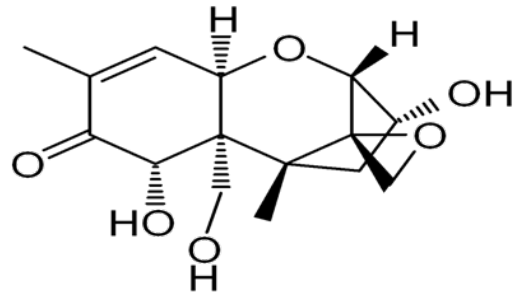


Figure 3 :

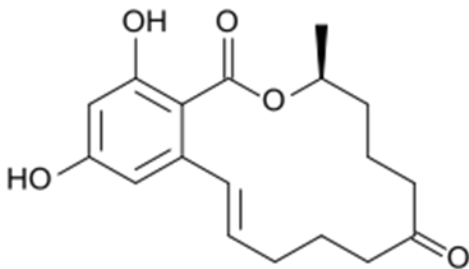
Diversité structurale des mycotoxines majeures



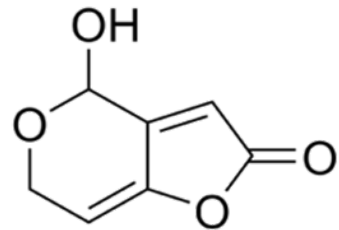
Fumonisine B1



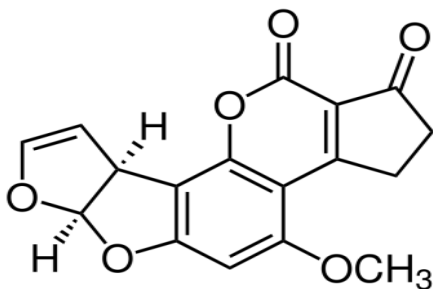
Déoxynivalénoï



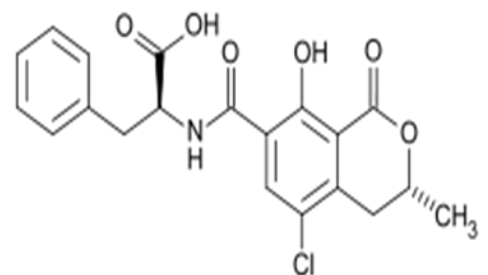
Zéaralénone



Patuline



Aflatoxine B1



Ochratoxine A

Elle se présentait sous la forme de délires, prostrations, douleurs violentes, abcès, gangrènes des extrémités aboutissant à des infirmités graves et incurables. Des épidémies ont sévi du 8ème au 16ème siècle en raison des conditions d'alimentation misérables des populations, en particulier la consommation de farines contaminées par les sclérotés de ces champignons. De la même manière, les fusariotoxines (toxine T-2 et zéaralénone) sont considérées comme responsables du déclin de la civilisation Étrusque et de la crise athénienne cinq siècles avant J.-C. Certains tombeaux égyptiens ont également été suspectés de renfermer des moisissures sécrétant une mycotoxine, l'ochratoxine A, qui aurait été responsable de la « malédiction des Pharaons ». La littérature vétérinaire rapporte de nombreux cas de mycotoxicoses, notamment le syndrome de Turkey X ou « maladie de la dinde », qui a décimé en Angleterre des milliers de dindes, de canetons et autres animaux domestiques dans les années 1960. Elle a permis de découvrir les aflatoxines, mycotoxines produites par *Aspergillus flavus* en quantités importantes dans la farine d'arachide importée d'Amérique latine dont se nourrissaient les volailles.

Mais de nos jours en Europe, il demeure exceptionnel d'être exposé à des doses toxiques en une seule ingestion d'aliments contaminés, provoquant ainsi une « mycotoxicose » aiguë. Les effets chroniques (exposition répétée à de faibles voire très faibles doses) sont les plus redoutés en raison des habitudes alimentaires et du pouvoir de rémanence de ces toxines.

Les mycotoxines peuvent être classées en polycétoacides, terpènes, cyclopeptides et métabolites azotés selon leur origine biologique et leur structure (**Figure 3**). On peut aussi classer les mycotoxines plus simplement selon leurs principaux effets toxiques. On distingue parmi les groupes de mycotoxines considérées comme importantes du point de vue agro-alimentaire et sanitaire les aflatoxines, les ochratoxines et l'ochratoxine A en particulier, la patuline, les fumonisines, la zéaralénone et les trichothécènes et tout spécialement le déoxynivalénole (**Figure 3**).

Un aspect important mais pourtant peu documenté concerne la présence simultanée de plusieurs mycotoxines dans les denrées alimentaires. En effet, une même moisissure a la capacité de produire différentes mycotoxines. A l'inverse, une même mycotoxine pourra être produite par plusieurs espèces et genres de moisissures. Ainsi, plusieurs toxines d'une même famille structurale ou présentant des structures différentes peuvent se retrouver dans le même produit alimentaire et, *a fortiori*, dans une ration composée de divers ingrédients alimentaires : on parle alors de multi-contamination. Cette situation naturelle pose des interrogations sur les interactions toxiques qui peuvent s'opérer et ainsi pourrait se traduire par un effet antagoniste ou additif ou encore synergique. De plus, les recommandations et régulations actuelles ne fixent des seuils que pour une mycotoxine et ne tiennent pas compte des contaminations avec plusieurs mycotoxines.

Une partie du travail de thèse a donc consisté à étudier l'interaction de deux mycotoxines, le deoxynivalenol et la fumonisine, à des faibles doses. En effet, la co-contamination par ces deux toxines majeures produites par des champignons du genre *Fusarium*, a été rapportée lors d'échantillonnage de denrées agricoles, et sont d'intérêt majeur en termes d'ubiquité et de toxicité. De plus, ces résultats s'inscrivent dans une étude bibliographique que nous avons réalisée et présentée dans ce mémoire, sur les données toxicologiques actuelles, rapportant les résultats d'expérience *in vivo* suite à l'exposition à des toxines seules ou en combinaison.

L'autre partie du travail de thèse a consisté à évaluer l'efficacité d'agents détoxifiants de mycotoxines, dans le cadre de notre collaboration avec l'industriel BIOMIN.

En effet, le risque mycotoxique étant d'origine naturelle, l'homme n'en maîtrise pas la survenue qui est notamment liée aux conditions climatiques, et ainsi la contamination fongique est difficilement contrôlable. Par conséquent, le contrôle du niveau de contamination des aliments exige l'emploi de stratégies variées et complémentaires. Des méthodes préventives telles que des pratiques culturales adaptées existent pour diminuer le risque de prolifération des moisissures. Cependant, l'élimination totale du risque fongique et mycotoxique est impossible.

Les stratégies impliquant le tri et/ou la destruction des denrées contaminées sont peu réalistes du fait de leur coût économique important. Par ailleurs, la dilution des aliments contaminés afin d'abaisser le niveau de contamination en-dessous des normes réglementaires, a été interdite en Europe à partir de juillet 2003. Ces méthodes d'élimination ou de réduction des teneurs en mycotoxines, directement sur les matières brutes, sont détaillées dans le présent manuscrit, suite à la publication d'un chapitre sur les méthodes physiques et chimiques existantes.

Une autre façon d'augmenter la qualité sanitaire des aliments est l'utilisation de ligands minéraux et organiques ou de micro-organismes/enzymes détoxifiant les mycotoxines afin de limiter l'absorption des mycotoxines dans l'organisme de l'animal. Cet aspect est également détaillé dans le mémoire de thèse, suite à un chapitre complémentaire sur les alternatives biologiques. Nous présenterons également les résultats d'une des méthodes développées par la société BIOMIN, concernant l'action d'une enzyme sur la fumonisine B1, mycotoxine prédominante de la famille des fumonisines et une des toxines les plus difficiles à éliminer.

Grâce à nos installations au sein du pôle ToxAlim, les phases animales expérimentales ont été conduites sur le porc. D'un point de vue agronomique, les animaux monogastriques d'élevage tels que le porc et la volaille sont particulièrement exposés aux mycotoxines du fait de l'importance de la part des céréales dans leur alimentation. De plus, ces animaux et en particulier le porc sont très sensibles aux mycotoxines, du fait de l'absence de réservoir ruminal, connu pour contenir des micro-organismes capables de dégrader les toxines avant leur absorption intestinale. Finalement,

l'utilisation de ce modèle animal permet aussi d'extrapoler nos données à l'homme, considérant la similarité de leurs systèmes immunitaire et digestif.

ETUDE BIBLIOGRAPHIQUE

L'étude bibliographique réalisée se présente sous la forme de deux grandes parties, précédées d'une brève introduction sur les mycotoxines majeures, en termes d'occurrence et de toxicité. La première grande partie est une revue exhaustive sur les expériences menées *in vivo* et qui caractérise les interactions des mycotoxines. La seconde partie présente les méthodes développées pour neutraliser, éliminer et décontaminer les denrées alimentaires contaminées en mycotoxines.

Comme indiqué précédemment, la présence de plusieurs mycotoxines en même temps n'est pas un cas isolé mais plutôt une situation fréquente. Les relevés de terrain et les études toxicologiques sur les multi-contaminations sont encore peu nombreux contrairement aux données individuelles, et par conséquent la connaissance du risque pour la santé humaine et animale est limitée. Nous présentons donc une synthèse des résultats d'expériences *in vivo*, où des animaux ont été exposés à deux toxines, seules ou en combinaison. A partir des données brutes, nous avons classé paramètre par paramètre l'effet des mycotoxines en association, tel que synergique, additif, moins qu'additif ou antagoniste. De par son aspect exhaustif, cette revue résume l'état actuel des connaissances sur l'interaction des mycotoxines chez l'animal.

La contamination en mycotoxines étant peu maîtrisable, de nombreuses approches pour réduire ou éliminer les mycotoxines ont été développées, et certaines intégrées dans les chaînes de production et/ou appliquées en aval dans les élevages. Ces stratégies sont présentées sous forme de deux chapitres en cours de publication dans un ouvrage scientifique. Le premier rapporte les méthodes physiques et chimiques qui agissent directement sur les denrées alimentaires contaminées. Nous présentons l'efficacité de ces techniques expérimentales ou appliquées, et aussi la toxicité potentielle des denrées modifiées physiquement et chimiquement. Le second chapitre traite des méthodes biologiques qui agissent directement dans le tractus gastro-intestinal des animaux, suite à l'ingestion d'aliments contaminés. Nous pouvons distinguer les approches basées sur la capacité d'adsorption des mycotoxines par des ligands minéraux ou organiques, et sur la capacité de biotransformation des toxines par voie enzymatique.

1. Les mycotoxines : généralités, métabolisation et effets toxiques

Les mycotoxines sont des molécules de faible masse moléculaire issues du métabolisme secondaire des moisissures. Plus de 300 métabolites secondaires ont été identifiés mais seule une trentaine possède de réelles propriétés toxiques préoccupantes.

Les effets toxiques sont de nature variée. Certaines toxines exercent un pouvoir hépatotoxique (aflatoxines), d'autres se révèlent oestrogéniques (zéaralénone), immuno/hématotoxiques (patuline, trichothécènes, fumonisines), dermonécrosantes (trichothécènes), néphrotoxiques (ochratoxine A) ou neurotoxiques (toxines trémorgènes). Certaines mycotoxines sont reconnues ou suspectées d'être cancérogènes.

Pour les consommateurs humains, un autre type de risque est indirect car induit par la présence possible de résidus dans les productions issues des animaux de rente exposés à une alimentation contaminée par les mycotoxines. Ces résidus correspondent à la toxine elle-même et/ou à des métabolites bioformés conservant les propriétés toxiques du composé parental. Les espèces d'élevage peuvent donc constituer un vecteur de ces toxines ou de leurs métabolites dans des productions telles que les abats, le lait ou le sang. C'est le cas notamment de l'aflatoxine B1, dont le métabolite l'aflatoxine M1 est retrouvé dans le lait des mammifères lorsque ceux-ci ont ingéré des aliments contaminés par l'aflatoxine B1.

Les mycotoxines sont généralement thermostables et ne sont pas détruites par les procédés habituels de cuisson et de stérilisation. Leur capacité à se lier aux protéines plasmatiques et leur lipophilie en font des toxiques capables de persister dans l'organisme en cas d'expositions répétées et rapprochées.

Les animaux monogastriques d'élevage, porcs et volailles sont particulièrement exposés aux mycotoxicoses du fait de l'importance de la part de céréales dans leur alimentation et de l'absence de réservoir ruminal contenant des micro-organismes capables de dégrader les toxines avant leur absorption intestinale.

En France, en dehors de cas sporadiques correspondant à des accidents aigus observables dans différentes espèces animales, l'essentiel des problèmes est lié à une contamination chronique par les fusariotoxines (trichothécènes, zéaralénone, fumonisines) des aliments produits en France ou importés. Les problèmes ponctuels dus à l'importation de matières premières contaminées justifient des procédures de surveillance et de contrôle.

a) Les aflatoxines (voir la revue de Meissonnier *et al.*, 2005)

Chez les animaux monogastriques, l'absorption pourrait représenter près de 90% de la dose administrée. Après absorption, les aflatoxines (AF) sont véhiculées dans l'organisme après fixation sur les protéines plasmatiques, c'est le cas de l'Aflatoxine B1 (AFB1) liée à l'albumine. La présence de cet adduit dans le sérum peut servir de bio-indicateur d'exposition.

La plus toxique des quatre aflatoxines naturelles est l'AFB1, viennent ensuite par ordre décroissant de toxicité l'AFG1 puis les AFG2 et AFB2. Les effets des aflatoxines sont principalement liés à l'action toxique des époxydes formés par l'action du système enzymatique réactionnel des mono-oxygénases à cytochromes P450. Ainsi l'AFB1 8,9-époxyde conduit à la formation d'adduits à l'ADN en créant des liaisons avec les bases guanines, et pouvant entraîner des effets carcinogènes.

En raison de ses capacités de bioactivation, le foie est la cible principale des aflatoxines. L'exposition à l'AFB1 peut aboutir à un cancer hépatique surtout lorsqu'elle est associée à une infection par le virus de l'hépatite B ou C. Chez l'animal, AFB1 exerce des propriétés immunosuppressives affectant en particulier l'immunité à médiation cellulaire par inhibition de la phagocytose, diminution de la production de radicaux oxygénés et altération de la production de cytokines. La réactivation d'infections parasitaires et la diminution de l'efficacité vaccinale ont été mises en évidence expérimentalement sur plusieurs modèles animaux après administration d'AFB1.

b) L'ochratoxine A (voir revue de Pfohl-Leskowicz & Manderville, 2007)

L'ochratoxine A (OTA) est connue pour sa néphrotoxicité. Elle serait l'un des facteurs potentiels à l'origine de troubles rénaux chez l'homme, connus sous le nom de Néphropathie Endémique des Balkans. Elle s'avère également immunotoxique, tératogène et neurotoxique.

L'OTA est d'abord absorbée dans l'estomac en raison de ses propriétés acides. Néanmoins, le site majeur d'absorption de l'OTA est l'intestin grêle avec une absorption maximale au niveau du jéjunum proximal. Elle est hydrolysée en OT α non toxique par la carboxypeptidase A et la chymotrypsine ainsi que par les flores microbiennes (rumen des polygastriques et gros intestin chez toutes les espèces). Au niveau hépatique, l'OTA est transformée en des métabolites mineurs qui permettent une détoxification partielle.

Au niveau immunitaire, l'un des effets les plus notables de l'OTA est la diminution de la taille des organes lymphoïdes, probablement par un mécanisme nécrotique ou apoptotique.

c) La zéaralénone (voir rapport de l'AFSSA, 2009)

Les propriétés physiques et chimiques de la zéaralénone (ZEA) et notamment son hydrophobicité sont des caractéristiques favorables à une large diffusion à l'intérieur des tissus. Les différentes études de pharmacocinétique montrent d'ailleurs que la ZEA est absorbée rapidement après administration orale et peut être métabolisée par le tissu intestinal. La ZEA et ses dérivés ont la capacité de se fixer de façon compétitive sur les récepteurs oestrogéniques cellulaires. La ZEA est donc un perturbateur endocrinien. Sa fixation est due à sa capacité à adopter une conformation similaire aux oestrogènes naturels tels que les 17 β -estradiol.

Chez l'homme, la ZEA est suspectée d'une vague de changements pubertaires chez des milliers de jeunes enfants à Porto-Rico. Des toxicoses aux Etats-Unis, en Chine, au Japon et en Australie ont été liées à la présence de ZEA dans les denrées alimentaires.

Les différentes propriétés d'absorption, de distribution, de biotransformations et d'excrétion de la ZEA chez différents animaux ont été résumées par Gaumy *et al.* (2001). Plusieurs métabolites tels que les α et β zéaralénols et α et β zéaralanols peuvent être formés. Ils peuvent ensuite subir une glucurono-conjugaison. L' α -zéaralénol est un métabolite 3 à 4 fois plus actif que la molécule initiale. Le porc est particulièrement sensible à la ZEA. Le taux de survie de l'embryon est notamment diminué pour les femelles en gestation ayant ingéré de la ZEA.

Plusieurs altérations des paramètres immunologiques ont été montrées *in vitro* après exposition de lymphocytes à la ZEA : inhibition de la prolifération lymphocytaire après stimulation par un mitogène, augmentation de la production d'IL-2 et d'IL-5. Par contre, aucune étude réalisée *in vivo* ne montre d'immunotoxicité de la ZEA.

d) Les trichothécènes (voir rapport de l'AFSSA, 2009)

➤ La toxine T-2

Chez l'animal, la toxine T-2 est rapidement absorbée après ingestion et distribuée dans l'organisme sans accumulation dans un organe spécifique. La toxine T-2 est rapidement métabolisée par déacétylation, hydroxylation, glucuronoconjugaison et dé-époxydation : la principale voie de biotransformation est une déacétylation qui aboutit à la formation de toxine HT-2.

La toxine T-2 est probablement à l'origine de l'Aleucie Toxique Alimentaire, maladie qui a touché des milliers de personnes en Sibérie pendant la seconde guerre mondiale. Elle provoque chez l'animal une perte de poids, des vomissements, des dermatoses sévères et des hémorragies pouvant entraîner la mort. La toxine T-2 possède des propriétés immunosuppressives intervenant à la fois sur

le nombre de macrophages, de lymphocytes et d'érythrocytes. Elle inhibe la synthèse protéique et la synthèse d'ADN et d'ARN.

➤ Le déoxynivalénol

La biodisponibilité du deoxynivalénol (DON) est extrêmement variable selon les espèces animales, allant de 10% chez les ovins à plus de 50% chez le porc. Le DON est un inhibiteur de la synthèse protéique, inhibant l'élongation de la chaîne protéique.

Le DON, communément appelé vomitoxine, provoque des vomissements chez le porc induisant une réduction de la consommation alimentaire, une diminution du gain de poids et des perturbations de certains paramètres sanguins. L'impact du DON sur le système immunitaire a été relativement étudié, montrant une augmentation de la sensibilité face à certains pathogènes ou encore une diminution de l'efficacité vaccinale. Par ailleurs, l'ingestion prolongée de DON provoque une augmentation de la concentration des IgA, pouvant induire une néphropatie à IgA.

e) Les fumonisines (voir rapport de l'EFSA, 2005)

Chez l'animal, après administration par voie orale, la fumonisine B1 (FB1), la plus toxique des fumonisines, est faiblement absorbée et se retrouve majoritairement dans les fecès. La biodisponibilité est évaluée de 1 à 6% selon l'espèce. La majeure partie de la toxine absorbée se retrouve dans le foie et les reins.

Les fumonisines agissent en particulier sur la synthèse des sphingolipides, en inhibant de façon compétitive l'activité de la céramide synthase. Cette perturbation de la synthèse des sphingolipides entraîne une accumulation de bases sphingoïdes (la sphinganine et la sphingosine) et une déplétion en céramide et en sphingolipides complexes. Ceci a de multiples conséquences sur la physiologie cellulaire, puisque les bases sphingoïdes et les céramides sont des second messagers impliqués dans de nombreuses fonctions telles que l'apoptose, la croissance et la différenciation cellulaire, l'inflammation ou encore la sécrétion protéique. Par ailleurs, les sphingolipides sont des constituants structuraux essentiels des membranes cellulaires et la FB1 peut provoquer une atteinte de l'intégrité membranaire.

Pour toutes les espèces animales étudiées, le foie est la principale cible de la FB1. Les reins sont également affectés chez de nombreuses espèces, notamment les rongeurs. Mais la particularité de cette toxine est la capacité d'induire lors d'intoxications aiguës des troubles spécifiques de l'espèce cible. Ainsi, à forte dose la FB1 est capable d'induire des oedèmes pulmonaires chez les porcins, des

leucoencéphalomalacies chez les équidés, des lésions rénales et hépatiques chez les rongeurs, ou encore d'induire des cancers de l'œsophage et des anomalies du tube neural chez l'homme.

2. La co-contamination en mycotoxines : méta-analyse des données publiées

Due à leur grande diversité structurale, la toxicité induite par les mycotoxines est très différente et varie en fonction de la cible cellulaire et tissulaire, mais aussi de l'espèce cible. Ainsi, il est très compliqué de prédire l'effet de leurs interactions, en se basant simplement sur les effets individuels. Néanmoins, lorsque certaines mycotoxines ou famille de mycotoxines ont des modes d'action similaires ou partagent des propriétés toxiques communes, telles que des effets immunosuppresseurs, mutagènes ou tératogènes, un effet au moins additif est attendu lors de leur association chez l'homme ou l'animal. Cette revue reporte l'analyse de plus de 100 expériences *in vivo* publiées dans la littérature, avec un plan factoriel 2 x 2, i.e. avec les effets individuels et combinés des mycotoxines. Pour chaque paramètre analysé, nous avons reporté l'effet de l'interaction tel que synergique, additif, moins qu'additif ou antagoniste. A noter que les interactions impliquant les aflatoxines représentent plus de la moitié des études reportées.

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Mycotoxins co-contamination : meta-analysis of published data

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ABSTRACT

Most fungi are able to produce several mycotoxins simultaneously; moreover food and feed can be contaminated by several fungi species at the same time. Thus, humans and animals are generally not exposed to one mycotoxin but to several toxins at the same time. Most of the studies concerning the toxicological effect of mycotoxins have been carried out taking into account only one mycotoxin.

In the present review, we analyzed 112 reports where laboratory or farm animals were exposed to a combination of mycotoxins, and we determined for each parameter measured the type of interaction that was observed. Most of the published papers concern interactions with aflatoxins and other mycotoxins, especially fumonisins, ochratoxin A and trichothecenes. A few papers also investigated the interaction between ochratoxin A and citrinin, or between different toxins from *Fusarium* species. Only, experiments with a 2 x 2 factorial design with individual and combined effects of the mycotoxins were selected. Based on the raw published data, we classified the interactions in four different categories: synergistic, additive, less than additive or antagonistic effects.

This review highlights the complexity of mycotoxins interactions which varies according to the animal species, the dose of toxins, the length of exposure but also the parameters measured.

Keywords : co-contamination, toxicological synergism, animal health, feeding trials, chronic toxicity.

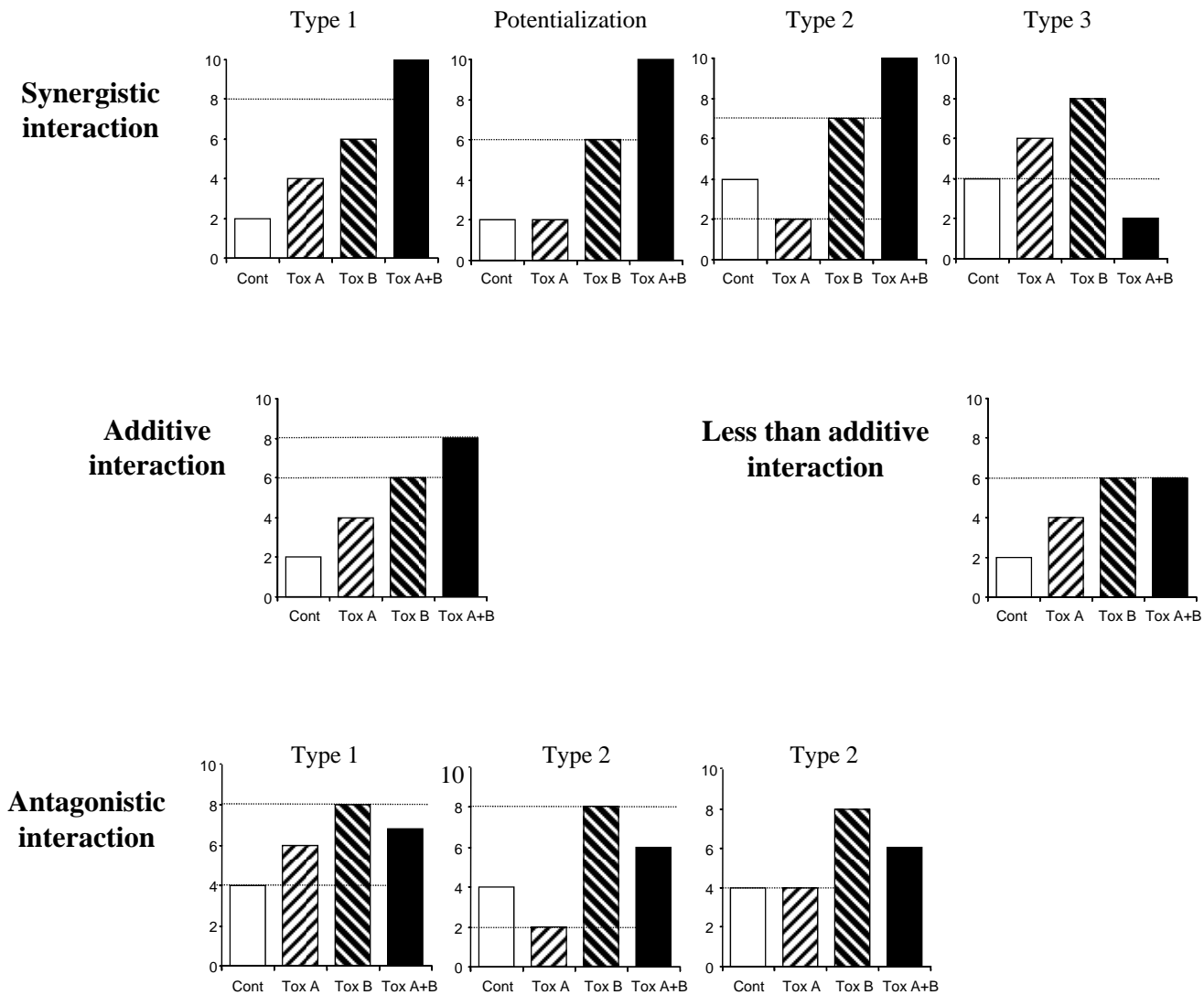
INTRODUCTION

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 fungi, mainly by species from the genus *Aspergillus*, *Fusarium* and *Penicillium*. Mycotoxins are very common contaminants of cereals. Furthermore, most mycotoxins are resistant to milling, processing and heating and, therefore, readily enter the food and feed chains (Bullerman and Bianchini, 2007). The toxicological syndromes caused by ingestion of such toxins range from acute mortality, to slow growth and reduced reproductive efficiency. Consumption of fungal toxins may also result in impaired immunity and decreased resistance to infectious diseases (Oswald and Comera, 1998).

Most fungi are able to produce several mycotoxins simultaneously in separate feedstuffs, and considering, it is a common practice to use multiple grain sources in animals diets, the risk to be exposed to several mycotoxins at the same time increases. This is supported by global surveys that indicate humans and animals are generally exposed to more than one mycotoxin (Bermudez *et al.*, 1997; Boeira *et al.*, 2000; Monbaliu *et al.*, 2010; Rodrigues and Griessler, 2010; Speijers and Speijers, 2004; Wangikar *et al.*, 2004b). The toxicity of combinations of mycotoxins cannot always be predicted based upon their individual toxicities. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive, or synergistic. The data on the *in vivo* combined toxic effects of mycotoxins are limited and therefore, the health risk from exposure to a combination of mycotoxins is incomplete.

The aim of this review is to summarize the published experiments, where laboratory and farm animals were exposed to a combination of mycotoxins and to describe, parameter by parameter, the type of interaction observed.

Figure 4 : characterization of the interaction between mycotoxins



CHARACTERIZATION OF THE DIFFERENT INTERACTIONS BETWEEN MYCOTOXINS

In the present review, in order to be consistent among the different papers, we went back to the original published raw data, and for each parameter that was measured, we classified the interaction in four different categories: synergistic, additive, less than additive and antagonistic effect.

- The “synergy” category was complex and we could differentiate 3 types of synergistic interaction (**Figure 4**) :

- ✓ Synergism type 1: contains experiments where the effect of the mycotoxins combination was greater than expected from the sum of the individual effects of the two toxins. We also included in this category, experiments where one toxin did not display any effect but where the effect of the co-contaminated treatment was greater than the effect of the other toxin alone (potentialization, P).
- ✓ Synergism type 2: the two mycotoxins induce opposite effects and the combined treatment induces an effect greater than the individual effect. So even though the toxins have opposite individual effects, the interaction led to an exacerbated effect.
- ✓ Synergism type 3: the two mycotoxins induce similar effects and the combined treatment induces an opposite effect than the individual effects.

- The “additive” category includes experiments where the effect of the combination could be calculated as the sum of the individual effects of the two toxins (**Figure 4**).

- The “less than additive” category includes experiments where the effect of the combined treatment mainly reflected the effect of only one of the toxin without additional effect of the other toxin (**Figure 4**).

- We also differentiated 2 types of antagonistic interaction (**Figure 4**) :

- ✓ Antagonism type 1: the two mycotoxins induce similar effects and the combined treatment induces a lower effect. So, the combination lowered the effect of the more potent toxin.
- ✓ Antagonism type 2: the two mycotoxins don’t induce the same effects and the combined treatment induces an effect intermediate between the two individual treatments. So, the combination lowered the effect of one of the toxin.

Results of the interaction were only reported, where significant differences were observed between the control group (no mycotoxin) and at least one of the mycotoxin-contaminated group (mycotoxin A, mycotoxin B or mycotoxin A+B), on parameters measured at the end of the experiment. Regarding histopathological analysis, few studies scored the incidence and severity of lesions; so we could not have analyzed the results based on the raw data, and include them in the different tables. Nevertheless, we included the conclusions as indicated by the authors in the text.

Table 1 : Interaction between Aflatoxins (AF) and Fumonisin (FB)

Mycotoxins Species (exposure)	Doses	SYNERGISTIC INTERACTION			ADDITIVE INTERACTION	LESS THAN ADDITIVE INTERACTION	ANTAGONISTIC INTERACTION		REF
		Type 1	Type 2	Type 3			Type 1	Type 2	
AF-FB <i>Fish</i> (322 d) ¹	0.1 – 3					- tumors in AF-initiated liver ↗			Carlson <i>et al.</i> , 2001
	0.1 – 23	- tumors in AF-initiated liver ↗ (P)							
	0.1 – 104	- tumors in AF-initiated liver ↗ (P)							
AF-FB <i>Mouse</i> (90 d)	0.01 – 10	- AST ↗ (P) - congestion, hemolysis in kidney ↗ - enlarged thymus ↗			- intensification of lesions in liver ↗	- feed intake ↗	- cholesterol ↘ - triglyceride → - ALP ↘	- calcium ↘ - congestion, hemolysis in spleen →	Casado <i>et al.</i> , 2001
AF-FB <i>Chicken</i> (21 d)	0.05 – 10	- heterophils ↘			- lymphocytes ↗				Del Bianchi <i>et al.</i> , 2005
	0.35 – 10						- lymphocytes ↗ - heterophils ↘		
	2.45 – 10	- albumin ↘					- lymphocytes ↗ - heterophils ↘		
AF-FB <i>Pig</i> (28 d)	0.05 – 10								Dilkin <i>et al.</i> , 2003
	0.05 – 30	- BWG ↘ (P) - feed intake ↘ (P)				- RW-L,H, Lg ↗			
AF-FB <i>Rat</i> (56 d) ²	0.017 bw/d – 250	- GSTP ⁺ lesions/foci ↗							Gelderblom <i>et al.</i> , 2002
AF-FB <i>Pig</i> (35 d)	2.5 – 100	- BWG, feed intake ↘ - AST, CHL, ALP ↗ - mortality ↗		- RW-L ↘	- triglycerides ↗ - RBC ↗ - lymphocytes stimulation ↘	- GGT ↗ - BUN ↘		- total iron ↗	Harvey <i>et al.</i> , 1995b
AF-FB <i>Turkey</i> (21 d)	0.75 – 200	- glucose, inorganic phosphorus ↘	- RW-L ↘		- BWG ↘ - RW-Pc ↗ - AST ↗	- RW-G ↗ - TP, cholesterol, triglycerides, calcium ↘ - LDH ↗ - lymphocytes stimulation ↘	- mortality ↗ - BUN ↗ - CK ↗	- RW-K ↗ - albumin ↘ - uric acid → - ALP ↘ - hemoglobin ↘	Kubena <i>et al.</i> , 1995b
AF-FB <i>Chicken</i> (27 d)	2.5 – 200	- BWG ↘ (P) - RW-L,K ↗ (P)				- TP ↘ - RW-S ↗		- albumin ↘ - RW-G ↗	Miazzo <i>et al.</i> , 2005
AF-FB	0.05 – 10	- feed intake ↘ - egg weight ↘				- egg production ↘			Ogido <i>et al.</i> ,

<i>Quail</i> (140 d)	0.2 – 10	- BW ↓					- feed intake ↓ - egg weight ↓ - BW ↓	- egg production ↓		2004
AF-FB <i>Rabbit</i> (21 d)	0.03 bw/d – 1.5 bw/d	- BWG ↓ (P) - mortality ↑ - AST, GGT ↑ - creatinine ↑ - Sa/So serum ↑ (P)	- RW-K ↑	- urea ↑	- RW-L ↓ - ALP ↑ - Sa/So urine ↑			- TP →	- albumin ↓ - Sa/So liver ↑	Orsi <i>et al.</i> , 2007
AF-FB <i>Rat</i> (21 d)	0.72 bw/d – 5 bw/d				- RW-L ↑ - AST, ALT ↑			- BWG ↑ - RW-K ↓ - ALP, GGT ↑ - TP, creatinine, albumin ↑	- urea →	Pozzi <i>et al.</i> , 2001
	0.72 bw/d – 15 bw/d	- GGT ↑	- BWG ↓ - RW-L ↓		- AST, ALP, ALT ↑ - TP, albumin ↑	- RW-K ↓		- creatinine ↑	- urea ↑	
AF-FB <i>Chicken</i> (33 d)	0.05 – 50	- AST ↑								Tessari <i>et al.</i> , 2010
	0.05 – 200				- AST ↑					
	0.2 – 50							- AST ↑		
	0.2 – 200					- TP ↑		- AST ↑		
AF-FB <i>Chicken</i> (33 d)	0.05 – 50	- BWG ↓ - Ab ND ↓ (P)				- RW-H,BF ↑				Tessari <i>et al.</i> , 2006
	0.05 – 200	- Ab ND ↓			- BWG ↓ - RW-H ↑			- RW-L ↑ - RW-BF →		
	0.2 – 50	- RW-BF ↑ - Ab ND ↓			- BWG ↓	- RW-L ↑		- RW-H ↑		
	0.2 – 200	- Ab ND ↓ (P)			- BWG ↓ - RW-H ↑	- RW-BF ↑		- RW-L ↑		
AF-FB <i>Rat</i> (90 d)	0.04 – 100	- SOD activity ↑					- DNA damage in SMC (ACA) ↑ - MDA, CAT activity ↑	- DNA damage in SMC (MN) ↑		Theumer <i>et al.</i> , 2010
AF-FB <i>Rat</i> (90 d)	0.04 – 100	- BW ↓ (P) - Incidence, severity of liver & kidney lesions ↑ - Sa/So kidney, urine, serum, liver ↑ (P)					- feed intake ↓ - Incidence, severity of lung & intestine lesions ↑			Theumer <i>et al.</i> , 2008
AF-FB <i>Turkey</i> (21 d)	0.2 – 75	- RW-Pc ↑			- Ab SRBC ↑	- BWG ↓ - RW-S ↑ - albumin, TP, cholesterol ↓	- AST ↑ - hemoglobin ↑	- RW-BF → - RW-L ↑ - Sa/So serum ↑		Weibking <i>et al.</i> , 1994

1) Carlson *et al.* (2001) : 30 min initiation by immersion with AF, followed by 4 weeks of recovery, and then, 42 weeks FB feeding

2) Gelderlom *et al.* (2002) : 14 days initiation with AF, followed by 3 weeks of recovery, and then 3 weeks FB feeding

INTERACTION BETWEEN THE DIFFERENT MYCOTOXINS

Most of the published papers, which are dealing with the effect of mycotoxin multi-contamination on animals, concern aflatoxins (62/112 reports). The main mycotoxins investigated in association with aflatoxins are fumonisins (16 reports), ochratoxin A (19 reports), and trichothecenes (16 reports), especially T-2 toxin (11 reports). The other important interactions involved ochratoxin A and citrinin (11 reports), and, fumonisins and moniliformin (7 reports). Despite the high occurrence of mycotoxins from *Fusarium spp.*, only few studies have investigated the interaction between Fusariotoxins (23 reports).

I. INTERACTIONS BETWEEN AFLATOXINS AND OTHER MYCOTOXINS

1) Interaction between Aflatoxins (AF) and Fumonisins (FB)

a) effects of AF and FB on zootechnical parameters

As displayed in the **Table 1**, this association of mycotoxins resulted most of the time in a synergistic effect on body weight gain (Dilkin *et al.*, 2003; Harvey *et al.*, 1995b; Miazzo *et al.*, 2005; Ogido *et al.*, 2004; Orsi *et al.*, 2007; Pozzi *et al.*, 2001; Tessari *et al.*, 2006; Theumer *et al.*, 2008). Surprisingly, Pozzi *et al.* (2001) reported an increase and an antagonistic interaction on body weight gain when rats were fed both AF and FB.

Reduced egg production and egg weight is one of the effects of aflatoxicosis in laying hens. In quails, one study reported an antagonistic effect of AF and FB on egg production (**Table 1**). Surprisingly, egg production was reduced in animals fed FB-contaminated diet, and this effect was partially spared when animals received AF+FB contaminated diet (Ogido *et al.*, 2004).

b) effects AF and FB on biochemical parameters and organs weight

Several experiments have measured enzyme concentrations in the serum of animals fed multi-contaminated diets, in order to investigate tissue damage, especially the liver. The levels of mycotoxins used in these reports were moderate to high, and therefore induced organ injuries, physiologically translated by increase of these biomarkers. The lack of response in some experiments is due to the low doses of mycotoxins used (Del Bianchi *et al.*, 2005). Overall, association of AF and FB induced a significant increase of the serum levels of AST, ALP, ALT and GGT (synergistic or additive effect, **Table 1**). Such increase in enzymatic activity can be attributed to cell necrosis, changes in cell membrane permeability or impairment of biliary excretion. This strong hepato-biliary dysfunction

due to the ingestion of co-contaminated feed is in accordance with the histopathological observations (*see below*).

Another parameter correlated with hepatotoxicity, is the relative weight of liver. Interestingly, either an increase or a decrease of the RW-L was observed in the experiments for the combined treatment (**Table 1**). This is attributed to the individual effect of each toxin, showing either an increase or a decrease for this parameter, according to studies. A synergistic interaction type 2 or 3 was recorded in pigs, turkeys, and rats exposed to both toxins (Harvey *et al.*, 1995b; Kubena *et al.*, 1995b; Pozzi *et al.*, 2001). It would be possible that the mixture induced enough hepatic cell wall damage to have a major effect on total hepatic parenchymal mass. By contrast, other studies have reported an increase in the RW-L, but leading to a less than additive (Dilkin *et al.*, 2003; Tessari *et al.*, 2006) or an antagonistic interaction (Tessari *et al.*, 2006; Weibking *et al.*, 1994) in animals receiving multi-contaminated feed.

In two studies, ALP concentration was decreased when animals were fed with AF (Casado *et al.*, 2001; Kubena *et al.*, 1995b). The authors suggested that it was due to the inhibition of protein synthesis by AF. The combination of AF with FB led to a lesser reduction, suggesting an antagonistic interaction (**Table 1**).

c) effects of AF and FB on lipids

Disruption of sphingolipid biosynthesis is the main mechanism involved in FB toxicity, with inhibition of ceramide synthase leading to accumulation of sphingoid bases (sphinganine, Sa and sphingosine, So). This inhibition is well documented and the Sa/So ratio is considered as a good indicator of FB exposure (Theumer *et al.*, 2008).

Three studies analyzed the effect of co-exposure to AF and FB on sphingoid bases in different biological samples, with the aim of investigating if AF could exacerbate the toxicity of FB (Orsi *et al.*, 2007; Theumer *et al.*, 2008; Weibking *et al.*, 1994). These studies gave fairly different results (**Table 1**). In contrast with the two other studies, Orsi *et al.* (2007) observed an increased Sa/So ratio in urine of animals exposed to AF alone. Both, Orsi *et al.* (2007) and Weibking *et al.* (1994) demonstrated an antagonistic interaction between AF and FB on the Sa/So ratio in the liver and in the serum of exposed animals. Conversely, Theumer *et al.* (2008) observed a synergistic effect of the two toxins when looking at the Sa/So ratio in the kidney, urine, and to a lesser extent in the serum and the liver of exposed rats. Of note, this study also described a synergistic interaction in the incidence and the severity of kidney and liver lesions (Theumer *et al.* 2008).

d) effects of AF and FB on microscopic lesions

Microscopic lesions following ingestion of AF and FB were mostly evaluated in the liver and kidneys, their respective target organs. In these studies, additive or synergistic interactions between the two toxins were observed (Casado *et al.*, 2001; Del Bianchi *et al.*, 2005; Dilkin *et al.*, 2003; Gelderblom *et al.*, 2002; Harvey *et al.*, 1995b; Orsi *et al.*, 2007; Pozzi *et al.*, 2001; Tessari *et al.*, 2010; Tessari *et al.*, 2006; Theumer *et al.*, 2008; Weibking *et al.*, 1994). Histopathological alterations in liver consisted mainly in vacuolar degeneration of hepatocytes, apoptotic and mitotic figures, dysplastic nodules, megalocytosis and fibrosis. In animals receiving both AF and FB, cirrhotic livers were also observed. Enlargement of gallbladder and duct proliferation were also reported (Orsi *et al.*, 2007). In kidney, apoptosis of tubular epithelial cells, glomerulonephritis and tubular epithelium with areas of degeneration and necrosis, as well as congestion and hemolysis were reported in animals fed AF and FB contaminated diet (Casado *et al.*, 2001; Del Bianchi *et al.*, 2005; Theumer *et al.*, 2008). These lesions were considered as moderate to severe, depending on the species and the doses used.

Lymphocytic infiltrates in the small intestine, and thickening of the alveolar walls and lymphocytic infiltrates, as well as apoptosis in the lungs, were observed in rats fed with diet containing AF (Theumer *et al.*, 2008). Incidence and severity of these alterations were similar in rats fed the co-contaminated diet, suggesting a less than additive interaction (**Table 1**).

e) effects of AF and FB on genotoxicity and carcinogenicity

Both AF and FB have carcinogenic effects, and besides, co-contamination of maize with AF and FB was related to a high-incidence area of human primary hepatocellular carcinoma in China (Li *et al.*, 2001). At the molecular level, the toxicology of AF involves its metabolic conversion by the cytochrome P450 system to the highly electrophilic AF-exo-8,9-epoxyde, which in turn binds to the DNA guanines to form adducts. Therefore, AF has been reported to be a good initiator and a proven complete carcinogen. On the other hand, FB has been described to be a potent tumor promoter but a weak initiator (Riley, 1998).

Two long-term trials, performed in rats and in rainbow trouts, initiated hepatocarcinogenesis with AF, and then treated the animals with high doses of FB (Carlson *et al.*, 2001; Gelderblom *et al.*, 2002) (**Table 1**). In trouts, FB promoted liver cancer in AF-initiated animals, but did not promote tumors in other tissues (Carlson *et al.*, 2001). Similarly, in rats, despite the fact that AF and FB were administered three weeks apart in a sequential model, they acted synergistically with respect to cancer initiation, as demonstrated by the number of hepatocytes nodules and foci (Gelderblom *et al.*, 2002).

Table 2 : Interaction between Aflatoxins (AF) and Ochratoxin A (OTA)

Mycotoxins <i>Species</i> (<i>exposure</i>)	Doses	SYNERGISTIC INTERACTION			ADDITIVE INTERACTION	LESS THAN ADDITIVE INTERACTION	ANTAGONISTIC INTERACTION		REF
		Type 1	Type 2	Type 3			Type 1	Type 2	
AF-OTA <i>Chicken egg</i> (10 d) ¹	0.025 – 1.15 (µg/egg)					- embryos mortality ↗		- abnormalities ↗	Edrington <i>et al.</i> , 1995
	0.05 – 2.3 (µg/egg)	- abnormalities ↗					- embryos mortality ↗		
AF-OTA <i>Chicken egg</i> (15 d) ¹	0.025 – 1.0 (µg/egg)	- abnormalities ↗			- embryos mortality ↗				Edrington <i>et al.</i> , 1995
	0.05 – 2.0 (µg/egg)	- abnormalities ↗			- embryos mortality ↗				
AF-OTA <i>Pig</i> (28 d)	2.0 – 2.0	- triglycerides ↗ (P)	- TP ↗ - RW-L ↗		- BW ↘ - ALP ↗ - inorganic phosphorus ↘	- BUN, cholesterol, glucose ↘	- calcium → - sodium ↘	- albumin → - hemoglobin → - RW-K →	Harvey <i>et al.</i> , 1989a
AF-OTA <i>Chicken</i> (21 d)	2.5 – 2.0	- BW ↘ - mortality ↗ - RW-L,K,G,S,Pv,Pc ↗						- liver lipid levels →	Huff and Doerr, 1981
AF-OTA <i>Chicken</i> (42 d)	2.5 – 2.0	- BW ↘ - prothrombin times ↗			- incidence and severity of breast bruises ↗		- incidence and severity of right thigh bruises ↗		Huff <i>et al.</i> , 1983
AF-OTA <i>Chicken</i> (42 d)	2.5 – 2.0	- live, dressed, eviscerated weight ↘ - carcasse yield, breast, drum, thigh, wing, back weights ↘ - plasma carotenoids ↘				- breast yield ↘		- liver lipid levels ↗	Huff <i>et al.</i> , 1984
AF-OTA <i>Chicken</i> (21 d)	3.5 – 2.0				- BW ↘ - RW-L,K,H,Pv ↗		- albumin, TP, cholesterol ↘	- uric acid ↗ - AST ↘	Huff <i>et al.</i> , 1992
AF-OTA <i>Chicken</i> (42 d)	0.2 – 0.2				- RW-S ↘ - DTH reaction ↘	- hemoglobin ↘ - albumin, cholesterol, triglycerides ↘ - creatinine, uric acid ↗ - ALP ↗ - RW-L ↗ - RW- BF,T ↘	- Ab ND ↘		Kalorey <i>et al.</i> , 2005
AF-OTA <i>Calve</i> (87 d)	0.013 – 0.450						- WBC ↘		Patterson <i>et al.</i> , 1981

AF-OTA <i>Chicken</i> (35 d)	0.3 – 2.0				- RW-G,A ↗ - GGT ↗ - hemoglobin ↘	- BW, feed intake ↘ - ALT ↘	- mortality ↗ - RW-L,K ↗ - TP, cholesterol, BUN ↘ -coagulation time →		Raju and Devegowda , 2000
AF-OTA <i>Chicken</i> (35 d)	0.3 – 2.0						- RW-BF,T ↘ - Ab ND, IBD ↘		Raju and Devegowda , 2002
AF-OTA <i>Guinea pig</i> (28 d)	0.01 mg/d – 0.45 mg/d					- TP, globulin ↘ - complement titer ↘			Richard <i>et al.</i> , 1975
AF-OTA <i>Chicken</i> (42 d)	0.2 – 0.2				- BW ↘ - cholesterol ↘ - RW-S,T ↘ - Ab ND, DTH reaction ↘	- triglyceride ↘ - creatinine, uric acid ↗	- hemoglobin ↘ - RW-L ↗, RW-BF ↘	- WBC →	Sakhare <i>et al.</i> , 2007
AF-OTA <i>Pig</i> (42 d)	0.375 – 1.0					- BWG ↘	- creatinine ↗	- BUN →	Tapia and Seawright, 1985
AF-OTA <i>Laying hen</i> (50 d)	0.5 – 1.0	- feed consumed for production of 1 dozen eggs ↗			- egg production ↘	- egg shape index ↘	- feed intake ↘ - egg shell thickness →		Verma <i>et al.</i> , 2003
	1.0 – 2.0				- egg production ↘ - feed consumed for production of 1 dozen eggs ↗ - egg shape index ↘		- feed intake ↘	- egg shell thickness ↘	
	2.0 – 4.0				- egg production ↘ - feed consumed for production of 1 dozen eggs ↗	- feed intake ↘ - egg shape index ↘	- egg shell thickness →		
AF-OTA <i>Laying hen</i> (50-75 d)	0.5 – 1.0	- metabolizable energy, protein retention ↘ - maintenance energy requirement ↗			- egg energy deposition ↘				Verma <i>et al.</i> , 2007
	1.0 – 2.0	- maintenance energy requirement ↗			- metabolizable energy, protein retention, egg energy deposition ↘				
	2.0 – 4.0				- metabolizable energy, protein retention ↘	- egg energy deposition ↘ - maintenance energy requirement ↗			
AF-OTA <i>Chicken</i>	0.5 – 1.0	- BWG ↘ - feed intake ↘			- RW-L ↗ - DTH reaction ↘		- Ab SRBC ↘		Verma <i>et al.</i> , 2004
	1.0 – 2.0	- feed intake ↘			- BWG ↘	- RW-BF ↘		- RW-K ↗	

(49 d)					- RW-L ↗ - Ab SRBC ↘	- DTH reaction ↘			
	2.0 – 4.0		- RW-K ↗		- BWG ↘ - feed intake ↘ - Ab SRBC ↘	- RW-BF ↘	- RW-L ↗ - DTH reaction ↘		

1) Edrington *et al.* (1995) : one administration and measurements 10 or 15 d post-administration

Cellular oxidative stress was also proposed as a possible mechanism of cancer initiation. However, Theumer *et al.* (2010) only observed a less than additive and an antagonistic interaction between the two toxins when looking at DNA lesions using the alkaline comet assay (ACA) and the micronucleus technique (MN), respectively (**Table 1**). Furthermore, in the same study, a less than additive interaction was recorded in the MDA and CAT activity, both are being biomarkers of oxidative stress.

f) effects AF and FB on immunity

Few studies investigated the combined effect of AF and FB on immunity (**Table 1**). After ingestion of the co-contaminated diet, the interaction on the reduced lymphocytes proliferation upon mitogenic stimulation, was described either as less than additive (Kubena *et al.*, 1995b) or additive (Harvey *et al.*, 1995b). Tessari *et al.* (2006) showed a synergistic decrease of the antibodies titers against Newcastle Disease. By contrast, Weibking *et al.* (1994) reported an unexpected increase and an additive effect of the two toxins when looking at the hemagglutination titers against SRBC in turkey poults.

2) Interaction between Aflatoxins (AF) and Ochratoxin A (OTA)

a) effects of AF and OTA on zootechnical parameters

As indicated in **Table 2**, the interaction between AF and OTA on animal performance has been studied in 11 publications. In most of these studies, the association induced a synergistic or an additive effect on body weight gain (Harvey *et al.*, 1989a; Huff and Doerr, 1981; Huff *et al.*, 1983; Huff *et al.*, 1992; Sakhare *et al.*, 2007; Verma *et al.*, 2004). In addition, Huff *et al.* (1983) observed that even four weeks after exposure to the co-contaminated diet had stopped, the animals did not recover their normal body weight. The same author also observed that in chicken, ingestion of feed co-contaminated with AF and OTA decreased carcass yield, breast weight and other processing parameters in a synergistic manner (Huff *et al.*, 1984) (**Table 2**).

Both AF and OTA affect egg production and hatchability, and one group investigated the combined effect of these two toxins on laying hens (Verma *et al.*, 2003; 2007) (**Table 2**). An additive interaction of AF and OTA was observed on egg production and on the feed efficiency (consumption for egg production) (Verma *et al.*, 2003). Regarding egg quality, less than additive and antagonistic interactions were observed on egg. Protein and energy utilization were also modulated by the consumption of AF and OTA. However the interaction between the two toxins varied according to their concentrations ranging from synergistic to less than additive effect (Verma *et al.*, 2007).

b) effects AF and OTA on biochemical parameters and organs weight

Regarding biochemical parameters, many studies reported a less than additive or an antagonistic interaction between AF and OTA (**Table 2**). These types of response were observed for serum concentrations of cholesterol, albumin, total proteins, creatinine, uric acid or blood urea nitrogen (Harvey *et al.*, 1989a; Huff *et al.*, 1992; Kalorey *et al.*, 2005; Raju and Devegowda, 2000; Richard *et al.*, 1975; Sakhare *et al.*, 2007; Tapia and Seawright, 1985).

Several studies have assessed the effect of the co-contaminated diet on relative weight of liver and on liver functions (**Table 2**). The type of interaction between the two toxins on the increased relative weight of the liver varied a lot from one experiment to another. Huff and Doerr (1981), and Harvey *et al.* (1989) observed a synergistic interaction, while Huff *et al.* (1992) and Verma *et al.* (2004) observed an additive interaction. Kalorey *et al.* (2005) noted a less than additive interaction, and with higher doses of toxins, Verma *et al.* (2004) described an antagonistic interaction. Antagonism was also observed in the experiments of Raju and Devegowda (2000) and Sakhare *et al.* (2007). Interestingly, OTA seems to inhibit or spare the effect of AF on the accumulation of lipid in the liver, leading to an antagonistic interaction between the two mycotoxins (Huff and Doerr, 1981; Huff *et al.*, 1984) (**Table 2**). Few data are available on the biomarkers of hepatotoxicity, but an additive or less than additive effect was observed for the increased concentrations of ALP (Harvey *et al.*, 1989a; Kalorey *et al.*, 2005) and GGT (Raju and Devegowda, 2000), and an antagonistic or less than additive effect was reported for the decreased concentrations of AST (Huff *et al.*, 1992) and ALT (Raju and Devegowda, 2000) (**Table 2**).

Concerning the increased relative weight of kidney, as already mentioned for the liver, the type of interaction of the two toxins on this organ range from synergism to antagonism (Harvey *et al.*, 1989a; Huff and Doerr, 1981; Huff *et al.*, 1992; Raju and Devegowda, 2000; Verma *et al.*, 2004) (**Table 2**).

Based on the relative weights of gizzard, proventriculus and pancreas, it seems that the upper alimentary tract is more sensitive to the combination than mycotoxins alone (Huff and Doerr, 1981; Huff *et al.*, 1992) (**Table 2**).

Of note, a lack of response for most of the variables studied was observed in calves fed any contaminated diets (Patterson *et al.*, 1981). This failure was probably attributable to the low doses of mycotoxins used, and to the detoxifying action of the rumen flora on OTA.

c) effects of AF and OTA on microscopic lesions

Microscopic lesions following ingestion of AF and OTA contaminated feed were mostly evaluated in the liver and kidneys, their respective target organs (Huff and Doerr, 1981). Unfortunately, the different studies are not in agreement one to each other. For example, in chicken, OTA in the diets prevented hepatic fatty infiltration that was caused by AF (Huff and Doerr, 1981). Pigs fed the co-contaminated diet, presented the same hepatic lesions than those fed diet contaminated with AF alone (Tapia and Seawright, 1985). By contrast, Sakhare *et al.* (2007) recorded more severe hepatic lesions in chicken receiving the co-contaminated diet, with granular and vacuolar degenerative changes, necrosis of liver parenchyma and areas of hemorrhages.

The same discrepancy was noticed for the histology of the kidney. In pigs, Tapia and Seawright (1985) observed less severe renal lesions in animal fed the co-contaminated diet than in animal receiving the OTA-contaminated diet. This is in agreement with the data obtained in pigs, by Harvey *et al.* (1989a) on the relative weight of kidney, and the data obtained by Tapia and Seawright (1985) on the creatinine and blood urea nitrogen concentrations (**Table 2**). Conversely, Sakhare *et al.* (2007) indicated that renal injuries appeared earlier and were more developed in chicken fed multi-contaminated diet than in animal receiving the mono-contaminated diets. This led to destruction of tubular epithelium, with detachment of tubular cells from basement membrane. The species used may explain these discrepancies.

One study focused on the effect of AF and OTA on bruising in broiler chickens (Huff *et al.*, 1983), as bloody thigh syndrome has been suspected to be associated with mycotoxicoses. This study revealed a severe coagulopathy, as measured by elevated prothrombin times, and, an additive and an antagonistic interaction between AF and OTA were observed for the incidence and severity of breast and right thigh bruises, respectively (**Table 2**).

d) effects of AF and OTA on immunity

Atrophy of lymphoid organs were observed in several studies (Kalorey *et al.*, 2005; Raju and Devegowda, 2002; Sakhare *et al.*, 2007; Verma *et al.*, 2004) (**Table 2**). This seems to be due to necrosis and cellular depletion, as suggested by microscopical observations with necrotic areas in germinal centre, depopulation of lymphocytes in spleen, or depletion and necrosis of lymphoid cells from follicle in bursa of Fabricius (Sakhare *et al.* 2007). In the bursa of Fabricius, these histopathological changes were more pronounced in animals fed the co-contaminated diet compared to animal receiving the mono-contaminated diets (Sakhare *et al.*, 2007).

The depletion of lymphocytes suggested a suppression of cell mediated immunity. Indeed, the contact sensitivity reaction, was reduced in chicken fed the co-contaminated diet with an additive interaction between AF and OTA (Kalorey *et al.*, 2005; Sakhare *et al.*, 2007; Verma *et al.*, 2004). However, Verma *et al.* (2004) observed that, with increasing doses of toxins, the interaction went from additive to less than additive and to antagonist (**Table 2**).

In order to analyze the effect of the mycotoxins on the humoral response, the antibodies titers against Newcastle disease, Sheep red blood cells or Infectious Bursal disease were measured (Kalorey *et al.*, 2005; Raju and Devegowda, 2002; Sakhare *et al.*, 2007; Verma *et al.*, 2004). Mono- and multi-contaminated diets decreased the humoral response, and interaction between AF and OTA showed either an additive (Sakhare *et al.*, 2007; Verma *et al.*, 2004) or antagonistic interaction (Kalorey *et al.*, 2005; Raju and Devegowda, 2002; Verma *et al.*, 2004) (**Table 2**).

Also reported the effect on complement activity, but a less than additive interaction was obtained between OTA and AF (Richard *et al.*, 1975) (**Table 2**).

e) effects of AF and OTA on teratology

Both AF and OTA are able to cross the placental barrier and demonstrated some teratogenic properties in some species (Wangikar *et al.*, 2004b; Wangikar *et al.*, 2005)

In two separate experiments, Edrington *et al.* (1995) showed that embryonic mortality increased following injection to combination of AF and OTA, but led to different types of interaction according to doses and experiments. He supposed these differences were related to variations in embryo sensitivities among batches of eggs. By contrast, exposure of chicken embryos to the combination of toxins did mostly result in a synergistic interaction in the number of abnormalities.

Wangikar in 2004b and 2005 performed two large studies in rats and rabbits, respectively, where mycotoxins were administered orally on days 6-18 of gestation. Both mycotoxins interfered with the body wall formation leading to gastroschisis as observed in both the high OTA and high AF combination groups (Wangikar *et al.*, 2004b). In the co-exposed group, more severe cardiac lesions were noticed (Wangikar *et al.*, 2004b; Wangikar *et al.*, 2005) but skeletal, and visceral anomalies were reduced or absent, when compared to the mono-exposed groups. Similarly, the presence of AF seems to prevent the exencephaly and incomplete closure of the skull caused by OTA; and the presence of OTA prevent the head abnormality and open eye caused by AF. Another study of the same group also indicates that brain, kidney, and liver lesions were less severe in rats fetuses co-exposed to AF and OTA than in mono-exposed animals (Wangikar *et al.*, 2004a). Two mechanisms were proposed to explain these observations. The first hypothesis is that AF may inhibit the metabolism of OTA in the liver and increase its excretion. The second hypothesis is based on the

Table 3 : Interaction between Aflatoxins (AF) and Trichothecenes (TCT)

Mycotoxins <i>Species</i> (<i>exposure</i>)	Doses	SYNERGISTIC INTERACTION			ADDITIVE INTERACTION	LESS THAN ADDITIVE INTERACTION	ANTAGONISTIC INTERACTION		REF
		Type 1	Type 2	Type 3			Type 1	Type 2	
AF-T2 <i>Chicken</i> (35 d)	2.0 – 1.0	- RW-K ↗ (P)			- BWG ↘ - RW-L ↗ - RW-T,BF ↘	- feed intake ↘ - RW-G,S ↗ - Ab IBD ↘	- Ab ND ↘		Girish and Devegowda, 2006
AF-T2 <i>Pig</i> (28 d)	2.5 – 10				- BWG ↘	- BUN ↘ - GGT ↗	- sodium, phosphorus, albumin ↘	- cholesterol, triglycerides, TP ↘ - ALP, CHL ↗, AST → - RBC, WBC ↗, hemoglobin → - RW-L ↗, RW-H,K →	Harvey <i>et al.</i> , 1990
AF-T2 <i>Chicken</i> (21 d)	2.5 – 4.0	- BWG ↘ - RW-L,K,Pv,H ↗ (P) - RW-G ↗ - TP, albumin, uric acid ↘ - cholesterol ↘ (P) - potassium ↘ (P) - CHL ↘ (P)	- CK ↗		- hemoglobin ↘ - triglycerides ↘ - calcium ↘ - LDH, ALP ↘	- oral lesions ↗ - RW-S,Pc ↗ - RBC ↘ - magnesium ↘	- glucose ↘	- sodium →	Huff <i>et al.</i> , 1988
AF-T2 <i>Chicken</i> (21 d)	3.5 – 8.0	- BWG ↘ - RW-L ↗ (P) - RW-G ↗ - hemoglobin ↘ - ALP ↘	- CK ↗		- RW-Pv ↗ - RW-BF ↘ - uric acid, triglycerides, TP ↘	- RW-K,Pc ↗ - cholesterol, albumin ↘	- mortality ↗ - LDH ↘ - CHL ↗	- oral lesions ↗ - RW-H,S ↗ - GGT ↗	Kubena <i>et al.</i> , 1990
AF-T2 <i>Quail</i> (35 d)	3.0 – 4.0	- GGT ↗ - calcium ↗			- TP, albumin, globulin ↘	- cholesterol ↘ - AST ↗ - potassium ↘	- glucose, BUN ↘ - ALT ↘	- ALP → - sodium →	Madheswaran <i>et al.</i> , 2004
AF-T2 <i>Quail</i> (35 d)	3.0 – 4.0				- BW, feed intake ↘	- hemoglobin, RBC ↘			Madheswaran <i>et al.</i> , 2005
AF-T2 <i>Hamster</i> (21 d) ¹	1.0 – 1.0	- GGT ↘	- LDH ↘ - bilirubin ↘	- ALT, AST ↘		- glucose ↘	- ALP ↗	- cholesterol ↗	Rajmon <i>et al.</i> , 2001
AF-T2 <i>Chicken</i> (35 d)	0.3 – 3.0	- RW-L,K ↗ (P) - ALT ↘ - hemoglobin ↘		- coagulation time ↘	- BW ↘ - GGT ↗	- feed intake ↘ - mortality ↗	- RW-A ↗ - BUN ↘	- RW-G → - TP, cholesterol ↘	Raju and Devegowda, 2000

AF-T2 <i>Chicken</i> (35 d)	0.3 – 3.0	- RW-T ↓			- RW-BF ↓	- Ab IBD ↓	- Ab ND ↓		Raju and Devegowda, 2002
AF-T2 <i>Rat</i> (140 d) ²	0.25 – 0.05						- RW-L,K →		Tamimi <i>et al.</i> , 1997
AF-DAS <i>Lamb</i> (34 d)	2.5 – 5.0	- TP ↑ - GGT ↑ (P)			- BWG, feed intake ↓ - BUN ↓	- CHL ↓		- cholesterol ↑	Harvey <i>et al.</i> , 1995a
AF-DAS <i>Pig</i> (28 d)	2.5 – 2.0				- BWG ↓	- ALP, GGT ↑ - AST ↓ - RW-S ↑	- CHL ↑ - creatinine ↑	- WBC, hemoglobin ↑ - RW-L ↑	Harvey <i>et al.</i> , 1991
AF-DAS <i>Chicken</i> (21 d)	3.5 – 5.0	- BWG ↓ - mortality ↑ - GGT ↑			- RW-G ↑ - LDH ↓	- oral lesions ↑ - RW-K,H,Pv,S,Pc ↑ - glucose, TP ↓	- triglycerides, albumin ↓ - AST ↓	- RW-L ↑ - cholesterol ↓ - calcium → - CK →	Kubena <i>et al.</i> , 1993
AF-DON <i>Pig</i> (28 d)	3.0 – 3.0	- cholesterol, glucose ↓ (P) - WBC ↑ (P)			- CK ↓ - TP ↓	- BWG ↓ - GGT, AST ↑ - BUN ↓ - calcium, magnesium ↓	- albumin ↓	- ALP ↑ - potassium, phosphorus ↓ - RBC, hemoglobin, prothrombin time ↑	Harvey <i>et al.</i> , 1989b
AF-DON <i>Chicken</i> (21 d)	2.5 – 16	- RW-Pv,G, L ↑ - ALP ↓			- BWG ↓ - hemoglobin ↓ - AST ↓	- RW-S,K ↑ - TP, albumin, uric acid, cholesterol, triglyceride ↓ - calcium ↓	- glucose ↓ - LDH ↓ - liver lipid ↑	- RBC → - phosphorus →	Huff <i>et al.</i> , 1986

1) Rajmon *et al.* (2001) : intragastrically administration, twice a week for 3 weeks

2) Tamimi *et al.* (1997) : intraperitoneally administration, twice a week for 20 weeks

property of OTA and AF to inhibit the protein synthesis. Indeed, OTA is known to limit the protein synthesis through competitive inhibition of phenylalanine-t-RNA-synthesis with phenylalanine, while AF prevented protein synthesis through an inhibition of RNA synthesis. Therefore, antagonistic effects of the mycotoxins might be due to inhibition of transcription by AF, with a concomitant increase in the cellular pool of phenylalanine available for translation, as inhibition of protein synthesis by OTA was inversely proportional to phenylalanine concentration (Wangikar *et al.*, 2004b).

3) Interaction between Aflatoxins (AF) and Trichothecenes (TCT)

3.1) Interaction between Aflatoxins (AF) and T-2 toxin (T2)

a) effects of AF and T-2 toxin on zootechnical parameters

Several studies have investigated the combined effect of AF and T-2 toxin on body weight gain (**Table 3**), and all of them demonstrated at least an additive, or a synergistic interaction between the two toxins (Girish and Devegowda, 2006; Harvey *et al.*, 1990; Huff *et al.*, 1988; Kubena *et al.*, 1990; Madheswaran *et al.*, 2005; Raju and Devegowda, 2000). A similar interaction was also observed when looking at the relative weight of several organs of poultry (Girish and Devegowda, 2006; Huff *et al.*, 1988; Kubena *et al.*, 1990; Raju and Devegowda, 2000; 2002). By contrast, in pigs and in rats, Harvey *et al.* (1990) and Tamimi *et al.* (1997) observed an antagonistic interaction on the relative weights of liver, heart and kidney (**Table 3**).

b) effects of AF and T-2 toxin on biochemical parameters

Regarding biochemical parameters, the combined treatment showed a significant decrease for several parameters, such as total proteins, albumin, cholesterol, triglycerides, as well as some enzyme concentrations (ALP, ALT, LDH) (**Table 3**). These reduced concentrations may be attributed to the property of both AF and T-2 toxin to inhibit the protein synthesis, during transcription and translation respectively. Nonetheless, among these studies, different types of interaction were observed for these biochemical parameters, ranging from synergistic to antagonistic interactions (**Table 3**).

c) effects of AF and T-2 toxin on microscopic lesions

Because T-2 toxin is a potent irritant of the buccal cavity, the effect of AF and T-2 toxin was investigated on the oral cavity. When chicken received mono-contaminated feed, oral lesions were

only seen in birds given T-2 toxin, and the combination with AF resulted in an antagonistic interaction (Kubena *et al.*, 1990) (**Table 3**). The authors suggested that the lower effect of T-2 toxin in the presence of AF was likely due to a decreased intake of T-2 toxin in chicks that received the multi-contaminated diet. In swine, Harvey *et al.* (1990) reported necrotizing contact dermatitis on the snout, buccal commissures, and prepuce of animals. However, in the publication, the authors indicated that these dermal lesions were observed in T-2 and T-2+AF groups, but did not mention the type of interaction.

The interaction of AF and T-2 toxin led to contradictory results concerning the lesions observed in the liver. Indeed, a synergistic effect was observed in rats with hepatic injuries characterized by congestions with fatty acid changes, and significant bile duct proliferation (Tamimi *et al.*, 1997). An additive effect was observed in quails characterized by fatty vacuoles, mitochondria degeneration, and indistinguishable rough endoplasmic reticulum (Madheswaran *et al.*, 2006). By contrast, less than additive and antagonistic effects were reported in chicken and swine fed with multi-contaminated diets respectively, in comparison to the lesions observed in animals exposed to AF-contaminated diet (Harvey *et al.*, 1990; Kubena *et al.*, 1990). These data correlate with the interactions observed on biochemical parameters for the two toxins.

A synergistic effect of the two toxins was also observed for renal lesions, with tubular epithelial degeneration, congestion, swelling of the glomureli, as well as hypercellularity (Tamimi *et al.*, 1997).

d) effect on immunity

One research group has investigated the interaction between AF and T-2 toxin on the immune system (Girish and Devegowda, 2006; Raju and Devegowda, 2002). Conclusions between these two studies were in agreement and showed different types of interaction of the two mycotoxins depending on the parameter : a synergistic or additive effect on the thymus weight, an additive effect on the bursa of Fabricius weight, a less than additive effect on the antibody titer against bursal disease infection, and an antagonistic effect on the antibody titer against Newcastle disease (**Table 3**).

3.2) Interaction between Aflatoxins (AF) and Diacetoxyscirpenol (DAS)

The interaction between AF and diacetoxyscirpenol (DAS) was reported in three studies performed in lamb, pig and chicken (Harvey *et al.*, 1995a; Harvey *et al.*, 1991; Kubena *et al.*, 1993). The levels of mycotoxins used in these studies were quite similar and the association of toxins led to a synergistic or additive interaction on body weight gain (**Table 3**).

Table 4 : Interaction between Aflatoxins (AF) and other mycotoxins

Mycotoxins <i>Species</i> (<i>exposure</i>)	Doses	SYNERGISTIC INTERACTION			ADDITIVE INTERACTION	LESS THAN ADDITIVE INTERACTION	ANTAGONISTIC INTERACTION		REF
		Type 1	Type 2	Type 3			Type 1	Type 2	
AF-CPA <i>Chicken</i> (28 d)	1.0 – 20				- RBC ↓	- albumin, glucose, hemoglobin ↓		- TP, cholesterol ↓	Kumar and Balachandran, 2005
AF-CPA <i>Rat</i> (3 d) ¹	0.1 bw/d – 0.1 bw/d					- incidence of lesions in liver ↑			Morrissey <i>et al.</i> , 1987
	2.0 bw/d – 0.1 bw/d	- mortality ↑ - incidence of lesions in kidney ↑				- BWG, feed intake ↓ - incidence of lesions in liver ↑		- glycocholic acid ↑	
	0.1 bw/d – 4.0 bw/d					- incidence of lesions in liver ↑			
	2.0 bw/d – 4.0 bw/d					- BWG, feed intake ↓ - mortality ↑ - incidence of lesions in kidney ↑ - glycocholic acid ↑	- incidence of lesions in liver ↑		
AF-CPA <i>Guinea pig</i> (20 d)	0.045 – 2.2	- BWG ↓ - mortality ↑				- hemolytic complement titers ↓		- DTH reaction →	Pier <i>et al.</i> , 1989
AF-CPA <i>Chicken</i> (21 d)	3.5 – 50	- mortality ↑ - AST ↑ (P)			- BW ↓ - RW-L, Pc ↑	- RW-K, Pv ↑ - RW-BF ↓ - phosphorus ↓ - BUN ↑	- albumin ↓	- RW-G → - TP, cholesterol, triglycerides → - uric acid ↑ - LDH →	Smith <i>et al.</i> , 1992
AF-CIT <i>Chicken</i> (28 d)	0.5 – 150				- RW-K ↑	- TP ↓	- BWG, feed intake ↓ - RW-L ↑ - RBC ↓ - albumin, cholesterol ↓	- hemoglobin ↓ - uric acid ↓	Ahamad <i>et al.</i> , 2006
AF-MON <i>Chicken</i> (21 d)	3.5 – 100	- inorganic phosphorus ↓			- BWG ↓ - RW-H ↑ - RBC ↓	- albumin ↓ - creatinine ↑ - ALT ↑	- RW-G ↑	- RW-K → - RW-BF ↓ - TP ↓ - cholesterol, calcium → - ALP ↓	Kubena <i>et al.</i> , 1997c
AF-STER <i>Guinea pig</i> (14 d)	0.01 mg/d – 4.2 mg/d	- albumin ↑ - globulin ↓			- BWG ↓ - complement titer ↓				Richard <i>et al.</i> , 1978
AF-RUB <i>Rat</i>	4.0 – 5.0	- BW ↓ (P)							Hayes <i>et al.</i> , 1977

(30 d) ²									
AF-RUB <i>Guinea pig</i> (14 d) ³	0.02 bw – 8.0 bw	- complement titer, bacteriostatic activity √ (P) - AST ↗ (P)						- ALP √	Thurston <i>et al.</i> , 1989
AF-RUB <i>Chicken</i> (21 d)	2.5 – 500					- BW √ - TP √		- cholesterol √	Wyatt <i>et al.</i> , 1973

1) Morrissey *et al.* (1987) : intragastrically administration

2) Hayes *et al.* (1977) : initial exposure to RUB, followed by AF on day 15

3) Thurston *et al.* (1989) : 11 administrations

Like T-2 toxin, DAS has been described as radiomimetic with regard to lymphoid tissues and gastrointestinal epithelium, as a contact necrotizing agent for lingual and buccosal mucosa. However, oral lesions were only observed in chicken, the most susceptible species (Kubena *et al.* 1993).

The association of AF and DAS led to a less than additive or an antagonistic interaction on most biochemical parameters investigated, and also with either increased or decreased values for the same compounds (cholesterol, TP, enzyme concentrations) depending on species (**Table 3**).

In liver, lesions (vacuolar changes accompanied by early portal fibrosis) were reported similar in nature and severity between animals exposed to AF and AF-DAS treatments (Harvey *et al.*, 1995a; Harvey *et al.*, 1991).

3.3) Interaction between Aflatoxins (AF) and Deoxynivalenol (DON)

Two experiments were conducted in pigs and chickens to study the interaction between AF and DON (Harvey *et al.*, 1989b; Huff *et al.*, 1986). In both experiments, the body weight gain was decreased with an additive or less than additive interaction between the two toxins (**Table 3**). The main difference between the two experiments was the individual effect of DON. Harvey *et al.* (1989b) only observed minor effects of DON, and for most parameters he measured, the interaction with AF was less than additive or antagonistic. Conversely, Huff *et al.* (1986), who used high concentration of DON, reported an important effect of the toxin. He also observed less than additive or antagonistic interaction between DON and AF for the different parameters he measured (**Table 3**).

At the histological level, mono-contaminated feed with either DON or AF induced edema of the gastric mucosa. By contrast, animals fed the multi-contaminated diet did not show any gastric lesions (Harvey *et al.*, 1989b). In liver, mild hepatic interlobular fibrosis, bile duct hyperplasia, and diffuse hepatocellular lipidosis were only observed in the groups fed AF, either mono- or multi-contaminated groups, and suggested less than additive interaction (Harvey *et al.*, 1989b).

4) Interaction between Aflatoxins (AF) and other mycotoxins

4.1) Interaction between Aflatoxins (AF) and Cyclopiazonic Acid (CPA)

The interaction between these two toxins was investigated as strains of *Aspergillus flavus* produce the toxins simultaneously. The experiments were performed on laboratory animals (rats or guinea pigs) or on chickens. The body weight gain was always decreased in animals fed the co-contaminated diet (**Table 4**). When rats were exposed for a short period to the toxins (3 days), a less than additive interaction was observed (Morrissey *et al.*, 1987). But in longer period of exposure, the interaction

between AF and CPA on the body weight of animals was qualified as synergistic or additive (Pier *et al.*, 1989; Smith *et al.*, 1992).

Biochemical and hematological alterations were also measured, and less than additive to antagonistic interaction between AF and CPA were reported (Kumar and Balachandran, 2005; Morrissey *et al.*, 1987; Smith *et al.*, 1992) (**Table 4**). Similar types of interaction were noted on immune parameters, with especially an antagonism action of CPA on the depressant effect of AF on cell-mediated immunity (Pier *et al.*, 1989) (**Table 4**). However, the author suggested that the reduced numbers of animals in the combination group, and that survivors had apparently greater resistance to the toxins may have influenced the data obtained.

Among these studies, a particular attention has been paid on microscopic lesions. When rats were shortly exposed to the mycotoxins, less than additive or antagonistic interaction between the two toxins were described for the incidence in liver lesions (Morrissey *et al.*, 1987) (**Table 4**). Conversely, when animals were exposed for a longer period to these toxins, the interaction was suggested to be additive in liver lesions, characterized by a marked cytoplasmic vacuolation (Kumar and Balachandran, 2009; Pier *et al.*, 1989). Likewise, this greater effect was noticed in the severity and incidence of kidney lesions in broiler chickens (Kumar and Balachandran, 2009), and inconsistently in rats, depending on the ratio of mycotoxins used (Morrissey *et al.*, 1987) (**Table 4**). Of note, lesions recorded in gizzard, crop and proventriculus suggested adverse effects on the digestive tract; the interaction leading either to additive (Kumar and Balachandran, 2009) or less than additive effects (Smith *et al.*, 1992).

4.2) Interaction between Aflatoxins (AF) and - Moniliformin (MON), - Sterigmatocystin (STER), - Citrinin (CIT), - Rubratoxin (RUB)

In order to give a complete picture of the published literature, we should mention that one study has investigated the combined effect of AF and moniliformin on chicken (Kubena *et al.*, 1997c), another one the combined effect of AF and sterigmatocystin on guinea pig (Richard *et al.*, 1978), and a third one the combined effect of aflatoxin and citrinin on chicken (Ahamad *et al.*, 2006). The combined effect of AF and rubratoxin has also been investigated (Hayes *et al.*, 1977; Thurston *et al.*, 1989; Wyatt *et al.*, 1973). The parameters measured and the type of interaction observed between AF and MON, STER, CIT and RUB are summarized in **Table 4**.

Table 5 : Interaction between Fusariotoxins

Mycotoxins <i>Species</i> (<i>exposure</i>)	Doses	SYNERGISTIC INTERACTION			ADDITIVE INTERACTION	LESS THAN ADDITIVE INTERACTION	ANTAGONISTIC INTERACTION		REF
		Type 1	Type 2	Type 3			Type 1	Type 2	
FB-MON <i>Turkey</i> (21 d)	200 – 100	- feed intake ↓ (P)			- RW-L ↑ - AST ↑	- BWG ↓ - RW-H ↑ - RW-BF ↓	- LDH ↑		Bermudez <i>et al.</i> , 1997
FB-MON: <i>Pig</i> (28 d)	100 – 100	- feed intake ↓ - creatinine ↑ (P)			- RBC ↓	- BWG ↓ - mortality ↑ - glucose ↓	- inorganic phosphorus ↓	- AST, ALP, LDH, GGT ↑ - total iron →	Harvey <i>et al.</i> , 2002
FB-MON <i>Laying hen</i> (420 d)	100 – 50	- RW-H ↑ (P) - albumin ↓	- AST ↑	- mortality ↓		- CK ↓	- RW-L,K ↓	- uric acid → - egg production ↓ - egg weight →	Kubena <i>et al.</i> , 1999
FB-MON <i>Chicken</i> (21 d) ¹	100 – 100				- RW-H ↑		- feed intake ↓ - mortality ↑ - AST ↑	- BWG ↓	Ledoux <i>et al.</i> , 2003
	100 – 200					- RW-H,K,L ↑ - albumin, TP ↑	- mortality ↑ - AST ↑		
	200 – 100	- RW-L ↑ - albumin, TP ↑			- RW-K ↑	- RW-H ↑	- feed intake, BWG ↓ - mortality ↑ - AST ↑		
	200 – 200				- RW-K,L ↑	- RW-H ↑ - albumin ↑	- mortality ↑ - AST ↑	- TP ↑	
FB-MON <i>Turkey</i> (21-28 d)	200 – 100				- Ab ND ↓	- feed intake, BWG ↓ - RW-T,BF,S ↓ - lymphocytes stimulation ↓ - bacteria in tissue & blood, mortality p.i ↑			Li <i>et al.</i> , 2000
FB-MON <i>Quail</i> (35 d)	200 – 100				- BW ↓ - mortality ↑	- TP, cholesterol ↑ - ALT, AST ↑	- albumin ↑ - LDH, CK ↑	- creatinine → - DTH reaction →	Sharma <i>et al.</i> , 2008
FB-MON <i>Fish</i> (70 d)	20 – 40				- size of hepatocyte nuclei ↓	- BWG, feed intake ↓ - Sa/So liver ↑			Yildirim <i>et al.</i> , 2000
	40 – 40	- BWG ↓ - serum pyruvate ↑ - size of hepatocyte nuclei ↓			- feed intake ↓	- Sa/So liver ↑			
FB-T2 <i>Turkey</i>	300 – 5.0	- RW-L ↑ (P) - RW-G ↑			- BWG ↓ - inorganic phosphorus	- oral lesions ↑ - RW-Pc ↑	- uric acid ↑		Kubena <i>et al.</i> , 1995a

(21 day)		- RBC, hemoglobin ↗ - AST ↗ - LDH ↗ (P)			↘	- cholesterol ↘			
FB-T2 <i>Chicken</i> (19 d)	300 – 5.0	- RW-Pc ↗ (P)			- BWG, feed intake ↘ - mortality ↗ - RW-G ↗	- RW-L,K ↗ - cholesterol ↗ - calcium ↗		- oral lesions ↗ - RW-S → - TP, albumin ↗ - AST, LDH, GGT ↗	Kubena <i>et al.</i> , 1997a
FB-DAS <i>Turkey</i> (21 d)	300 – 4.0	- inorganic phosphorus ↘ - RBC ↗ (P)			- BWG, feed intake ↘	- RW-L,G ↗ - RW-S,H ↘ - AST, LDH ↗	- triglycerides ↘ - uric acid ↗	- oral lesions ↗ - RW-Pc → - cholesterol ↘	Kubena <i>et al.</i> , 1997b
FB-DON <i>Pig</i> (28 d)	50 – 4.0	- BWG ↘ - AST, CHL, ALP ↗ (P) - lymphocytes stimulation ↘ (P)		- RW-L ↘	- feed intake ↘ - RW-Lg ↗	- creatinine ↗	- albumin ↘	- cholesterol ↗ - GGT ↗	Harvey <i>et al.</i> , 1996
FB-DON <i>Chicken</i> (21 d)	300 – 15	- mortality ↗ - RW-Pv ↗ (P) - cholesterol ↗ (P) - AST ↗ (P) - LDH ↗	- feed intake ↗		- RW-G ↗ - BUN ↗ - GGT ↗	- BWG ↘ - RW-L,K ↗ - TP ↗	- RW-H →	- RW-BF →	Kubena <i>et al.</i> , 1997a
FB-DON <i>Pig</i> (35 d)	6.0 – 3.0				- severity, extent of liver & lung lesions ↗ - specific Ab IgG ↘ - cytokines expression ↘	- neutrophils ↘ - severity, extent of kidney lesions ↗ - specific lymphocytes stimulation ↘		- creatinine, albumin → - specific Ab IgA →	Grenier <i>et al.</i> (2011)
FB-FA <i>Chicken egg</i> (21 d)	1.0 – 1.0 (µg/egg)	- embryos mortality ↗							Bacon <i>et al.</i> , 1995
	5.0 – 5.0 (µg/egg)	- embryos mortality ↗							
	25 – 25 (µg/egg)	- embryos mortality ↗							
	50 – 50 (µg/egg)	- embryos mortality ↗							
T2-DAS <i>Laying hen</i> (24 d)	2.0 – 2.0	- egg production (at end of the trial) ↘			- oral lesions ↗ - feed intake ↗	- LDH ↗	- egg production (at intermediate time) ↘ - GDH ↘		Diaz <i>et al.</i> , 1994
DON-T2 <i>Pig</i> (35 d)	2.5 – 0.4					- BWG, feed intake ↘			Friend <i>et al.</i> , 1992
	2.5 – 0.8					- BWG, feed intake ↘			
	2.5 – 1.6					- BWG, feed intake ↘			
	2.5 – 3.2	- BWG, feed intake ↘							
DON-T2 <i>Chicken</i> (21 d)	16 – 4.0				- BW ↘ - cholesterol ↘	- RW-G,BF ↗ - TP, albumin ↘ - LDH ↘ - oral lesions ↗			Kubena <i>et al.</i> , 1989b
DON-NIV	0.071 bw – 0.071 bw	- uric acid ↗			- TP ↗ - PROD ↗	- IgA ↗ - CDNB ↗	- total CO2 ↘ - EROD ↗		Gouze <i>et al.</i> ,

<i>Mouse</i> (28 d) ²	0.071 bw – 0.355 bw	- uric acid ↗ (P)			- TP ↗ - IgA ↗ - DCNB ↗	- total CO2 ↘ - PROD ↗ - DCNB ↗	- EROD ↗	- feed intake ↘	2005
	0.355 bw – 0.071 bw	- phosphorus ↗ - uric acid ↗			- total CO2 ↘ - TP ↗	- IgA ↗ - DCNB ↗	- EROD, PROD ↗		
	0.355 bw – 0.355 bw	- IgA ↗ - DCNB ↗			- TP ↗ - phosphorus ↗	- total CO2 ↘ - uric acid ↗ - DCNB ↗	- EROD, PROD ↗	- feed intake ↘	
DON-ZEA <i>Mouse</i> (56 d)	5.0 – 10			- RBC ↘			- BW ↘ - RW-L → - IgA ↗		Forsell <i>et al.</i> , 1986
DON-ZEA <i>Mouse</i> (14-21 d)	25 – 10				- bacteria in spleen ↗			- DTH reaction ↘	Pestka <i>et al.</i> , 1987
DON-MON <i>Turkey</i> (21 d)	20 – 100		- feed intake ↘ - globulin ↘		- RW-K ↗	- BWG ↘		- RW-H ↗ - calcium →	Morris <i>et al.</i> , 1999

1) Ledoux *et al.* (2003) : for the highest dose of MON in the combination, feed intake and BWG not took into account due to high mortality

2) Gouze *et al.* (2005) : administration thrice a week for 4 weeks

II. INTERACTIONS BETWEEN FUSARIOTOXINS

1) Interaction between Fumonisin (FB) and other Fusariotoxins

1.1) Interaction between FB and MON

Several studies reported the effects of interaction between FB and MON, and mainly with highly contaminated feeds and in poultry (**Table 5**).

A less than additive and an antagonistic interactions in turkeys (Bermudez *et al.*, 1997; Li *et al.*, 2000) and in broiler chicks (Ledoux *et al.*, 2003), respectively, were observed on the growth animals depression. In pigs, quails and catfish, the effect of the multi-contaminated diet on BWG ranged from less than additive to additive and to synergistic (Harvey *et al.*, 2002; Sharma *et al.*, 2008; Yildirim *et al.*, 2000) (**Table 5**). However, the interaction on feed consumption was not consistently associated with the interaction on BWG.

Some authors reported animals mortality, especially due to MON presence in chickens (Ledoux *et al.*, 2003). Surprisingly, no mortality in laying hens fed combination treatment has been observed compared to individual treatments (Kubena *et al.*, 1999). But as suggested by the authors, the higher mortality in FB group alone may be attributed to the increased egg weights because 4 of the deaths were attributed to uterine prolapses in the later stages of production. In the same study, antagonistic interaction between MON and FB was reported, with a sparing action of MON on the FB effects on eggs performance (**Table 5**).

Interestingly, in comparison to other mycotoxin interactions both FB and MON induced an increase of serum TP and albumin concentrations. Other mycotoxins mainly reported a decrease of these biochemical compounds, likely due to their property to inhibit the synthesis of proteins.

Serum AST concentrations increased following exposure to the combined treatment, but different types of interaction, from synergism type 2 to antagonism were recorded (Bermudez *et al.*, 1997; Harvey *et al.*, 2002; Kubena *et al.*, 1999; Ledoux *et al.*, 2003; Sharma *et al.*, 2008) (**Table 5**). Of note that Yildirim *et al.* (2000) analyzed the serum pyruvate concentrations in animals exposed to contaminated diets, as MON is known to inhibit the pyruvate dehydrogenase. At the higher inclusion of FB in the co-contaminated diet, he reported a synergistic interaction on the level of serum pyruvate (**Table 5**), suggesting a stronger inhibition of this enzyme.

This enzyme inhibition could be related in the increase of relative weight of hearts in animals exposed to MON, as this enzyme is involved in the production of ATP and that the heart may have to increase the blood flow to supply more oxygen to the body in order to increase ATP production (Ledoux *et al.*, 2003). Cardiomegaly was thus observed, and MON combined with FB led either to

synergistic and additive effects (Kubena *et al.*, 1999; Ledoux *et al.*, 2003) or less than additive effects (Bermudez *et al.*, 1997; Ledoux *et al.*, 2003) (**Table 5**). A lack of cardiomegaly was indicated by Harvey *et al.* (2002), showing that swine is less sensitive to MON than poultry.

Cardiac lesions, mainly characterized by a diffuse loss of cardiomyocyte cross striations were recorded in the MON and MON+FB groups, with the same degree of severity between these groups (Bermudez *et al.*, 1997; Harvey *et al.*, 2002; Ledoux *et al.*, 2003). Similarly, liver lesions described by these authors were attributed to FB and were not more severe in the FB+MON diet. In other hand, combined mycotoxins revealed smaller hepatocellular nuclei in fish, indicating an additive or synergistic interaction according to the concentration of FB in the combination (Yildirim *et al.*, 2000).

Two studies focused on the interaction effect on the immune system (Li *et al.*, 2000; Sharma *et al.*, 2008) (**Table 5**). An additive effect was observed in the reduced antibodies against Newcastle Disease. Li *et al.* (2000) indicated that after an *E. coli* challenge, the ability of animals to eliminate bacteria from the blood system was diminished in animals exposed to mycotoxins. However, a less than additive interaction was reported in the multi-contaminated diet, as shown by the increased numbers of bacterial colonies in blood and tissues. Likewise in this study, a less than additive effect was reported in the reduced proliferation of lymphocytes upon mitogenic activation. By contrast, Sharma *et al.* (2008) observed that MON reversed the immunosuppressive effects of FB, in quails undergoing skin hypersensitivity test, suggesting an antagonistic interaction on the cellular immune response.

1.2) Interaction between FB and TCT

1.2.1) with TCT type A

Interaction between FB and T-2 toxin was reported in turkeys and in chickens (Kubena *et al.*, 1997a; Kubena *et al.*, 1995a) (**Table 5**).

Both experiments reported an additive effect on the BWG depression. Oral lesions, caused by T-2 toxin, were also recorded, but effect on scores differs between studies. The antagonist effect was likely due to the greater reduction in feed consumption, thereby diminishing the T-2 toxin ingestion in the combination group (Kubena *et al.*, 1997a). In both studies, the increased RW-G was related to the irritant property of T-2 toxin, typically described as focally reddened mucosa (Kubena *et al.*, 1995a).

The decreased or increased effect on cholesterol concentration reflects the individual effect of FB in each study. An important difference between these two studies is the activity of hepatic enzyme. In one hand, T-2 toxin seems to potentiate the FB effect (Kubena *et al.*, 1995a) on AST and LDH,

whereas in the experiment of Kubena *et al.* (1997a), the effect on these enzymes was lesser than FB alone. No histological analysis on liver was done to find out a possible link, but a partial explanation could also be due to the greater reduction in feed intake in the combination group (Kubena *et al.*, 1997a). Another reason may be the type of birds used and the sex, sensitivity being different between male and female.

However, the likelihood of encountering concentrations of 300 mg FB/kg in finished feed is very small.

One study investigated the interaction of DAS with a very high content of FB in diets (Kubena *et al.*, 1997b), and reported additive effect on performance and antagonistic effect for oral lesions (**Table 5**). Hematological and biochemical values showed different types of interaction, from synergism to antagonism.

1.2.2) with TCT type B

Three experiments, performed in pigs and in chickens, investigated the combined effect of DON and FB, the most frequently detected fusariotoxins (Grenier *et al.*, 2011; Harvey *et al.*, 1996; Kubena *et al.*, 1997a) (**Table 5**).

The type of interaction on body weight gain differed depending on the experiment. A synergistic interaction was reported by Harvey, no change in this parameter whatever the toxins included in the diet was observed by Grenier. In chicken, Kubena *et al.* (1997a) observed a less than additive effect, mainly due to FB in the combination group. Interestingly, in this latter study, the feed intake was slightly increased in comparison to the other treatments (**Table 5**).

An antagonistic interaction has been observed for albumin. DON in the multi-contaminated diet seems to potentiate the FB effects, such as reflected by the hepatic enzymes concentrations (Harvey *et al.*, 1996; Kubena *et al.*, 1997a). It could be linked to the unexpected effect on the RW-L in the experiment conducted by Harvey *et al.* (1996), where the combination led to an opposite effect compared to individual effects (**Table 5**). The large and synergistic decrease in the RW-L may be due to an important hepatic damage, as already mentioned in this review for AF-FB interaction. However, histologically in this experiment, hepatic lesions observed (necrosis) in animals fed the combined mycotoxins, were not more severe than those observed in animals fed the FB diet. By contrast, Grenier *et al.* (2011) showed a greater effect in the severity and extent of lesions (mainly hepatocytes vacuolization and megalocytosis) (**Table 5**), suggesting that DON, known to alter the intestinal permeability, had enhanced the intestinal absorption of FB, well known to be poorly absorbed. The differences in these two studies are most likely due to the different concentrations of

FB used. These two studies are in agreement regarding the kidney lesions, where pigs exposed to the DON+FB diet did not have an increased frequency of lesions compared to individual diets (**Table 5**).

Immune system was also evaluated following the exposure to both DON and FB (**Table 5**). Harvey *et al.* (1996) recorded a potentialization of the effect of FB by DON in the reduced lymphocytes proliferation upon mitogenic stimulation. Grenier *et al.* (2011) reported a less than additive effect on the decreased index when lymphocytes were stimulated by ovalbumin. In their experiment, pigs were immunized with ovalbumin and the specific immune response was thus impaired, as also displayed by the additive effect on the content of specific IgG. On contrary, antagonistic interaction was recorded in the levels of specific IgA, showing a sparing effect of FB on the DON-induced IgA elevation. Also in this study, an additive effect on the decreased expression of cytokines in spleen was noted.

1.3) Interaction between FB and Fusaric Acid (FA)

A synergistic interaction was reported when FB and Fusaric Acid (FA) were combined in equal concentrations and injected into chicken eggs (**Table 5**), resulting in increased toxicity, as shown by the percentage of dead embryos (Bacon *et al.*, 1995). In the same study, when a relatively non-toxic concentration of FA was combined with graded doses of FB, a synergistic response was also obtained.

2) Interaction between Trichothecenes (TCT)

2.1) TCT type A

One study investigated the interaction between the two type A trichothecenes, T-2 toxin and DAS. Both toxins inhibit protein synthesis in eukaryotic cells and are highly toxic to poultry (Diaz *et al.*, 1994). In laying hens, Diaz *et al.* (1994) observed an additive effect of T-2 toxin and DAS on feed intake and oral lesions (**Table 5**). In the experiment, the authors recorded egg production throughout the trial. The lower egg production observed in hens receiving either T-2 toxin or DAS recovered gradually during the experiment. Interestingly, the decrease in egg production of hens fed the co-contaminated diet became progressively worse (**Table 5**). The authors suggested that whereas hens exposed to a single mycotoxin at low concentrations may be able to sustain a satisfactory rate of egg production, they may not be able to tolerate a combination of mycotoxins at similarly low concentrations.

2.2) TCT type A and B

Two reports focused on the interaction between type B and type A trichothecenes, DON and T-2 toxin (Friend *et al.*, 1992; Kubena *et al.*, 1989b). In swine, except for the highest dose of T-2 toxin that interact synergistically with DON in the decreased BWG and feed intake, a less than additive effect was observed on these zootechnic parameters following the combination exposure, reflecting predominantly the DON effect (Friend *et al.*, 1992) (**Table 5**). By contrast, in broiler chicks, most of the observed effects (oral lesions, total protein, albumin and LDH) were due to T-2 toxin in the combined treatment (Kubena *et al.*, 1989b). However, an additive effect was obtained on the body weight gain and on the serum cholesterol concentration (**Table 5**). Decreased cholesterol levels could suggest inhibition of biosynthesis, with liver involvement and perhaps a shift of concentration from blood to the liver.

2.3) TCT type B

One study investigated the interaction between the two type B trichothecenes, deoxynivalenol and nivalenol (NIV), using two different low doses of both toxins (Gouze *et al.*, 2005) (**Table 5**). Although the lowest dose of NIV and the two doses of DON used did not affect the plasmatic levels of uric acid, when used in combination the toxins act in a synergistic manner. Whatever the dose of NIV and DON used, when the toxins were used in combination an additive effect was observed for the total protein level.

Ingestion of DON or NIV induces IgA nephropathy in mice. Depending on the doses of two toxins, when used in combination the interaction range from synergistic to additive or less than additive on IgA synthesis. The hepatic drug metabolism activity was also assessed. When mice were exposed to DON and NIV, the toxins showed an antagonistic interaction on the activities of two monooxygenase (EROD and PROD). By contrast, for the highest doses of DON and NIV, a synergistic effect was observed on the elevated activity of glutathione S-transferase (DCNB as substrates).

3) Interaction between Deoxynivalenol (DON) and other Fusariotoxins**3.1) Interaction between DON and Zearalenone (ZEA)**

Three publications investigated the interaction between DON and zearalenone (ZEA) (Boeira *et al.*, 2000; Forsell *et al.*, 1986; Pestka *et al.*, 1987). Forsell *et al.* (1986) showed in mice that exposure to the combined treatment resulted mainly in antagonistic interaction (**Table 5**). The author observed an antagonistic effect of ZEA on the DON-induced elevation of serum IgA. Similarly,

antagonism was noted in the ability of DON to inhibit the delayed hypersensitivity response in presence of ZEA (Pestka *et al.*, 1987). However, the resistance to *Listeria monocytogenes* was reduced in an additive manner by co-administration of DON and ZEA (Pestka *et al.*, 1987) (Table 5).

Interestingly, DON+ZEA combination was assessed on growth of brewing yeast, considering that grains contaminated with mycotoxins are used for beer production, and may be introduced at different steps of the brewing process (Boeira *et al.*, 2000). Despite the effect caused by combination of DON and ZEA at high concentrations, shown to pass from antagonism to synergism depending on the ratio of toxins in the mixture, when low concentrations were used, inhibition of yeast growth was not observed.

3.2) Interaction between DON and MON

Due to the relatively tolerance of DON in poultry, its interaction with MON reflected mostly the MON effects (Harvey *et al.*, 1997; Morris *et al.*, 1999). Poult fed diets containing MON alone and the DON-MON combination exhibited an increased incidence of variable sized cardiomyocyte nuclei, with numerous large, giant nuclei and a generalized loss of cardiomyocyte cross striations (Morris *et al.*, 1999). Isolated renal tubules in sections of kidney were noted to have diffuse mineralization in animals fed MON and DON-MON (Harvey *et al.*, 1997; Morris *et al.*, 1999), and also extensive tubular epithelial degeneration (Harvey *et al.*, 1997). But Harvey *et al.* (1997) indicated a moderation of the severity of lesions in the tissues of chicks fed DON-MON, suggesting an antagonistic effect.

Table 6 : Interaction between Ochratoxin A (OTA) and other mycotoxins; interaction between T-2 toxin (T2) and cyclopiazonic acid (CPA) is also included

Mycotoxins <i>Species</i> (<i>exposure</i>)	Doses	SYNERGISTIC INTERACTION			ADDITIVE INTERACTION	LESS THAN ADDITIVE INTERACTION	ANTAGONISTIC INTERACTION		REF
		Type 1	Type 2	Type 3			Type 1	Type 2	
OTA-CIT <i>Rabbit</i> (60 d)	0.75 – 15					- lymphocytes stimulation ↘	- Ab SRBC ↘		Kumar <i>et al.</i> , 2008
OTA-CIT <i>Chicken</i> (21 d)	3.0 – 300				- RW-L ↗ - potassium ↘	- RW-K ↗ - glucose ↗ - calcium ↘ - lesion score in kidney ↗	- feed intake, BW ↘ - water consumption ↗ - triglycerides ↗ - phosphorus ↘	- TP, albumin, globulin ↘ - cholesterol → - uric acid ↗	Manning <i>et al.</i> , 1985
OTA-CIT <i>Rat</i> (20 d) ¹	1.0 – 30	- maternal mortality ↗ - resorptions ↗ - live fetuses ↘ - % malformed ↗							Mayura <i>et al.</i> , 1984
OTA-CIT <i>Rat</i> (21 d)	0.026 – 0.1	- DNA adducts in kidney ↗ (P)					- BWG →		Pfohl-Leszkowicz <i>et al.</i> , 2008
OTA-CIT <i>Rat</i> (1 d) ²	1.0 – 25	- renal Na ⁺ -K ⁺ -ATPase activity ↘	- NADPH dehydrogenase, NADPH cytochrome c reductase ↘	- cytochrome P-450 content ↘		- renal Mg ²⁺ -ATPase activity ↘	- aniline oxidation ↘		Siraj <i>et al.</i> , 1981
OTA-T2 <i>Chicken</i> (21 d)	0.567 – 0.927	- % repairing tissue in liver ↗			- BWG, feed intake ↘	- TP, albumin, globulin ↘ - % tumid, necrotic cells in liver ↗	- RW-L ↗	- uric acid → - RW-K ↗ - % necrotic tubular cells in kidney ↗	Garcia <i>et al.</i> , 2003
OTA-T2 <i>Pig</i> (30 d)	2.5 – 8.0	- GGT ↘			- BWG, feed intake ↘ - hemoglobin ↘ - lymphocytes stimulation ↘	- cholesterol ↘ - creatinine ↗ - ALP ↘ - phagocytosis activity ↘	- inorganic phosphorus ↘	- TP →	Harvey <i>et al.</i> , 1994
OTA-T2 <i>Chicken</i> (21 d)	2.0 – 4.0	- RW-G ↗ - BUN ↘ - triglycerides ↗ - AST ↘ (P)		- calcium →	- BWG ↘ - hemoglobin ↘ - TP ↘ - CK ↘ - LDH ↘	- oral lesions ↗ - RW-L,K,Pv ↗ - creatinine ↗ - cholesterol, albumin ↘	- phosphorus ↘	- RW-Pc ↗ - magnesium ↘ - uric acid ↗ - GGT → - ALP ↘	Kubena <i>et al.</i> , 1989a
OTA-T2 <i>Chicken</i>	2.0 – 3.0				- RW-G ↗ - hemoglobin ↘	- BW, feed intake ↘ - mortality ↗		- RW-L ↗ - TP, BUN →	Raju and Devegowda,

(35 d)						- RW-K,A ↗ - GGT ↗ ALT ↘		- cholesterol ↘ - coagulation time ↗	2000
OTA-T2 <i>Chicken</i> (35 d)	2.0 – 3.0				- RW-T ↘		- RW-BF → - Ab ND,IBD ↘		Raju and Devegowda, 2002
OTA-DAS <i>Chicken</i> (19 d)	2.0 – 6.0		- mortality ↘	- RW-G ↗	- BW ↘ - oral lesions ↗ - RW-K ↗ - TP, albumin ↘ - CK ↘	- RW-Pv ↗ - uric acid → - cholesterol ↘	- RW-L ↗ - triglycerides ↗		Kubena <i>et al.</i> , 1994a
OTA-DON <i>Chicken</i> (21 d)	2.0 – 16				- RW-G ↗ - RBC ↗	- BWG ↘ - RW-L,K,Pv ↗ - glucose, TP, albumin ↘	- BUN →	- uric acid ↗ - creatinine → - triglycerides ↗ - cholesterol ↘ - inorganic phosphorus →	Kubena <i>et al.</i> , 1988
OTA-FB <i>Rat</i> (15 d) ³	5.10 ⁻⁶ bw/d – 2.10 ⁻⁴ bw/d	- MDA, PC in liver ↗ - CAT in kidney ↘			- PC in kidney ↗		- MDA in kidney ↗		Domijan <i>et al.</i> , 2007
	5.10 ⁻⁶ bw/d – 0.05 bw/d	- MDA, PC in liver ↗ - MDA in kidney ↗ - CAT in kidney ↘				- PC in kidney ↗			
	0.05 bw/d – 0.05 bw/d	- PC in liver ↗ - PC in kidney ↗ - CAT in kidney ↘				- MDA in liver ↗ - MDA in kidney ↗			
OTA-FB <i>Turkey</i> (21 d)	3.0 – 300	- BWG, feed intake ↘ - RW-H ↘ - cholesterol ↘ (P) - creatinine ↗ - AST ↗ (P) - ALT ↗	- uric acid ↗	- BUN ↗	- RW-K ↗	- RW-L,Pc,G ↗ - RW-S ↘ - RBC ↗	- hemoglobin ↗	- triglycerides → - LDH ↗	Kubena <i>et al.</i> , 1997b
OTA-FB <i>Rabbit</i> (45 d)	2.0 – 10	- TP ↘			- albumin, globulin ↘ - ALP ↗	- creatinine ↗ - ALT, AST, LDH ↗	- glucose ↘		Sivakumar <i>et al.</i> , 2009
OTA-PA <i>Chicken</i> (28d) ⁴	1.0 bw – 60 bw	- mortality ↗				- BW ↘			Kubena <i>et al.</i> , 1984
OTA-PA <i>Mouse</i> (21 d)	10 – 40	- RW-S ↘ (P) - mortality ↗				- RW-K ↘ - BW ↘			Shepherd <i>et al.</i> , 1981
OTA-CPA <i>Chicken</i> (21 d)	2.5 – 34	- RW-Pc ↗ - RW-L,K ↗ (P) - TP, cholesterol ↘ (P) - albumin ↘			- BW ↘	- uric acid, triglycerides ↗	- RW-Pv ↗		Gentles <i>et al.</i> , 1999

		- CK ↗ (P)							
T2-CPA <i>Chicken</i> (28 d)	1.0 – 10				- % CD ₄ ⁺ & CD ₈ ⁺ in thymus ↓ - % CD ₈ ⁺ in spleen ↓ - lymphocytes stimulation ↓		- % CD ₄ ⁺ in spleen ↓ - Ab ND ↓		Kamalavenk atesh <i>et al.</i> , 2005
T2-CPA <i>Chicken</i> (21 d)	6.0 – 34	- RW-K ↗ - triglycerides ↗	- RW-L ↗ - cholesterol ↗		- RW-Pc ↗ - albumin ↓	- BW ↓ - mortality, oral lesions ↗ - RW-Pv,G ↗ - RW-BF ↓ - TP ↓ - GGT ↓	- glucose ↓		Kubena <i>et al.</i> , 1994b

1) Mayura *et al.* (1984) : subcutaneously administration during the 20 d-period of pregnancy

2) Siraj *et al.* (1981) : one administration intragastrically and measurements 12 d post administration

3) Domijan *et al.* (2007) : 15 d-treatment with OTA, and treatment with FB for the last 5 days of OTA treatment

4) Kubena *et al.* (1984) : 14 administrations

III. INTERACTIONS BETWEEN OCHRATOXIN A AND OTHER MYCOTOXINS

1) Interaction between OTA and Citrinin (CIT)

Regarding zootechnical parameters, Manning *et al.* (1985) observed an antagonistic interaction for body weight gain when chickens were fed with OTA and CIT (**Table 6**). This response was most likely the result of the improved feed intake in the OTA+CIT group. Birds receiving the co-contaminated feed also showed an antagonistic effect in the increase of water consumption that is normally associated with CIT toxicosis.

Changes in serum total protein, cholesterol, triglycerides, and uric acid concentration also demonstrated an antagonistic effect between the two toxins (**Table 6**), and suggested that the combination of mycotoxins gave some advantage to the birds and allowed them to maintain biochemical parameters close to the to normal ranges. A proposed mechanism would be that the cytoplasmic accumulation of smooth endoplasmic reticulum may be indicative of enzyme induced by the exposure to CIT and would protect against the toxicity of OTA (Manning *et al.* 1985; Brown *et al.* 1986).

Less than additive and antagonistic interactions between the two toxins were also observed in rabbits for lymphocytes proliferation and specific antibody response (Kumar *et al.*, 2008) (**Table 6**).

Because both OTA and CIT target the kidney and have been implicated as causal agents for the Balkan endemic nephropathy, most of the experiments conducted with both OTA and CIT investigated their effects on renal function and structure (Brown *et al.*, 1986; Glahn *et al.*, 1988; Kitchen *et al.*, 1977a; b; Kumar *et al.*, 2007; Manning *et al.*, 1985; Siraj *et al.*, 1981). A synergistic interaction between the two toxins was observed in dog when looking at the severity of clinical signs and mortality (Kitchen *et al.*, 1977a). Concentrations of urinary GOT and LDH were significantly increased in animals exposed to both toxins, signing renal damages. A synergistic interaction was also observed for the inhibition of the renal Na⁺-K⁺-ATPase activity in neonatal rats (Siraj *et al.*, 1981) (**Table 6**). An experiment also reported that the OTA pretreatment blunted the diuretic effect of CIT on kidney of pullets (Glahn *et al.*, 1988). But as indicated by the author, the sequential use of toxins and mode of administration may create conditions that differ significantly from situations in which OTA and CIT are present simultaneously.

Ultrastructural assessment of kidney showed mainly degenerative and necrotic changes in the proximal and distal tubules. The nephrotoxicity was related with mitochondria damage (Kumar *et al.* 2007; Brown *et al.* 1986). In the animal group receiving both toxins, these lesions were more severe and intense (Kitchen *et al.*, 1977b; Kumar *et al.*, 2007), similar to the ones observed in the OTA-

exposed group (Brown *et al.*, 1986; Manning *et al.*, 1985) or less severe than the ones observed in the animal group receiving the highest dose of CIT alone (Kitchen *et al.*, 1977b).

An increased genotoxicity of OTA was also observed in the presence of CIT, with increased DNA-adducts in kidney, suggesting synergism (Pfohl-Leszkowicz *et al.*, 2008) (**Table 6**).

Two studies investigated the interaction between OTA and CIT on the teratogenesis and led to different results. Mayura *et al.* (1984) observed synergism between the toxins when looking at fetal malformations in rats (**Table 6**). By contrast, Vesela *et al.* (1983) observed the same teratogenic effects in chicken embryos exposed to either OTA alone or OTA+CIT.

2) Interaction between OTA and Fusariotoxins

2.1) Interaction between OTA and TCT

2.1.1) with TCT type A

Five studies focused on the interaction between OTA and T-2 toxin (Garcia *et al.*, 2003; Harvey *et al.*, 1994; Kubena *et al.*, 1989a; Raju and Devegowda, 2000; 2002) (**Table 6**).

Except the study of Raju and Devegowda (2000), the experiments showed that the association of OTA and T-2 toxin act in an additive manner to depress the body weight gain (**Table 6**). The serum concentrations of albumin, creatinine, cholesterol, uric acid and phosphorus were affected in these studies, representing mostly the effect of OTA alone, but less than additive or antagonistic interactions were reported. These studies noted an additive interaction on the decreased hemoglobin concentration. Effect of both OTA and T-2 toxin ingestion on enzyme concentrations induced different type of interactions between these studies, but in most of the case a significant decrease was reported (**Table 6**). It was suggested that the property to inhibit the protein synthesis by these mycotoxins was involved.

It seems from these studies that T-2 toxin may interfere in the OTA-induced renal impairment, as shown by the antagonistic interaction on the RW-K, uric acid, phosphorus or also the percentage of necrotic tubular cells (**Table 6**). Nonetheless, microscopic lesions showing degenerative changes in renal tubular epithelium and proximal convoluted tubules were similarly reported for OTA and OTA+T2 groups (Harvey *et al.*, 1994; Kubena *et al.*, 1989a). In liver, T-2 toxin appeared to slightly potentiate the hepatotoxicity of OTA, visible on the percentage of repairing tissue (Garcia *et al.*, 2003) (**Table 6**).

Finally, their interaction on immune system was reported by Harvey *et al.* (1994) and Raju and Devegowda (2002). Although an additive effect was observed on the lymphocytes proliferation upon

mitogenic stimulation, the reduced activity of phagocytosis in the combined treatment was similar to the individual OTA treatment (Harvey *et al.*, 1994), and an antagonistic effect was reported for the level of specific antibodies (Raju and Devegowda, 2002) (**Table 6**).

One experiment investigated the interaction between OTA and DAS in chicken (**Table 6**), and observed in general less than additive or antagonistic effect (Kubena *et al.*, 1994a). Surprisingly, no mortality was recorded in comparison to the individual treatments. A less than additive effect was observed for the oral lesions, induced by DAS. Like T-2 toxin, in association with OTA, DAS seems to spare the OTA-induced renal dysfunction, as shown with the antagonistic effect on uric acid concentration.

2.1.2) with TCT type B

One study focused on the interaction between OTA and DON in chicken (Kubena *et al.*, 1988). For many parameters, such as BWG, relative weight of organs and biochemistry, the interaction was less than additive or antagonistic in nature (**Table 6**). Similarly, lesions in liver and kidney were only due to the OTA presence in the contaminated diet.

2.2) Interaction between OTA and FB

Three experiments were conducted in rats, turkey poults and rabbits to analyze the interaction between these two toxins (**Table 6**); however the authors used very different doses of mycotoxins (Domijan *et al.*, 2007; Kubena *et al.*, 1997b; Sivakumar *et al.*, 2009). Using very high concentrations of FB, Kubena *et al.* (1997b) showed synergistic effects in association with OTA compared to mycotoxins alone (performance, some biochemical parameters and enzyme levels). Using lower doses of toxins, Sivakumar *et al.* (2009) observed less than additive or additive effects on biochemistry and enzyme levels. Interestingly, in both studies, the concentrations of serum enzymes were increased compared to the interaction between OTA and T-2 toxin (**Table 6**).

Using doses of toxins reflecting the European-type diet, Domijan *et al.* (2007) analyzed the interaction between these two toxins on the oxidative stress, in liver and kidneys of rats (**Table 6**). They observed that although the lowest doses of OTA and FB given separately did not increase the concentration of malondialdehyde (MDA) and protein carbonyls (PCs) in the liver, their combination produced a synergistic effect. Similarly, in the kidney, the combination further increased the PCs concentration, ranging from synergistic to additive or to less than additive effect, depending on the doses used. The catalase activity in rat kidney decreased in a synergistic manner after exposure to both OTA and FB, while this parameter was not affected by separate OTA or FB treatments.

2.3) Interaction between OTA and ZEA

Halabi *et al.* (1998) studied the histopathological effects of OTA and ZEA, in liver and kidneys of rats. Small amounts of mycotoxins, over a long period were given intraperitoneally to rats. The authors indicated that ZEA antagonizes the toxic effects of OTA for body weight gain and relative weight of kidney, leading to no changes in the combination group. Likewise, less severe lesions in kidneys were observed for ZEA and ZEA+OTA in comparison to the severity induced by OTA.

3) Interaction between OTA and *Aspergillus/Penicillium* toxins

3.1) Interaction between OTA and Penicillic Acid (PA)

Combination of OTA and PA showed less than additive effects on body weight in chickens (Kubena *et al.*, 1984) and in mice (Shepherd *et al.*, 1981), but act in a synergistic manner in the increase of the mortality in both species (**Table 6**). Shepherd *et al.* (1981) also indicated that the combination produced more extensive lesions in kidney within the proximal convoluted tubules, but with less renal damages at day 21 than at day 10, showing a recovery from the initial shock.

3.2) Interaction between OTA and CPA

In chicken, synergistic interactions were often recorded after combination of both mycotoxins (**Table 6**), and some of the deleterious effects induced by OTA were exacerbated by the presence of CPA (Gentles *et al.*, 1999).

Another interaction, that cannot be classified in these different sections, concern the association between T-2 toxin and CPA. This interaction was investigated in chicken either on general parameters (Kubena *et al.*, 1994b) or on immune parameters (Kamalavenkatesh *et al.*, 2005) (**Table 6**). Kubena *et al.* (1994b) showed less than additive effects on animal performances and synergistic effects on relative weight of liver and kidney, as well as lipid compounds. Kamalavenkatesh *et al.* (2005) reported some additive interactions in the reduced subpopulations of lymphocytes in lymphoid organs, and in the lymphocytes stimulation. By contrast, he observed an antagonistic interaction in the specific antibodies content.

CONCLUSION

In the present review, we have analyzed the data published in more than 100 papers, to characterize for different parameters the interaction between mycotoxins. More than half of the published papers investigated the interactions between aflatoxins with other mycotoxins. Although these experiments are relevant in terms of toxicity and natural co-occurrence, we were surprised that only few published papers investigated the interactions between other mycotoxins, especially interaction between toxins from *Fusarium spp.* which are of major concern worldwide.

Overall, the review highlights the complexity of the interaction between mycotoxins. Indeed, even within the same experiment the type of interaction varies according to the parameter measured. Although, most of the studies have shown a synergistic or additive interaction on the adverse effects of the animals performance, the results on other parameters, especially on biochemical compounds, led to different type of interaction, going from synergistic to antagonistic for a same association. Many contributing factors could explain these discrepancies: sensitivity of animal model to mycotoxins, age and sex, nutritional status, as well as the duration and the route of exposure to the toxin. In addition, as reflected in the tables, the levels of mycotoxins used in the experiments may change the type of interaction observed.

Very few studies investigated the effect of mycotoxins, alone or in combination on immune parameters, while in farms vaccination or response to pathogens are common situation. Histopathological analyses provide informations on the organs and cells injuries, but would need to be related with physiologically consequences, especially on functions, not enough studied (oxidative stress, hepatic drug metabolizing enzymes, intestinal permeability...).

Finally, the number of studies investigating effect of low doses of toxins, representative of field situation is very low. Although the use of moderate to high concentrations of toxins provides information on the type of interaction, these doses are not expected in environmental conditions and are largely over the limit set by regulation/recommendation in different countries. It has been noted in this review that a combination of mycotoxins at low concentration may have negative effects, even though the concentrations of individual mycotoxins are below the concentrations reported to cause negative effects (Domijan *et al.*, 2007). Although not reported in this review, the analysis of interaction with more than two mycotoxins would be also relevant and useful in terms of risk assessment.

Nonetheless, it can be concluded that exposure to a co-contaminated food/feed result in a greater risk to human and animal health. The co-exposition to two toxins led finally to greater total

effects in comparison to the total effect of each individual toxin, even in cases categorized less than additive or antagonistic.

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TABLES LEGEND

- ↗, ↘, → : evaluation at the end of each experiment, of the effect of the co-contaminated treatment in comparison to the control treatment.

- (P), potentialization

- unless stated, doses are expressed in mg of toxins/kg of feed. In few experiments, doses are expressed in mg of toxins/kg of body weight/day and in mg of toxins/day, and indicated as bw/d and mg/d, respectively.

- **zootechnical parameters** : BW(G), body weight (gain)

- **RW, relative weight of organs** : RW-L, liver; -K, kidney; -G, gizzard; -Pv, proventriculus; -Pc, pancreas; -H, heart; -Lg, lung; -A, adrenals; -S, spleen; -T, thymus; -BF, bursa of Fabricius.

- **biochemical and hematological parameters** : TP, total proteins; BUN, blood urea nitrogen; RBC, red blood cells; WBC, white blood cells; Sa, sphinganine; So, sphingosine; MDA, malondialdehyde; PC, protein carbonyl; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene.

- **enzymatic parameters** : ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; GGT, gamma-glutamyltransferase; LDH, lactate dehydrogenase; GDH, glutamate dehydrogenase; CK, creatine kinase; CHL, cholinesterase; CAT, catalase; SOD, superoxide dismutase; GSTP⁺, glutathione-S-transferase placental form positive; EROD, ethoxyresorufin O-dealkylase; PROD, pentoxyresorufin O-depentylase

- **immune parameters** : SMC, spleen mononuclear cells; Ab, antibodies; ND, Newcastle disease; IDB, infectious bursal disease; SRBC, sheep red blood cells; DTH, delayed-type hypersensitivity.

3. Procédés de décontamination des denrées contaminées et réduction des mycotoxines chez l'animal

En conséquence des préjudices causés par les mycotoxines (sanitaire, pertes économiques), de nombreuses stratégies ont été développées pour empêcher la croissance des champignons toxigènes et pour décontaminer et/ou détoxifier les aliments contaminés en mycotoxines. Ces stratégies incluent : 1) la prévention de la contamination en mycotoxines, 2) la décontamination des mycotoxines présentes dans les aliments et 3) l'inhibition de l'absorption des mycotoxines dans le tractus gastro-intestinal.

Nous présentons ci-après les approches mises en œuvre pour satisfaire les points 2 et 3. Les techniques décrites ont principalement été rapportées en termes d'efficacité pour éliminer ou réduire la teneur en mycotoxines, mais d'autres critères ont également été pris en compte. En effet, la FAO (Food and Agriculture Organization of the United Nations) a établi certaines lignes directrices dans l'évaluation de ces procédés de détoxification et décontamination. Le procédé doit (i) inactiver, détruire, ou éliminer la ou les mycotoxines ; (ii) ne pas aboutir à la production de substances toxiques, métabolites ou sous-produits dans l'alimentation ; (iii) conserver les valeurs nutritives et l'acceptabilité des denrées alimentaires ; (iiii) ne pas altérer les propriétés technologiques des produits ; et si possible (iiii) détruire les spores fongiques. En plus de ces critères, les procédés doivent être facilement accessibles, facile d'utilisation et peu coûteux.

Cette étude bibliographique fait l'objet de deux chapitres dans l'ouvrage [Mycotoxin Reduction in Grain Chains: A Practical Guide](#) édité par Antonio F. Logrieco, et paraîtra courant 2011.

3.1. Méthodes physiques et chimiques pour la décontamination du maïs contaminé

Le premier chapitre de cet ouvrage traite des techniques permettant une décontamination des denrées contaminées, et plus particulièrement celles évaluées sur le maïs. D'une part, nous reportons les traitements dits physiques, tels que le tri, le tamisage ou le lavage, et également des procédés industriels, initialement non développés pour la lutte contre les mycotoxines, tels qu'en amidonnerie, les radiations ou l'extrusion. D'une autre part, nous présentons les traitements dits chimiques, tels que l'ammoniation, l'ozonation ou la nixtamalization. A noter que, l'ammoniation est

un procédé largement connu et utilisé dans certains pays d'Amérique, d'Europe ou d'Afrique, contre la contamination en aflatoxines.

Nous avons également indiqué dans la mesure du possible, la toxicité résiduelle des aliments traités par ces méthodes, en particulier par les méthodes chimiques. Des tests biologiques, ainsi que des expériences animales ont été menés à cet effet.

Physical and chemical methods for mycotoxins decontamination in maize

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ABSTRACT

Mycotoxins are fungal secondary metabolites that have been associated with toxic effects in human and animal. The contamination of food and feed with mycotoxins is a worldwide problem. Because of the detrimental effects of these mycotoxins, a number of strategies have been developed to prevent the growth of mycotoxigenic fungi and to decontaminate and/or detoxify mycotoxin contaminated food and animal feed. These strategies include : 1) the prevention of mycotoxin contamination, 2) detoxification of mycotoxins present in food and feed and 3) inhibition of mycotoxin absorption in the gastrointestinal tract. The present chapter mainly highlights processes involved in the second point, commonly classified as physical and chemical methods, and which have been evaluated in the maize grain chain. Efficiency and safety of these approaches are the main criteria reported. Only a few of these methods are in practical use; this is probably due to the difficulty to comply with the FAO requirements. In addition, no single method has the ability to remove simultaneously the wide variety of mycotoxins which may co-occur in maize.

INTRODUCTION

Consumption of mycotoxin-contaminated food or feed lead to many different toxic effects such as cancer, acute mortality, reproduction disorder or growth impairment in humans and/or animals (Oswald and Comera, 1998). The Food and Agricultural Organization (FAO) estimated that as much as 25% of the world's agricultural commodities are contaminated with mycotoxins. The economic losses due to mycotoxins contamination are estimated in billions dollars annually worldwide (Kabak et al, 2006).

Due to the multiple possible origins of fungal infection, any prevention strategy for fungal and mycotoxin contamination must be carried out at an integrative level all along the food production chain. Three steps of intervention have been identified. The first step in prevention should occur before any fungal infestation; the second step is during the period of fungal invasion of plant material and mycotoxin production; the third step is initiated when the agricultural products have been identified as heavily contaminated. Such hazard analysis has some similarity with the HACCP management system of food safety (Jouany, 2007).

The efforts must be concentrated on the two first steps since, once mycotoxins are present, it is difficult to eliminate them in a practical way. However, the prevention of mycotoxin contamination prior to harvest or during post-harvest and storage is not always possible necessitating decontamination before the use of such materials for food and feed purposes. Consequently, developing and implementing efficient detoxification methods became very important.

Guidelines for evaluating mycotoxin detoxification and decontamination procedures have been established by the FAO. The process should (1) inactivate, destroy, or remove the mycotoxin; (2) not result in the deposition of toxic substances, metabolites, or byproducts in the food or feed; (3) retain nutrient value and feed acceptability of the product or commodity; (4) not result in significant alterations of the product's technological properties; and if possible, (5) destroy fungal spores. In addition to these criteria, the process(es) should be readily available, easily utilized, and inexpensive (CAST, 2003).

This chapter will present and discuss some of the physical and chemical methods, which combined most of the points mentioned above, especially the effectiveness of mycotoxins decontamination, the safety of these treatments, and the ability to be used on a commercial scale as well. Nonetheless, in the following sections are only reviewed approaches assessed in the maize grain chain and thereby, their efficiencies on mycotoxins that are of the greatest concern for this cereal : **aflatoxins, fumonisins, trichothecenes, zearalenone and ochratoxins.**

I. PHYSICAL METHODS

1) Removal from contaminated maize

a) Sorting

Since the majority of mycotoxin contamination usually occurs in a relatively small number of seeds or kernels, the segregation and sorting of damaged, discolored, crops containing visible mould growth can lead to the removal of significant quantities of mycotoxins from these crops (Kabak *et al.*, 2006). Sorting of the maize by fluorescence can achieve a partial removal of **aflatoxins**. Contamination can be observed by fluorescence following illumination with UV light (365 nm wavelength). The Bright Greenish Yellow (BGY) fluorescence obtained, is not caused by aflatoxin itself but by a peroxidase transformation product of kojic acid, a metabolite of *Aspergillus flavus* and other fungi (Scott, 1998). This test, known as the black light test, is considered as a presumptive indicator of aflatoxin, but as mentioned by Bothast and Hesseltine (1975) both false positives (BGY fluorescence and no toxin detected) and false negatives (toxin detected and no BGY fluorescence) may occur during this evaluation, and results must be interpreted with caution.

More recently, Pearson *et al.* (2004) proposed another sorting method, based on the fact that mycotoxin-producing molds initially infect the oil-rich germ using grain lipids for their growth and metabolism. Therefore, the free fatty acid content of grain has been proposed as a sensitive index of incipient grain deterioration. Using optimal pair of filters (spectral absorbances at 750 and 1200 nm) the authors could distinguish kernels with aflatoxin contamination at >100 ppb from kernels with no detectable aflatoxin, with >98% accuracy. When these two spectral bands were applied to sorting corn at high speeds, reductions in aflatoxin averaged 82% for corn samples with an initial level of aflatoxin >10 ppb and by removing ≈5% of the incoming grain (Pearson *et al.*, 2004).

In the same study, the method was applied for **fumonisin**-contaminated corn. The high-speed dual-wavelength sorter reduced fumonisin by an average of 88%, including those with low levels of fumonisin.

b) Sieving-cleaning

Cleaning grains removes kernels with extensive mold growth, broken kernels and fine materials such as dirt and debris, which helps to reduce mycotoxin concentrations. Murphy *et al.* (1993) reported the sieving of corn screenings (160 samples) into eight different particle sizes and concluded that corn screenings or broken corn kernels usually contain **fumonisin** levels about 10 fold higher than intact corn kernels. Similarly, fractionation of maize samples by sieving through a 3

mm screen shows that fractions termed 'fines' (< 3 mm) had significantly higher total fumonisins concentrations and accounted for between 4.7 and 20.0% of the samples by mass (Sydenham *et al.*, 1994). The data indicated that removal of the 'fines' resulted in overall reductions in total fumonisin levels of between 26.2 and 69.4%.

Regarding **deoxynivalenol (DON)** and **zearalenone (ZEA)**, Trenholm *et al.* (1991) investigated the efficacy of sieving with a series of screens, on corn contaminated with 23 mg of DON/kg and with 1.2 mg of ZEA/kg. Reduction of 73% and 79% of the total DON and ZEA respectively has been reported.

c) Flotation and density segregation

Mould-damaged, mycotoxin-contaminated kernels may exhibit different physical properties than non-damaged kernels. So they can be separated by density segregation in certain liquids, or fractionation according to specific gravity tables. Because fungi deriving nutrients from grains through metabolism may alter the density of grains, it seems possible that fungal-infected grains can be segregated from uninfected grains without specificity for any given fungus or grain. A 67% reduction in **aflatoxin** concentration of corn was obtained by removal of corn buoyant in water (Huff, 1980; Huff and Hagler, 1982). The efficiency of density segregation was enhanced by increasing the specific gravity of the suspending liquid. With the use of a 30% sucrose solution, the aflatoxin concentration of corn was reduced by 87% (Huff, 1980). In another study, only 4 of 54 aflatoxin-contaminated samples remained aflatoxin-positive after density segregation with 30% sucrose (Huff and Hagler, 1982). Similarly, density segregation using a saturated sodium chloride solution was effective in reducing the aflatoxin concentration of contaminated corn (Huff and Hagler, 1985). Corn kernels which were buoyant in saturated sodium chloride represented 3% of the total sample, yet contained 74% of the aflatoxin.

Besides aflatoxin-contaminated grains removal, Huff and Hagler (1985) assessed their method on **deoxynivalenol**-contaminated corn kernels and obtained for two naturally contaminated corn samples, a reduction of 53% and 77% in DON concentration with water and 30% sucrose.

The same principle was applied for **fumonisins**-contaminated maize (Shetty and Bhat, 1999). With increasing concentrations of NaCl, more maize entered the buoyant fraction and the removal of fumonisins can reach 86% with saturated NaCl solution. The authors suggested washing grains with water in order to remove the salty taste (Shetty and Bhat, 1999).

d) Washing with sodium carbonate solution

Washing methods take advantage of the fact that mycotoxins are primarily found on the outer surface of grains, but also involve contamination of commodities by soluble mycotoxins. In addition, sodium carbonate is inexpensive, widely available, and nontoxic to humans and animals. A simple method to reduce *Fusarium* mycotoxins such as **deoxynivalenol** and **zearalenone**, has been proposed by Trenholm *et al.* (1992), where simple washing procedures, using distilled water or sodium carbonate solutions were applied in corn contaminated samples. Authors reported for corn contaminated with **deoxynivalenol** at 25 mg/kg, a removal from the grain (69-95% reduction) after soaking in a solution of sodium carbonate (0.1M) for 24-72h. Mycotoxin removal increased with increased soaking time. A substantial reduction in **zearalenone** levels (87%) was also observed on contaminated corn washed with 1M sodium carbonate solution for 30 minutes (Trenholm *et al.*, 1992). ZEA is a weak phenolic acid whose solubility is greatly enhanced in alkaline conditions as sodium carbonate solution.

In a feeding trial, this procedure was shown to be effective in reducing the toxicity of *Fusarium*-contaminated corn when fed to growing pigs, as measured by the increase in daily feed consumption and weight gains (Rotter *et al.*, 1995). By contrast, Bennet *et al.* (1980) reported that sodium bicarbonate had no effect on the ZEA concentration of naturally or artificially contaminated corn.

e) Dehulling

Dehulling is a milling technique that removes the outer layers of grain by abrasion. One of the early practices of African food preparation was manual dehulling of grain before grinding. This practice is no longer in common use except in few countries, and has been replaced by use of mechanical disc dehullers on a commercial and semi-commercial scale. The efficacy of this procedure depends on the degree of fungal penetration into the kernel.

The outer layers are most susceptible to fungal attack and **aflatoxins** accumulation. A reduction of 92% in aflatoxin levels was reported after a physical dehulling procedure in contaminated maize (Siwela *et al.*, 2005). The same reduction level of aflatoxin content in naturally contaminated maize was obtained using “*muthokoi*”, a traditional dehulled maize dish prepared in Kenya (Mutungi *et al.*, 2008).

f) Milling

Milling is traditionally used for grain processing. We can distinguish two milling processes : dry and wet milling, the latter being the major milling process used for maize. This method allows to

separate the grain into different fractions. It is therefore, important to identify the fractions where the mycotoxin remains, so that they can be diverted to lower-risk uses or subjected to decontamination procedures.

During the dry milling process, mycotoxins tend to be concentrated in germ and bran fractions. By contrast, during the wet milling process, mycotoxins may be dissolved into the steep water or distributed among the byproducts of the process (Bullerman and Bianchini, 2007).

After wet milling of **aflatoxin**-contaminated corn samples, the steepwater-solubles fractions contained the highest concentration of aflatoxin (about 40% of the recovered aflatoxin). The fiber fraction accounted for about one-third (30 and 38%) of the total aflatoxin, the germ for 6 and 10% and 1% for the starch. Thus, after wet-milling of aflatoxin-contaminated corn, most of the aflatoxin will ultimately end in the feed fractions. Thus, feed by-products from the milling industry need to be decontaminated by others processes such as ammonia treatment (Bennett and Anderson, 1978).

In the dry milling process, **fumonsins** tend to be concentrated in the bran and germ fractions of corn (used as animal feed or for oil extraction) and produced grits are relatively free of contamination (Katta *et al.*, 1997).

Bennett and Richard (1996) found that during wet milling, **fumonisin** (water-soluble) in contaminated corn was dissolved into the steep water or distributed to the gluten, fiber and germ fractions, leaving no detectable amounts in the starch.

Using a laboratory stimulated process of wet milling, Collins and Rosen (1981) found that 66% of **T-2 toxin** in corn was removed by the steep and process water, 4% was found in the starch, and approximatively 30% was in the germ.

For **ochratoxin A**, concentration in cleanings, bran and other fractions derived from the seed coat were the outcomes reported after maize milling (Scudamore, 2005).

Dry milling was also an effective method for salvaging **zearalenone** contaminated corn (Bennett *et al.*, 1976). The starch fraction, the largest and most important for food purposes, was essentially free of ZEA. However, the germ and bran fractions, which are used as animal feed contained much higher ZEA concentrations than the original corn.

g) Steeping

The corn steeping process is the first step of the industrial wet-milling of corn, which involves soaking the corn in water containing SO₂ to facilitate germ separation. Preliminary work by Canela *et al.* (1996) has shown that steeping in water may reduce the **fumonisins** content of naturally contaminated corn, where FB1 migrates from kernels to the steeping water as a result of its high

polarity. Pujol *et al.*, (1999) concluded that steeping corn kernels in 0.2% SO₂ solution at 60°C for 6 h is very effective to decrease the amount of FB1.

Ochratoxin A is distributed differently in the corn steeping process, and the toxin remains in the steeped corn, and do not reach into the steep water (Wood, 1982).

2) Inactivation in contaminated maize

a) Heat treatment

Most of mycotoxins are heat-stable within the range of conventional food/feed processing temperatures (80-121°C), so little or no destruction occurs under normal cooking conditions such as boiling and frying, or even following pasteurization. The sensitivity of mycotoxins to heat treatment is affected by many different factors including moisture, pH, and ionic strength of food. Degradation by heat treatment also depends on the type of mycotoxin, its concentration, the extent of binding between the mycotoxin and the food constituents, the degree of heat penetration, and the processing time (Kabak *et al.*, 2006). A heat treatment which has been widely studied for the destruction of some naturally occurring food toxins, is extrusion cooking process. The reduction of the mycotoxin in the finished products depends on several factors including extruder temperature, screw speed, moisture content of the extrusion mixture, and transit time in the extruder. Of these factors, extrusion temperature and transit time seem to have the greatest effect. The highest reductions in mycotoxin concentrations in extruded products occur at temperatures of 160°C or greater, and when time of transit is increased. During extrusion cooking, the raw material is subjected to high temperatures, high pressure, and severe shear forces. Two major types of extruder are used in the food industry : single-screw and twin-screw extruders.

Hameed (1993) showed that extrusion alone was able to reduce **aflatoxins** content in naturally-contaminated corn by 50-80%, and with addition of ammonia, either as hydroxide (0.7 and 1.0%) or as bicarbonate (0.4%) the aflatoxin reduction achieved was greater than 95%. However, Cazzaniga *et al.*, (2001) emphasizes the importance of the extruder design, which could be a significant factor affecting the residence time and the degree of mixing.

In addition to this heat process, roasting is also effective in the detoxification of **aflatoxin**-contaminated corn. Reduction in aflatoxin content was higher when roasting was combined with ammonia treatment (Conway *et al.*, 1978).

Extrusion appears to be an effective method for reducing **fumonisin**s levels in corn (Castelo *et al.*, 1998a; Katta *et al.*, 1999). In the study of Katta (1999), the FB1 losses in corn grits at different

Table 7 : toxicological evaluation of effective processes on maize decontamination

Processing methods	Mycotoxins	Toxicity assessment		Toxicity outcomes after treatments	References
		Bioassay	Feeding trial		
<i>Physical methods</i>					
Extrusion	FUM		x	in rats – decrease of the kidney lesions	Bullerman et al. (2007)
	DON	x		loss of toxicity assessed by the MTT assay on CHO-K1 (Chinese hamster ovary) cell line	Cetin and Bullerman (2006)
	ZEA	x		loss of biological activity assessed by the MTT assay on MCF-7 (human breast cancer) cell line	Cetin and Bullerman (2005)
<i>Chemical methods</i>					
Ammoniation	AF	x		metabolites less toxic than AFB1 in the <i>Salmonella</i> microsomal assay – assessment of the mutagenic activity	Park et al. (1993)
				x	absence of toxicity in some species (<i>rats, ducks, swine, chickens, trouts</i>) – performance, biochemistry & histopathology verified
	FUM		x	in rats - reduced weight gains, elevated serum enzyme levels and histopathological lesions	Norred et al. 1991
Ozonation	AF	x		absence of toxicity of metabolites in the <i>Hydra attenuata</i> (HA) assay – toxicity endpoint determined by the “tulip” or “disintegration” stage of HA	McKenzie et al. (1997)

			x	in turkeys - improvement of body weight gain and relative organs weights, no liver discoloration and positive effect on biochemistry	McKenzie et al. (1998)
	FUM	x		toxicity of metabolites in the <i>Hydra attenuata</i> assay and Sa/So ratio still elevated	McKenzie et al. (1997)
	OTA	x		absence of toxicity of metabolites in the <i>Hydra attenuata</i> assay	McKenzie et al. (1997)
	ZEA	x		uterine weights of mice not affected	Lemke et al. (1999)
Nixtamalization	AF	x		mutagenic activity higher for extracts of acidified tortillas	Price and Jorgensen (1985)
	FUM		x	in rats - hepatotoxicity and nephrotoxicity of nixtamalized culture material (biochemistry and histopathology)	Voss et al. (1996)
Modified nixtamalization	FUM	x		toxicity reduction in the brine shrimp assay and no positive mutagenic potential in the <i>Salmonella</i> microsomal assay	Park et al. (1995)
Sodium bisulfite	DON		x	in pigs - improvement of feed intake and body weight gain, and no emesis	Young et al. (1987)
Reducing sugars	FUM		x	in pigs - improvement of body weight gain and feed intake, positive effect on biochemistry, no lesion in liver and kidney, and decrease of Sa/So ratio	Fernandez-Surumay et al. (2005)
Citric acid	AF	x		mutagenic activity of acidified samples greatly reduced in the <i>Salmonella</i> microsomal assay	Mendez-Albores et al. (2005)
			x	in ducks – improvement of body weight gain, positive effect on biochemistry and decline in the severity of lesions in liver	Mendez-Albores et al. (2007)

AF = Aflatoxins; **FUM** = Fumonisin; **DON** = Deoxynivalenol; **ZEA** = Zearalenone; **OTA** = Ochratoxin A

extrusion parameters ranged from 34 to 95%. Another approach proposed by Castelo *et al.* (2001), was to evaluate the effect of sugars on the stability of fumonisins during extrusion processing of corn grits. Both the screw speed and glucose concentration significantly affected the extent of FB₁ reduction in extruded grits, with greater reductions of FB₁ (up to 92.7%) observed at low screw speeds and high glucose concentrations. However, extrusion may lead to modifications of the mycotoxin structure, or interactions with the matrix, that may not be detectable or quantifiable by either HPLC or ELISA methods. Identification and toxicological assessment of degradation products is therefore necessary. For example, Bullerman *et al.* (2007) showed for FB₁ that extrusion with 10% glucose led to the formation of *N*-(1-Deoxy-D-Fructos-1-γ1) FB₁, the main FB₁ derivative detected. When the rat kidney was used as a biosensor to detect residual toxicity, the authors observed that *N*-(1-Deoxy-D-Fructos-1-γ1) FB₁ was less toxic than unmodified FB₁ (see Table 7).

Other thermal processes have also been studied in the **fumonisin** inactivation. Castelo *et al.* (1998b) showed that roasting corn meal spiked with 5 ppm FB₁ at 218°C for 15 min resulted in almost complete loss of fumonisins. Corn flakes processing in the presence of glucose, gave 86-89% reduction of FB₁ after cooking and toasting (Castelo, 1999). When canning was applied to whole kernel corn, a significant decrease in fumonisins content occurred as well (Castelo *et al.*, 1998b). Finally, Scott and Lawrence (1994) reported that heating dry corn meal spiked with FB₁ and FB₂ at 190°C (60 min) and 220°C (25 min) resulted in 60 and 100% loss of both toxins, respectively.

Cazzaniga *et al.* (2001) studied the effect of extrusion cooking on the stability of **deoxynivalenol** in maize in the presence or not of additives (sodium metabisulphite). In all conditions applied, which included moisture contents of 15% and 30%, temperatures of 150 and 180°C, and sodium metabisulphite concentrations of 0 and 1%, the detoxification achieved was greater than 95%. Toxicity assessment of degradation products following extrusion-processed corn grits contaminated with DON has been reported (Cetin and Bullerman, 2006; see also Table 7).

Extrusion cooking of corn grits resulted in reductions of **zearalenone** ranging from 77 to 83%, 74 to 83% and 66 to 77% at 120°C, 140°C and 160°C, respectively (Ryu *et al.*, 1999). More recently, Cetin and Bullerman (2005) reported in an *in vitro* study that the extrusion process at temperatures of 150, 175, and 200°C resulted in a reduction of ZEA ranging from 60 to 78%. In this study, the authors developed a bioassay in order to determine the toxicity obtained after extrusion processing (see Table 7).

b) Irradiation treatment

Ionizing radiation is currently used to eliminate pathogenic microorganisms in foods. Herzallah *et al.* (2008) reported that the solar radiation was more effective in **aflatoxins** reduction when

compared with γ -irradiation and microwave heating. More than 60% of the aflatoxins were found to be degraded after 30h of exposure to sunlight. The degree of aflatoxin reduction was found to be dependent on the duration of exposure to sunlight. But solar radiation has potential as an inexpensive means of degrading aflatoxins in tropical countries. Regarding γ -irradiation treatment, 40% of aflatoxin content was destroyed and these results were in agreement with Farag *et al.* (1995), who found that an 83% reduction of aflatoxin after a 20-kGy dose of γ -irradiation of yellow corn was achieved. On the contrary, Aziz and Youssef (2002) found that the dose of 20 kGy was sufficient to completely destroy AFB1 in yellow corn.

The effect of γ -irradiation on **fumonisin B1** and **fumonisin B2** occurring in maize materials has been investigated together with the stability of fumonisins in γ -irradiated maize stored at different temperatures (-18 to +40°C) for different periods (2, 4, 13 and 26 weeks). Fifteen KGy γ -irradiation was required to sterilize efficiently maize flour. This process caused a decrease in fumonisin content of about 20%. The stability studies showed that fumonisins are stable in γ -irradiated maize for at least 6 months at 25°C or at least 4 weeks at 40°C (Visconti *et al.*, 1996).

Paster *et al.* (1985) indicated that pure **ochratoxin A (OTA)** is stable even at 75 kGy but Refai *et al.* (1996) showed that a dose of 15 or 20 kGy is sufficient for complete destruction of OTA in yellow corn. Similarly, Aziz and Youssef (2002) reported 75% of detoxification in yellow corn at a radiation dose of 20 kGy.

For **zearalenone**, complete destruction was obtained when a dose of 20 kGy was applied in yellow corn (Aziz and Youssef, 2002).

Other ionizing radiations such as microwave and convection heating have been reported by Young (1986) to be partially successful in lowering **deoxynivalenol** level in naturally contaminated corn.

In conclusion, physical methods outlined in this first part can be divided in two categories with regard to their initial applications. Some methods have been specifically developed for mycotoxins decontamination (such as sorting, cleaning or washing); whereas others are industrial processes (such as milling, irradiation, ethanol fermentation or extrusion) initially developed for other purposes than mycotoxins reduction, that have shown an interest in the fighting strategy against mycotoxins. The first category can be considered as a first step in the removal of mycotoxins from maize. Besides the substantial efficiency of these approaches, most of the criteria listed by the FAO guidelines are fulfilled : cheap and simple, no production of toxic metabolites, and no change in the nutritional value and properties of raw materials. Nonetheless, application of these techniques on a commercial scale raises some problems :

- an important loss of grain material has been reported in some cases for sieving and cleaning procedures. In their study, Trenholm *et al.* (1991) observed a 73% and 79% reduction for DON and ZEA respectively, however in these conditions up to 69% of the total weights of the corn were removed as well.
- after flotation and washing, the cost of drying grains is a significant problem; unless these methods are used prior manufacturing processes that require the grain to be wetted or tempered such as wet milling or alkaline processing of corn.
- the duration of grains treatment is also a parameter to take into account.

It is also important to stress that contaminated waste (broken kernels, residues, dust...) resulting from these decontamination procedures are highly contaminated, thus they must be destroyed and not used in animal feed.

Processes (milling, thermal treatments) from the second category are commonly used in the industry for food production, intended to the human consumption. Many reports have also shown their ability to reduce mycotoxins content. Of note, a few methods are already used in feed industry, such as extrusion in pet food manufacturing.

The problems met with milling process, concern the toxicity of the different fractions obtained after grain separation. Indeed, mycotoxins tend to be concentrated in these fractions (bran and germ) that are used in animal feed. Accordingly, decontamination procedures need to be applied on these fractions.

Regarding heat treatments, it is important to emphasize that this process may lead to the formation of unknown biologically active mycotoxin degradation products or, to the reversible binding of the toxin to sugar or proteins in the food/feed matrix (Humpf and Voss, 2004). Additional investigations using an integrated approach combining chemical studies and appropriate bioassay methods are needed to identify and characterize these “hidden” mycotoxins.

Finally, another point to mention is the blend of contaminated crops with batches of good-quality material. Despite the effectiveness of this method in decreasing mycotoxins concentration, the practice is prohibited within the European Union (Commission Regulation, 2001).

II. CHEMICAL METHODS

1) Ammoniation

Treatment with ammonia in the gaseous phase, or with substances capable of releasing it, achieved optimum results in detoxifying corn meals, especially for aflatoxins reduction. It has been widely demonstrated that the efficacy of mycotoxins detoxification with ammonia is positively correlated with the quantity used, reaction time, temperature and pressure levels. Since few data on ammonia treatment have been reported for other mycotoxins, the section on aflatoxin decontamination is presented in more details.

First outcomes on the chemical inactivation of **aflatoxin** by ammoniation were reported for cottonseed and peanut meals (Dollear *et al.*, 1969). Nowadays, it is an approved procedure and a method of choice for the detoxification of aflatoxin-contaminated agricultural commodities and feeds, in some of the North American states (Arizona, California, Georgia, Alabama), in France, in Senegal, in Sudan, in Brazil, in Mexico and in South Africa.

While at least five types of ammoniation processes have been described or patented, the two procedures considered most practical are a high temperature/high pressure process used by commercial treatment plants and an atmospheric pressure/ambient temperature process for in situ farm usage. Both procedures have the advantages of removing up to 90% of aflatoxin contamination in the matrix.

Aflatoxin B1 degradation by ammonia proceeds through hydrolysis of the lactone ring followed by decarboxylation to aflatoxin D1 and loss of the cyclopentone ring to give a compound of a molecular weight of 206 daltons. It was also observed that the AFB1 molecular structure is irreversibly altered if exposure to ammonia lasts long enough. In contrast, if exposure is not sufficiently protracted, the molecule can revert to its original state. To note that the addition of formaldehyde in the process, allows to reduce the use of ammonia, to break more easily the lactone ring and to avoid the reversibility of the reaction.

The studies concerning the safety of ammoniation process began in 1983, where no signs of toxicosis occurred in rats fed ammoniated aflatoxin-contaminated corn, in contrast to rats fed with untreated aflatoxin-contaminated corn (Norred and Morrissey, 1983). A comprehensive review on feeding trials carried out to evaluate the safety of ammoniated feed, was published by Park *et al.* (1988; **see Table 7**). In addition, toxicity assessment of metabolites obtained after aflatoxin processing was reported (Park, 1993; **see also Table 7**). More recently, an experiment on broiler chicks suggests that replacement of aflatoxin-containing maize with ammoniated grains significantly suppress aflatoxicosis, leading to an improvement in production parameters in broilers (Allameh *et*

al., 2005). The fate of ammonia products in lactating cows was also investigated, and several studies concluded that the efficient reduction of aflatoxin levels by ammoniation, resulted in a strong reduction of aflatoxin-related residues in milk (Fremy *et al.*, 1988; Hoogenboom *et al.*, 2001).

Fumonisin B1 is also degraded by ammoniation with a high pressure/ambient temperature or atmospheric pressure/high temperature. Ammonia treatment resulted in 79% of FB1 destruction on contaminated maize (Park *et al.*, 1992). However, Norred *et al.* (1991) found that ammoniation procedure on fumonisins content of corn did not reduce the toxicity of the materials when fed to rats (see Table 7).

Exposing corn to ammonia (100%) for at least 18h resulted also in substantial reductions (85%) in **deoxynivalenol** levels (Young, 1986). Toxicological studies are needed to evaluate the safety of ammoniated DON-contaminated corn.

In barley, **ochratoxin A** was nearly completely degraded after ammonia treatment. However, in studies in which the ammoniated ochratoxin-contaminated barley was fed to pigs, some toxicity and lower nutritional value was observed (Madsen *et al.*, 1983). Residues of OTA were also found in the kidneys, and it was thought to be reformed in the animals by recyclization.

In 1981, Chelkowski *et al.* reported that addition of ammonia to corn in a final concentration of 2% inactivates partially **zearalenone** at temperatures of 20-50°C.

2) Ozonation

Ozone is a powerful oxidizing agent capable of reaction with numerous chemical groups, though it has an affinity for double bonds. Its industrial application as a disinfectant is already known and currently used. In addition, ozone decomposes to form oxygen and therefore, can be classified as a nonpersistent chemical. Thus, several research studies have been undertaken to evaluate the effect of ozone in reducing mycotoxins in agricultural products. Ozone has a high potential for degrading **aflatoxins**. It reacts across the 8-9- double bond of the furan ring through electrophilic attack. In 1997, McKenzie developed a novel and continuous source of O₃ gas through electrolysis. Total degradation of AFB1 in solution was obtained at 15 sec using 20 weight% ozone, and the same year, the author claimed using O₃ generated by electrolysis, the processing and the decontamination of bulk quantities of corn that was contaminated with high levels of AFB1. Furthermore, the toxicity resulting from processing of aflatoxin by ozonation, was evaluated in a bioassay (see Table 7). In a similar study, McKenzie *et al.* (1998) found that aflatoxins could be reduced by 95% in corn treated with 14 wt% ozone for 92h at a flow rate of 200 mg/min. Turkeys fed with ozone-treated contaminated corn did not show harmful effects as compared to animals fed with untreated contaminated corn (see Table 7). Similarly, phagocytosis by rat peritoneal macrophages, which was

found to be suppressed in the presence of aflatoxin, remained unimpaired when the applied AFB1 was pretreated with 1.2 mg/L ozone for 6 min at a flow rate of 40 mL/min (Chatterjee and Mukherjee, 1993). However, the ozonation process may cause formation of fat-soluble reaction products with low mutagenic potential (Prudente and King, 2002).

McKenzie *et al.* (1997) found that treating an aqueous solution of **fumonisin B1** with 10 wt% ozone gas for 15 sec resulted in the conversion of the parent compound to the 3-keto FB1 derivative. In two separate toxicity tests, 3k-FB1 was found to retain most of the toxicity present in the parent compound (**see Table 7**). Therefore, a further degradation of FB1 to a product less active than 3k-FB1 is needed (i.e. removal of primary amine).

Moisture appears to be essential in the reaction between **deoxynivalenol** and ozone. Moist ozone (1.1 mol %) in air resulted in a 90% reduction in DON-contaminated corn, at 1000 ppm, after 1h, while dry ozone resulted only in a 70% reduction (Young, 1986). For DON treatment with ozone, differences also occur between wheat and corn. A matrix effect was suggested (Young *et al.*, 2006), the ground corn being more porous, whereas ozone may not be able to penetrate the whole wheat kernels as readily. Young *et al.* (2006) reported the degradation of several **trichothecenes** mycotoxins by aqueous ozone and on the basis of UV and MS data, proposed that the degradation begins with attack of ozone at the C9-10 double bond with the net addition of two atoms of oxygen. However, toxicity of the breakdown products and efficiency of this technology in maize need to be evaluated.

Ozone treatment induces a rapid degradation of **zearalenone** level in water solutions containing 12 ppm of ZEA (Lemke *et al.*, 1999), and prevents its estrogenic effects (**see Table 7**). In an *in vitro* study a reduction to undetectable levels was observed after ozone treatment for 15 s in ZEA solution. No new products were observed after the treatment (McKenzie *et al.*, 1997).

The same study showed treatment of aqueous solution of **ochratoxin A** with 10 wt% O₃ for 15 seconds reduced the mycotoxin level to undetectable levels, and a bioassay revealed a decrease in the toxicity (**see Table 7**).

Using ozone to purify commodities, the commercial Oxygreen[®] process claims the destruction of mycotoxins found on grain by bringing them to levels tolerated by legislation. This procedure lead to a 94% reduction of OTA in grains. Toxicity of this process was evaluated by a four-week study in rats by dietary administration of treated wheat, and the treated products were considered as safe for the consumer (Gaou *et al.*, 2005).

3) Nixtamalization

Nixtamalization, a Mexican traditional process for making tortillas, consists of the cooking of maize grain in abundant water and lime (calcium hydroxide), at temperatures near boiling, followed

by a steeping period. Several studies reported a significant reduction in **aflatoxins** content (90-95%) in corn with this alkaline treatment. However, the apparent aflatoxin reduction in contaminated maize was not permanent, since it was reverted by an acidic treatment, which probably caused aflatoxin reformation by closing the open lactone ring (Mendez-Albores *et al.*, 2004; Price and Jorgensen, 1985). Acidification of the aflatoxin extracts, with a pH similar to the stomach as occurs during digestion, lead to a rebuilding of the aflatoxin molecule (**see Table 7**).

Under alkaline conditions, **fumonisin**s in contaminated corn are converted to the so-called hydrolyzed fumonisins (HFB1). Dombink-Kurtzman *et al.* (2000) showed that nixtamalization reduced the FB1 concentration in tortillas by 81.5% and that the FB1 and HFB1 were mainly found in the steeping and washing water. Cortez-Rocha *et al.* (2002) observed a 39% reduction of FB1 concentration when raw corn was nixtamalized. Others found that the traditional nixtamalization method used by the Mayan communities in Guatemala reduced total fumonisins (FB1 and HFB1) by 50% and that the residual lime and washing water also contained 50% of the fumonisins initially present in the corn (Palencia *et al.*, 2003). However, when fed to rats, Voss *et al.* (1996) showed that nixtamalized corn culture materials that contained HFB1, still induce toxicity (**see Table 7**). This toxicity was originally attributed to the hydrolyzed fumonisins, but the same author in 2009 disclaimed the HFB1-induced toxicity, suggesting these effects were mediated by residual or “hidden” FB1 (matrix bound forms not detected by HPLC) remaining in the nixtamalized preparations, rather than HFB1.

Park *et al.* (1995) observed that further decontamination and detoxification of FB1 contaminated corn was achieved by using a modified nixtamalization procedure. This procedure consisted in a combination of heat treatment with hydrogen peroxide/sodium bicarbonate with calcium hydroxide and gave up to 100% reduction of FB1 in contaminated maize (100 ppm). The best procedure involved treating corn with a combination of H₂O₂/NaHCO₃ alone for one hour. This is a simple treatment that could be integrated into industrial processing procedures for corn. Furthermore, treatments of FB1-contaminated corn simulating modified nixtamalization have shown a reduction of toxicity (**see Table 7**).

Abbas *et al.* (1988) indicated that boiling **deoxynivalenol**-contaminated corn in lime-water removed 72-82% of DON and 100% of 15-acetyl-DON. In the same study, the authors observed a reduction of 59-100% for **zearalenone**-contaminated corn. No data on the safety of treated feed, contaminated with either DON or ZEA, has been reported.

4) Hydrogen peroxide/sodium bicarbonate

As mentioned above, the addition of oxidizing agents, such as hydrogen peroxide, is an effective help in nixtamalization. Some studies have shown that hydrogen peroxide/sodium bicarbonate alone is effective for simultaneous degradation/detoxification of **aflatoxins** and **fumonisin**s, thereby reducing toxicity (Lopez-Garcia *et al.*, 1999).

Abd Alla (1997) investigated the efficiency of hydrogen peroxide for detoxification of contaminated corn with **zearalenone**. A degradation level of 84% was achieved with a treatment with 10% H₂O₂ for 16 hours at 80°C.

5) Sodium bisulfite

The use of sodium bisulfite as a detoxification reagent is worth special mention as it is a common food additive. In maize, sodium bisulfite treatments used at 0.5% and 2% destroy 80% and 90% of **aflatoxin B1** respectively (Doyle *et al.*, 1982). The main product of reaction is a sulfonate, AFB1-S, formed by addition of bisulfite to the furan ring present in AFB1 and AFG1, but not in AFB2 and AFG2. Piva *et al.* (1995) considered that although this process is less efficient than ammoniation, it overcomes some of the typical disadvantages of ammonia method (less hazardous to handle for the staff, no undesirable brown color of treated feed or no impact on nutrient value) and is much cheaper.

Sodium bisulfite also destroys **deoxynivalenol** in maize. Indeed, Young *et al.* (1987) reported that DON in contaminated maize was reduced by 85% through treatment with sodium bisulfite solutions. The greatest reductions (up to 95%) were achieved when the contaminated corn was autoclaved for 1h at 121°C in the presence of 8.33% aqueous sodium bisulfite. In the same study, the authors performed two toxicological trials in order to assess the safety of this process; the first one consisted to a 7 days feeding trial in pigs with treated-contaminated corn (7.2 ppm DON), and the second one with DON-sulfonate (the by-product obtained after treatment) which was administered orally to swine at the same level (molar equivalent) at which nonderivatized DON caused severe emesis (see **Table 7**). However, some authors indicated that DON-sulfonate is stable in acid but hydrolyzed to DON under alkaline conditions.

6) Reducing sugars

The primary amine group in the **fumonisin B1** molecule is responsible for its toxicity (Fernandez-Surumay *et al.*, 2004). Transformation of FB1 into a FB1-reducing sugar adduct through a nonenzymatic browning reaction (65 °C for 48 h) reduces significantly the toxicity of the native FB1

on cell tissue cultures, on mice, on rats and on swine (Lu *et al.*, 1997; Liu *et al.*, 2001; Fernandez-Surumay *et al.*, 2004). The products of FB1-reducing sugar reaction were characterized as *N*-(carboxymethyl) FB1, *N*-(1-deoxy-d-fructos-1-yl) FB1, *N*-methyl-FB1, *N*-(3-hydroxyacetyl) FB1, and *N*-(2-hydroxy-2-carboxyethyl) FB1. In 2005, Fernandez-Surumay *et al.* examined the effects of fumonisin B-glucose reaction products in swine fed with treated maize (see **Table 7**). The author suggested that dietary FB1-glucose might provide a detoxification approach in instances of widespread fumonisins grain contamination.

7) Citric acid

Up to 96% degradation of **aflatoxin B1** occurred in maize contaminated with 93 ppb when treated with an aqueous citric acid (Mendez-Albores *et al.*, 2005). In addition, they evaluated the toxicity of acidified samples obtained in a bioassay (see **Table 7**). These results show the efficacy and safety of the acidification procedure in reducing aflatoxins levels in maize. The author suggested also that for whole grain, it is likely that acid treatment may be less effective than for ground maize, since toxins deposited inside whole kernels are less likely to be exposed to acidic treatment than toxins in small maize particles. Toxicity of untreated aflatoxin-contaminated feed was reduced in ducks when aqueous citric acid was applied to feed (Mendez-Albores *et al.*, 2007; see **Table 7**).

8) Other chemical treatments

A complete destruction of **deoxynivalenol** was reported in corn contaminated with 1000 ppm of DON, treated with 30% chlorine (v/v) for 30 min (Young, 1986). The author suggested that the treatment might be too drastic for grains destined for human consumption but could be used in animal feed production.

Calcium hydroxide monomethylamine has been used successfully to decontaminate **T-2** and **HT-2 toxin** in maize (Bauer, 1994).

Butylated hydroxytoluene (BHT) treatment significantly reduced the hepatocellular necrosis, biliary hyperplasia, and elevated serum enzymes commonly caused by **aflatoxin B1** in turkeys. This diminished pathology demonstrates a physiologic protective effect (Coulombe *et al.*, 2005). These results were supported by the fact that dietary BHT can cause significant reductions in AFB1 bioavailability, AFB1-DNA adduct formation in the liver, and reduced AFB1 residues in tissues (Guarisco *et al.*, 2008).

In conclusion, although the chemical detoxification methods could greatly reduce mycotoxin contamination, they do not seem able to fulfill all the FAO requirements; thus limiting their widespread use. Critical points remains :

- identification of mycotoxin degradation/transformation products with suitable methods of detection. For example, problems to detect the presence of “hidden” mycotoxins, such as for nixtamalization of FB1-contaminated feed (Voss *et al.*, 2009).
- bioassay and toxicological studies in target species to assess the safety of the treatment. Importance to evaluate the fate of processed contaminated food/feed after ingestion in animals. For example, mycotoxin reversion into its native toxic form may occur, as revealed after nixtamalization of aflatoxin-contaminated feed (Price and Jorgensen, 1985).
- impact on the nutrients content or on the fate of nutrients after processing. For example, reduction in the content of some amino acids (cystine, methionine and especially lysine) and increase in total nitrogen and non protein nitrogen, are some of the effects reported for ammoniation (Piva *et al.*, 1995).

In addition, the handling of chemical products implies risks for the workers. It should be noted that chemical treatment is not allowed within the European Community for commodities destined for human food.

Finally, an approval procedure should also act efficiently on several types of mycotoxins simultaneously, without eliciting residual toxicity. According to criteria listed above and to studies presented in this chapter, ozonation and modified nixtamalization seem to be the most suitable.

CONCLUSION

Detoxification efforts for mycotoxins have focused primarily on the aflatoxins; many strategies to decontaminate aflatoxin-contaminated crops and products have been reported and reviewed. By contrast, informations on methods detoxifying other mycotoxins are limited. Multi-contamination commonly occurs in commodities and as the impact of these hazardous toxins is being recognized, successfully removing them from the food/feed supply represents an emerging area of interest and research. The effectiveness of a method in the detoxification of mycotoxins depends on 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. Although a number of effective processes have been developed, no single physical or chemical method can remove simultaneously all mycotoxins from feed.

Other approaches based on mycotoxins adsorption and biotransformation, which act directly in the gastrointestinal tract of animals, have been reported. Consequently, when prevention of mycotoxins contamination prior to harvest or during post-harvest and storage is unavoidable, an integrated multipronged approach seems to be the most suitable strategy for controlling mycotoxins contamination in feed : physical and/or chemical methods to lower the mycotoxins content in raw materials, and adsorption and/or biotransformation to reduce the bioavailability of mycotoxins during the digestive process of animals.

To conclude, mycotoxin contamination of food/feed will remain a global problem in the future, especially with the global warming. The decontaminated or detoxified crops are mostly considered to be a lower quality, and thus fetch a lower price than a normal uncontaminated crop. This is a serious constraint on the practical use of decontamination and detoxification methods, and the processed crops are mainly used for feed production and animal feeding. Therefore, a partial solution to mycotoxins would be to use contaminated crops in more cases for purposes other than direct food and feed, such as biofuel industry.

3. Procédés de décontamination des denrées contaminées et réduction des mycotoxines chez l'animal

3.2. Adsorption et détoxification biologique

Le deuxième chapitre de cet ouvrage présente des méthodes alternatives mais complémentaires à celles décrites dans le chapitre précédent, et à la différence, consistent en une action directe dans l'organisme de l'animal. Ces approches plus récentes sont en plein essor, et nous pouvons distinguer dans ces catégories d'additifs, les adsorbants et les micro-organismes/enzymes.

Les adsorbants sont des molécules permettant de séquestrer les mycotoxines et ainsi d'éviter l'absorption intestinale des toxines. Le complexe adsorbant-toxine est finalement excrété *via* les fèces de l'animal. On distingue deux classes d'adsorbants, inorganiques et organiques. La première classe concerne essentiellement les argiles, tels que les aluminosilicates de sodium ou les bentonites, qui sont largement utilisés contre les aflatoxines. Les ligands organiques sont plus nombreux, tels que le charbon activé, les polymères ou les parois de levures et de bactéries.

La détoxification biologique par des micro-organismes entiers ou des enzymes purifiées repose sur une dégradation par voie enzymatique des mycotoxines dans le système digestif de l'animal, et aboutit à des métabolites moins ou non toxiques. De nombreux micro-organismes (incluant les levures, bactéries et champignons) ont été isolés à partir du sol, de compost, de rumen, ou encore d'insectes, et ont été screenés pour leur capacité à modifier ou inactiver les mycotoxines.

Mycotoxin Reduction in Animals – Adsorption and biological detoxification

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ABSTRACT

Mycotoxins are toxic chemical products formed by fungal species that colonize crops in the field or after harvest and thus pose a potential threat to human and animal health. Even though recommended agricultural practices have been implemented to decrease production of mycotoxins during crop growth, harvesting and storage, the potential for significant contamination still exists. The significance of these unavoidable, naturally occurring toxicants to human and animal health, the increase in mycotoxin regulations and global trans-shipment of agricultural commodities highlight the need to provide successful counteracting strategies. The approaches to this are varied and may be categorized as physical methods, chemical decontamination, treatments with adsorbents (organic or inorganic) and biological detoxification to decrease the bioavailability of mycotoxins to the host animal.

Certain treatments have been found to reduce levels of specific mycotoxins, however, no single method has been developed that is equally effective against the wide variety of mycotoxins which may co-occur in different commodities.

INTRODUCTION

Mycotoxin-producing fungi grow on staple raw materials used for animal feeds and human foods. Subsequently, mycotoxins must be considered persistent and common contaminants of feed and foods that world-wide affect both, animal and human health (Adams, 2004; Galvano *et al.*, 2005). Extreme vigilance and careful management is vital to ensure that levels of mycotoxins are kept acceptably low (Adams, 2001).

The complex processes involved in mycotoxin production by moulds make it difficult to predict which toxin will be produced, when and in what concentration.

Unfortunately it is not possible to entirely prevent the production of mycotoxins before harvest of agricultural crops, in storage, or during processing operations. Thus, numerous strategies are evolving for the management and field-practical control of mycotoxins. Some are clearly more practical and effective than others.

A variety of physical, chemical and biological approaches to counteract the mycotoxin problem have been reported, but large-scale, practical, and cost-effective methods for a complete detoxification of mycotoxin-containing feedstuffs are currently not available. Substances used to suppress or reduce the absorption of mycotoxins by the animal should either promote the excretion of mycotoxins in feces (in case of binders) or modify their mode of action (in case of biological detoxifiers).

One of the most promising approaches to the problem has been the use of sorbents in the diet that sequester mycotoxins and reduce the absorption of these toxins from the gastrointestinal tract, avoiding the toxic effects for livestock and the carryover of these fungal metabolites into animal products. For an adsorbent to successfully prevent the absorption of mycotoxins from the gastrointestinal tract, it should have a high binding capacity and affinity for the mycotoxin, avoid dissociation and thus reduce the bioavailability. Adsorbents that may appear effective *in vitro* do not necessarily retain their efficacy and safety when tested *in vivo*.

Besides, biological detoxification has become stronger and more important during the last decade and it is nowadays an emerging area of interest and research in animal feed production. Isolation and characterization of microorganisms or enzymes that are able to biotransform mycotoxins can possibly be the breakthrough for the practical application of biotechnology in specific decontamination processes taking place directly in the intestinal tract of animals. This biological decontamination may become a technology of choice, as enzymatic reactions offer a specific, efficient and environmentally friendly way of detoxification.

Independent of the kind of product one of the main targets is to evaluate efficacy in multi-contamination conditions which is closer to reality.

Thus, this chapter comments on the main binders used in mycotoxin adsorption, their *in vitro* efficacy and *in vivo* applications in a range of animals. Moreover, the biological detoxification is described and presented as a promising strategy in counteracting mycotoxins that cannot be inhibited with inorganic and organic adsorbents.

I. ADSORPTION OF MYCOTOXINS

The ever-increasing number of reports on the presence of mycotoxins in foods and feed dictates the necessity for practical and economical deactivation procedures.

The high costs and limitations of physical and chemical treatments prompted a search for other solutions to the mycotoxin hazard. Up to now, the most widely investigated method in this field is the addition of nutritionally inert sorbents with the capacity to tightly bind and immobilize mycotoxins in the gastrointestinal tract of animals, resulting in a major reduction in toxin bio-availability.

1) Use of inorganic adsorbents

In several scientific studies clay and zeolitic minerals, hydrated sodium calcium aluminosilicates (HSCAS) and other similar montmorillonite and bentonite clays have proven to be the most promising adsorbents for aflatoxins (AF) (Grant and Phillips, 1998; Ramos and Hernandez, 1996; Phillips *et al.*, 1988, 1994; Vekiru *et al.*, 2007). Mixed into feed they markedly diminished AF uptake by the blood and distribution to target organs, thus avoiding aflatoxin-related diseases and the carryover of aflatoxins into animal products such as milk (Phillips *et al.*, 1990 and 1991). These binders have the property of adsorbing organic substances either on their external surfaces or within their interlaminar spaces, by the interaction with or substitution of the exchange cations present in these spaces. However, clay and zeolitic minerals, which comprise a broad family of diverse aluminosilicates, are not produced equally thus do not possess the same physical properties.

While good and scientifically explained results were obtained in counteracting aflatoxins, adsorption of other mycotoxins (e.g. zearalenone, ochratoxin A, fumonisins) was limited or even failed under field conditions (e.g. trichothecenes such as deoxynivalenol, T-2 toxin) (Friend *et al.*, 1984; Kubena *et al.*, 1990; Huff *et al.*, 1992; Kubena *et al.*, 1993; Ramos *et al.*, 1996).

a) Hydrated sodium calcium aluminosilicate (HSCAS)

HSCAS is a phyllosilicate clay. Its discovery as an effective enterosorbent of **aflatoxins**, its chemical composition and its mechanism of aflatoxin sorption at interlayer surfaces is described in numerous scientific publications by the T.D. Phillips group. The stability of the aflatoxin-HSCAS complexes may explain the *in vivo* effectiveness of the adsorbent in preventing the toxic effects of aflatoxins in several animal species (Pettersson, 2004).

Since early studies, HSCAS has been reported to diminish the effects of aflatoxins in a variety of young animals including rodents, chicks, broilers, turkey poults, ducklings, lambs, pigs, minks and

Table 8 : Outcome of HSCAS effects in AFB₁ contaminated diets in different species

Animal species	AFB ₁ concentration	HSCAS concentration	Outcome of HSCAS effects	References
Chickens	1000 ppb	0.5%	Performance: improvement of feed intake and body weight Organs: reduction of toxic effects on liver weight, no cell damage and decline in the severity of lesions in liver Systemic response: positive effects on serum chemical parameters	Gowda <i>et al.</i> 2008
	4000 ppb	1%	Performance: improvement of feed intake and body weight Organs: protection against changes in organ weights (liver, kidney, proventriculus and pancreas) and decline in the severity of lesions Systemic response: positive effects on serum chemical parameters	Ledoux <i>et al.</i> 1998
	7500 ppb	2.5 g/kg	Performance: improvement of feed intake and body weight Organs: reduction of toxic effects on liver weight and decline in the severity of lesions in liver Systemic response: no data	Dixon <i>et al.</i> 2008
	7500 ppb	0.5%	Performance: decrease the growth inhibitory effect Organs: protective effects on gross hepatic changes Systemic response: no data	Phillips <i>et al.</i> 1988
	2500-5000 ppb	0.5%	Performance: decrease the growth inhibitory effect Organs: reduction of toxic effects on liver, kidney and proventriculus weights Systemic response: protection against changes in serum biochemical parameters	Kubena <i>et al.</i> 1993
	5000 ppb	0.375%	Performance: improvement in body weight gain Organs: reduction of toxic effects on liver, kidney, pancreas and proventriculus weights Systemic response: protection against changes in serum biochemical parameters	Kubena <i>et al.</i> 1998
Turkey poults	500-1000 ppb	0.5%	Performance: 68% decrease in mortality, improvement in body weight gain Organs: protection against changes in organ weights Systemic response: improvement in hematological and biochemical parameters	Kubena <i>et al.</i> 1991
Pigs	840 ppb	0.5%	Performance: decrease the growth inhibitory effect Organs: improvement in liver functions	Lindemann <i>et al.</i> 1993

			Systemic response: restored cell-mediated responsiveness	
	3000 ppb	0.5 %	Performance: improvement in body weight Organs: no data Systemic response: positive effect on lymphocytes proliferation, on macrophage activity and function	Harvey <i>et al.</i> 1994
Rats	2500 ppb	0.5 %	Performance: no data Organs: any microscopic lesions observed in liver and in kidney Systemic response: improvement in hematological and biochemical parameters	Abdel-Wahhab <i>et al.</i> 2002
	2500 ppb	0.5%	Performance: improvement of feed intake and body weight, ability of reproduction warranted Organs: no data Systemic response: positive effects on serum chemical parameters	Abdel-Wahhab <i>et al.</i> 1999
Lambs	2600 ppb	2.0%	Performance: improvement in body weight Organs: no data Systemic response: positive effects on serum biochemical parameters	Harvey <i>et al.</i> 1991
Minks	34-102 ppb (AF B ₁ + B ₂)	0.5%	Performance: prevention of mortality, improvement of feed intake and body weight Organs: decline in the severity of lesions in liver Systemic response: no data	Bonna <i>et al.</i> 1991

Chickens included broiler chicks, laying hens.

Rats included male and female.

Pigs included piglets, sow, barrow.

trouts (**Table 8**). Levels of aflatoxin M₁ in milk from lactating dairy cattle and goats decreased with HSCAS clay presence (CAST, 2003) and, also, when present at 0.5% in the diet it significantly reduced aflatoxins (M₁ + B₁ + B₂) in liver, kidney, muscle and fat (Beaver *et al.*, 1990).

Several authors found limited effects of HSCAS against zearalenone and ochratoxin A and proved even their ineffectiveness against trichothecenes (Huwig *et al.*, 2001; Satin *et al.*, 2002a; Santin *et al.*, 2002b; CAST, 2003; Pettersson, 2004). This can be explained by the HSCAS-mycotoxin-binding mechanism: a β -keto-lactone or bilactone system, which no other mycotoxins but aflatoxins have, is thought to be essential for chemisorption to HSCAS. Results have shown that **ochratoxin A (OTA)** in the diet impaired the productivity indexes (heavier kidneys, enlarged livers) and that HSCAS did not improve these parameters (Santin *et al.*, 2002b). Additionally, according to Bursian *et al.* (1992) HSCAS did not significantly alter the hyperestrogenic effects of **zearalenone (ZEA)**. The increase in gestation length, decrease in litter size and increase in kit mortality of mink were some of the effects reported.

Adding HSCAS at either 0.5 or 1.0% did not positively influence the average daily gain of pigs exposed to **deoxynivalenol (DON)** (CAST, 2003).

b) Bentonite (montmorillonite)

Bentonites are clay minerals which result from the decomposition of volcanic ash consisting mainly of phyllosilicate minerals belonging to the smectite group; in 70% of the cases this smectite is montmorillonite.

Promising *in vitro* results were obtained with bentonites concerning effective adsorption of aflatoxins, fumonisins and ergot alkaloids (ergin, ergotamine, ergovalin). Vekiru *et al.* (2007) proved their high aflatoxins adsorption capacity and affinity. Bentonites were also proven to be able to adsorb fumonisins at an extent of 90% in a complex media such as gastrointestinal fluid (CAST, 2003). According to Lindemann *et al.* (1993) and Schell *et al.* (1993b), an inclusion rate of 0.5% sodium or calcium bentonite seemed to be beneficial in restoring growth performance and serum profiles of growing pigs fed diets containing 800 ppb of **aflatoxin B₁**. Further feeding trials suggested that bentonites and thus montmorillonite can effectively reduce the toxicity of aflatoxins in broiler chicks, poultry and pigs, improve growth performance, average daily weight gain, average daily feed intake, feed conversion ratio and reduce the negative immunosuppressive effect (Ibrahim *et al.*, 2000; Rosa *et al.* 2001; Gupta and Gardner, 2005; Shi *et al.*, 2006; Yu *et al.*, 2008). The addition of montmorillonite in aflatoxin-contaminated diets diminished the adverse effects on relative organ weights, hematological, serum and liver biochemical values as well as enzymatic activities associated with aflatoxicosis (Shi *et al.*, 2006).

In studies of the potential of bentonites to reduce the carry-over of aflatoxins into milk of dairy cows variable reductions have been observed (Veldman, 1992). Petterson (2004) thus concluded that it will be hazardous to trust the efficiency of these adsorbents at the low EU tolerance limits for aflatoxins in feed and milk. However, significant reduction of milk aflatoxin M₁ content was verified by Pietri *et al.* (2009) using a blend of bentonites. Regarding **trichothecenes**, in 1983 Carson and Smith observed general improvements in performance parameters and an increase in the toxin excretion in rats fed a diet with **T-2 toxin** and bentonite incorporation. However, these results could only be achieved when the incorporation level in the diet was about 10-20 times higher (10 %) than the efficient level for aflatoxins.

Contradictory, bentonites have shown low or no effect against **zearalenone** and although they may be useful in situations where moulds cause non-specific unpalatability, bentonites appear ineffective where feed refusal is due to direct effects on appetite as appears to be the case with trichothecenes such as **nivalenol** (Williams *et al.* 1994; Guerre, 2000).

c) Zeolite

Zeolites, other aluminosilicate clays, are similar to molecular sieves as well as to ion exchange resins and are suitable for the distinction of different molecules by size, shape and charge. Depending on dosage, physical structure, type of zeolite and respective concentration of aflatoxins in the contaminated feed, the ability of zeolites in reducing their toxicity can differ widely.

Zeolites have been studied as adsorbents for **aflatoxins**. They normally have less capacity than HSCAS or bentonites to adsorb aflatoxins *in vitro*, but showed some *in vivo* efficacy under practical conditions (Pettersson, 2004). Natural zeolite (1% in the diet) given to broilers consuming 2.5 ppm AFB₁ has alleviated the growth depression and reduced the increase in liver lipid concentration caused by aflatoxins (Scheideler, 1993). Indeed, several studies with natural zeolite, the clinoptilolite, which is frequently added to animal feed, confirmed improvement on performance, decline of liver and kidney lesions, heterophilia and lymphopenia reduction and improvements on humoral immunity (Sova *et al.*, 1991, Parlat *et al.*, 1999; Oguz and Kurtoglu, 2000; Oguz *et al.*, 2000; Oguz *et al.*, 2003; Ortatatli *et al.*, 2001; Ortatatli *et al.*, 2005).

However, other studies reported contradictory results being ineffective and causing organ lesions (Mayrúa *et al.*, 1998; Harvey *et al.*, 1993). Incorporation of a synthetic anion-exchange zeolite into rat diets at 5% completely eliminated the deleterious effect of **zearalenone** on body weight, feed consumption and feed efficiency. In opposite, the use of a synthetic cation-exchange zeolite was not able to protect rats against ZEA toxicosis (Smith, 1980), presenting higher uterus weight in piglets fed

diets containing ZEA and zeolite than in piglets fed diets with ZEA and without the zeolite (Guerre, 2000; Coenen and Boyens, 2001).

This observation supported the hypothesis that an anion-exchange zeolite, which together with zearalenone is anionic at intestinal pH, treated zearalenone toxicosis by reducing absorption of the toxin and increasing excretion of zearalenone in feces (Ramos, 1996a).

d) Other clays

To some extent other clays such as diatomaceous earth have also been studied for their ability to bind mycotoxins *in vitro* and *in vivo*.

Some commercial products have been reported to have positive effects against **aflatoxins** in broilers (Denli *et al.*, 2009) and **ochratoxin A** in hens leading to an increase in egg albumen height, redness color of egg yolk and serum calcium concentration (Denli *et al.*, 2008).

e) Nutrient interactions in animals

The adsorbents' activity has raised many questions regarding their influence on the utilization of nutrients such as carbohydrates, proteins, vitamins and minerals and controversy results have been reported.

No nutrient interactions have been reported with HSCAS at the 0.5% level in the diet (Phillips, 1999); it did not impair phytate or inorganic phosphorous utilization. In other studies, the addition of HSCAS to basal diets at concentrations of 0.5% or 1% did not impair the utilization of riboflavin, vitamin A or manganese; however there was a slight but significant reduction in zinc utilization in the presence of 1% clay (Chung *et al.*, 1990). According to Schell *et al.* (1993a) significant interactions for many minerals indicated that the effects on mineral metabolism were more pronounced when aflatoxin-contaminated corn was fed. Feeding bentonite with aflatoxin-contaminated corn resulted in partial restoration of performance and liver function without greatly influencing mineral metabolism (Schell *et al.*, 1993a). Nonetheless, zeolites seemed to have a significantly negative effect on vitamin and mineral uptake and on their distribution in the body of sows (Papaioannou *et al.*, 2002). Thus, one of the most important criteria to take into account when evaluating a potential binder is the absence of affinity for vitamins, minerals or other nutrients (Huwig *et al.*, 2001).

2) Use of organic adsorbents

Substances investigated as potential organic mycotoxin-binding agents include among others activated charcoal, alfalfa, canola oil bleaching clays, organic polymers such as cholestyramin, polyvinylpyrrolidone (PVPP), yeast cell walls and components thereof as well as bacterial cells.

a) Activated charcoal

Activated charcoal is known as one of the most effective and non-toxic, but also most unspecifically acting group of sorbents with a high surface to mass ratio (500-3500 m²/g). They are formed by pyrolysis of several organic compounds. They can efficiently adsorb most of the mycotoxins in aqueous solution, whereas different activated charcoals have less or even no effects against mycotoxicosis (Kolossova *et al.*, 2009).

Activated charcoal has been investigated for its ability to adsorb mycotoxins both, *in vitro* and *in vivo*, being verified to adsorb zearalenone, deoxynivalenol and nivalenol *in vitro*, using a gastrointestinal model, and aflatoxins, ochratoxin A and fumonisins rather efficiently *in vivo* (CAST, 2003, Pettersson, 2004; Kolossova *et al.*, 2009).

In vitro **aflatoxin** adsorption tests with different activated charcoals showed good results at different pH values (Huwig *et al.*, 2001). *In vivo*, certain granulated activated carbons (GACs) decreased conversion of aflatoxin B1 to aflatoxin M1 in Friesian cows by 40.6 to 73.6% when included in the diet at concentrations of 2.0% (CAST, 2003). Improvements in body weight gains and feed intake of chickens were verified when activated charcoal was added to AF-contaminated diets (Edrington *et al.*, 1997; Dalvi and Ademoyero 1983; Dalvi and McGowan 1984 and Jindal *et al.*, 1993). However conflicting results have been verified, possibly due different types of activated charcoals used.

Successful *in vitro* **OTA** adsorption tests with activated charcoal showed that 1% product addition leads to complete adsorption of ochratoxin A from aqueous solutions not being influenced by pH-values ranging from 3-8 (Plank *et al.*, 1990).

In addition, some trials have been performed with superactivated charcoal which differs from activated charcoal by its reduced particle size, higher surface area and a chemical modification during the manufacturing process. Superactivated charcoal was evaluated for its effectiveness in preventing death in rats given an oral lethal dose of 8 mg/kg body weight of **T-2 toxin**. The median effective dose of oral superactive charcoal in preventing deaths in rats was found to be 0.175 g/kg body weight. At the end of the trial it was concluded that one gram per kilogram body weight oral superactive charcoal enhanced survival times and survival rates in rats given T-2 toxin (Galey *et al.*, 1987). *In vivo*, orally administered activated charcoal was assessed for treatment of acute oral or parenteral exposure to T-2 toxin in mice. Charcoal treatment (7 g/kg) either immediately or 1 hr after

toxin exposure resulted in a significant improvement of survival rate with values of 100% and 75%, respectively (Fricke *et al.*, 1990). This is also in agreement with Bratich *et al.* (1989) who verified no lesions in rats fed diets contaminated with about 25 mg/kg T-2 toxin.

No adsorbent materials, with the exception of activated carbon, showed relevant ability in binding **deoxynivalenol** and **NIV** (Binder, 2007). At 2% inclusion level the absorption with respect to the intake was lowered from 51% to 28% for DON and from 21% to 12% for NIV (Avantaggiato *et al.*, 2004). However, the binding activity of activated carbon for these trichothecenes was lower than that observed for **zearalenone** (Avantaggiato *et al.*, 2004).

In vitro efficacy of activated carbon toward **fumonisin**s was not confirmed *in vivo* using respective biomarkers (Binder, 2007).

In a study of Vekiru *et al.* (2007) activated charcoal was investigated with regard to aflatoxin binding and adsorption of vitamin B5 (Panthothenic acid), B12 and vitamin H (biotin). These water-soluble vitamins play an important role in animal growth and productivity. Since they are not stored in the tissues in appreciable amounts they have to be regularly added to animal diets. When compared to other binders, charcoal presented a very high unspecific binding, with a high adsorption of nutrients. Charcoal absorbed 100% AFB1 and extremely high amounts of vitamin B12 (99%) and biotin (78%).

b) Cholestyramine

Cholestyramine is an anion exchange resin pharmaceutically used to decrease cholesterol. It has been shown to efficiently bind zearalenone, ochratoxin A and fumonisins *in vitro* and *in vivo* (CAST, 2003; Pettersson, 2004; Binder, 2007). Only a small number of adsorbent materials possessed the ability to bind more than one mycotoxin.

In *in vitro* tests, cholestyramine has shown to be the best adsorbent for **zearalenone** followed by crospovidone, montmorillonite, bentonite, sepiolite and magnesium trisilicate (Ramos *et al.*, 1996a). ZEA was tested in experiments using a dynamic gastrointestinal model. It is known that formation of cholestyramine-ZEA complexes occurs rapidly (within 1 min) being stable for 24 h and is not influenced by pH or temperature (Ramos *et al.*, 1994).

Studies carried out in mice and rats fed diets containing different amounts of cholestyramine resulted in a reduction of estrogenic effects, toxin urinary excretion as well as renal and hepatic residues and also in a reduction of 19 to 52% of intestinal absorption of ZEA (Underhill *et al.*, 1995; Guerre, 2000; Avantaggiato *et al.*, 2003; CAST, 2003).

Table 9 : Outcome of yeast cell walls effects in mycotoxin contaminated diets in different species

Animal species	mycotoxin concentration	Additives concentration	Outcome of feed additive effects	References
Chickens	AF (5 ppm)	0.05-0.1%	Performance: improvement in body weight Organs: restoration of relative weights of liver, proventriculus and heart Systemic response: positive effects on serum biochemical parameters	Stanley <i>et al.</i> (1993)
Chickens	AF (2 ppm)	0.05-0.1%	Performance : no data Organs : decline in the number and severity of pathological lesions in liver, bursa of Fabricius, thymus and spleen Systemic response : no data	Karaman <i>et al.</i> (2005)
Chickens	AF (2 ppm)	0.075 %	Performance: no data Organs: Subacute dietary intake of AF altered nonenzymatic components (CP, alb, vit A, â-carotene, Vit E) of antioxidant defense systems. Systemic response: yeast glucomannan was not sufficient to ameliorate the oxidative damage	Çinar <i>et al.</i> (2008)
Chickens	T-2 (8.1 ppm)	0.1%	Performance: no data Organs: partial protection of the feed additive alone and further protection in combinaison with organic selenium against antioxidant depletion and lipid peroxydation in liver Systemic response: no data	Dvorska <i>et al.</i> (2007)
Pigs	T-2 (up to 2.1 ppm)	0.2%	Performance: no difference on performance whatever the diets Organs: no lesions were observed regardless of T-2 toxin dose or glucomannan supplementation Systemic response: protective effect against AFB ₁ immunotoxicity during the vaccinal response	Meissonnier <i>et al.</i> (2009)
Pigs	DON (4.44 ppm)	0.2 %	Performance: Feeding of the mycotoxin-contaminated diets resulted in a decrease in feed intake and live weight gain by 28% and 14% when compared to the control group. Organs: no data Systemic response: The addition of glucomannan mycotoxin adsorbent to piglet	Dänicke <i>et al.</i> (2007)

			diets which are mainly contaminated with DON could not be recommended	
Cows	AF (100 ppb)	1%, 5%	Performance: evaluation of milk aflatoxin concentrations in lactating Holstein cows with the inclusion of non-digestible yeast oligosaccharides Organs: no data Systemic response: No significant changes ($P>0.25$) in AFM ₁ concentrations in response to non-digestible yeast oligosaccharides were verified.	Hopkins <i>et al.</i> (2008)
Cows	AFB ₁ (112 ppb)	0.56%	Performance: reduction of AFM ₁ concentrations in dairy cows Organs: no data Systemic response: the commercial product was not effective in reducing milk AFM ₁ concentrations (4%), AFM ₁ excretion (5%), or AF transfer from feed to milk (2.52%)	Kutz <i>et al.</i> (2009)

Chickens included broiler chicks, laying hens.

Rats included male and female.

Pigs included piglets, sow, barrow.

Furthermore, cholestyramine has shown to increase **ochratoxin A** excretion in rats (Madhyasta *et al.*, 1992), to inhibit the enterohepatic circulation of OTA in mice (Roth *et al.*, 1988) and to reduce OTA absorption in the gastrointestinal tract of rats (Kerkadi *et al.*, 1998).

However, cholestyramine demonstrated just a little effect on the reduction of ochratoxin A concentration in blood, bile and tissues (Huwig *et al.*, 2001).

Several adsorbent materials were tested for their *in vitro* capacity to adsorb **fumonisin B₁** and cholestyramine showed to be an effective binder with best adsorption capacity (Solfrizzo *et al.*, 2001; Avantaggiato *et al.*, 2005).

c) Polyvinylpolypyrrolidone (PVPP)

Adsorption of mycotoxins by PVPP is based on the hydration hull formed by the adsorbent. Particles must be polar to be attracted towards this hull and consequently to be bound to the adsorbent. PVPP reduces the toxicity of **aflatoxins** by decreasing its absorption in the gastrointestinal tract thus preventing negative effects on aflatoxicosis (Pasteiner *et al.*, 1994 and Kiran *et al.*, 1998). In fact, results of broiler chickens fed a contaminated diet with 2.5 ppm AF and PVPP (3 g/kg diet) suggested a prevention of depressive effects on peritoneal macrophage functions (Celik *et al.*, 1996) and also some immune organ functions (Celik *et al.*, 2000).

In vitro 0.3 mg/g **zearalenone** were adsorbed by PVPP. Regarding respective *in vivo* efficacy, however, this polymer has rarely been tested. Pettersson (2004), Friend *et al.* (1984) and Ramos (1996) did not find any effects of PVPP in pigs fed **deoxynivalenol**-contaminated feed.

d) Yeast cell walls and components thereof (Table 9)

Yeasts, and particularly the cell wall of *Saccharomyces cerevisiae*, are an environmentally friendly alternative to inorganic adsorbents, which are not extensively biodegradable and are associated with the risk of contaminants (Yiannikouris *et al.*, 2007).

The structure of the cell wall of *S. cerevisiae* and the nature of its polysaccharide (glucan, mannan) components was subject of intensive scientific research, however, their role in the adsorption of mycotoxins is a concept that has been little studied and has shown conflicting results.

The walls of yeast are used to form complexes with dietary toxins which limit their absorption in the digestive tract and consequently their negative impact on the animal and the quality of animal products used in food. Their ability to bind is due to their large surface of exchange. Recently, Yiannikouris *et al.* (2004; 2006) demonstrated that the β -D-glucan fraction of yeast cell wall is directly

involved in the binding process of mycotoxins and that the structural organisation of β -D-glucans modulates the binding strength (Jouany, 2007).

Beneficial effects were verified in counteracting aflatoxins in several animal species with live yeast culture preparations based on a *Saccharomyces cerevisiae* strain. Yeast glucomannan showed binding ability for various mycotoxins *in vitro and/or in vivo* (Stanley, 2004; Meissonnier *et al.*, 2009, Pettersson, 2004). A binding capacity trial for **T-2 toxin** concluded that supplementation of modified glucomannan at 1 kg/t of feed is beneficial in preventing the absorption of T-2 toxin. Up to 35% T-2 binding capacity was observed in the gastro-intestinal tract of broilers (Reddy *et al.*, 2003). Contrary, yeast glucomannan investigated by Cinar *et al.* (2008) was not sufficient to prevent or ameliorate the oxidative damage caused by **aflatoxins** in broilers.

An experiment, conducted to determine the effect of non-digestible yeast oligosaccharides (NYO) on milk aflatoxin concentrations in lactating Holstein cows consuming aflatoxins, concluded that there were no significant ($P>0.25$) changes in AFM₁ concentrations in response to NYO (Hopkins *et al.*, 2008). These results are in accordance with a study of Stroud *et al.* (2006).

Several studies have been carried out in order to assess the efficacy of yeast glucomannans against multi-mycotoxin contaminations. *In vitro* mycotoxin binding capacity by a commercial esterified glucomannan on **aflatoxins, ochratoxin A and T-2 toxin** was performed by Raju and Devegowda (2002). Results showed that the binding values of the mycotoxins decreased significantly when they were present in combination. The cumulative binding of the mycotoxins by the product was dependent on the mycotoxin present.

Esterified glucomannan (0.05-0.1%) in chickens reduced or eliminated many of the combined toxic effects (on performance, serum biochemistry and haematology) of AF, OTA and T-2 toxin (Raju and Devegowda, 2000; Aravind *et al.*, 2003). Using 0.5% of the same product also alleviated growth depression in broilers associated with naturally contaminated diets containing **aflatoxins, ochratoxin A, zearalenone and T-2 toxin** (Bursian *et al.*, 2006). According to a pig trial carried out by Meissonnier *et al.* (2009) glucomannan dietary supplementation demonstrated protective effects against **AFB₁ and T-2 toxin** immunotoxicity. According to Bursian *et al.* (2004), however, a glucan polymer product (0.2%) did not alleviate the toxic effects on mink consuming diets contaminated with **fumonisin B₁, ochratoxin A, moniliformin and zearalenone**.

e) Adsorption by bacteria

Until now, most of the research regarding the use of bacteria as adsorbents has been focused on *in vitro* tests, and only a few studies have been performed *in vivo*. Trials with some dairy strains of lactic acid bacteria and bifidobacteria showed that they are able to effectively bind **aflatoxins**. Cell

wall peptidoglycans and polysaccharides have been suggested to be the two most important elements responsible for the binding by lactic acid bacteria. According to Haskard *et al.* (2001) and Peltonen *et al.* (2001), *Lactobacillus amylovorus* strains and *Lb. rhamnosus* strains removed more than 50% AFB₁.

Furthermore, probiotics such as *Lactobacillus rhamnosus* strain GG and a mixture of *Lactobacillus rhamnosus* LC705 and *Propionibacterium freudenreichii* ssp. *shermanii* JS (LC705+JS) were investigated in order to know whether these probiotic bacteria bind AFB₁ under physiological conditions in the gut. Results concluded that both probiotic preparations were able to bind AFB₁ *in vitro* (Gratz, 2007). Studies carried out by Pierides *et al.* (2000) suggested that six dairy strains of lactic acid bacteria can reduce AFM₁ content in liquid media. Their ability to bind AFM₁ gave a potential approach for either decontamination of foods and feeds or reduction of the bioavailability of aflatoxins in the diet, presuming that the bacteria are able to remove the toxin from the gastrointestinal tract.

Several successful studies were conducted with bacteria regarding binding of **zearalenone**. The interaction between two *Fusarium* mycotoxins, zearalenone and its derivative zearalenol with two strains of *Lactobacillus* was investigated by El-Nezami *et al.* (2002b). After mycotoxin incubation (2 mg/ml) with two strains of *Lb. rhamnosus*, approximately 55% of the toxins were bound instantly after mixing with the bacteria. The mechanism linking these toxins to the surface of bacteria was shown not to be due to metabolic activity of living cells. In addition, removal of ZEA from medium by five *Propionibacterium*, three *Lactobacillus* and two *Bifidobacterium* strains has shown to be possible. All examined strains caused a decrease of toxin concentration in medium after incubation with both, viable and nonviable cells. Usually higher efficiency was observed with *Propionibacteria* (El-Nezami *et al.*, 2002a; Gwiazdowska *et al.*, 2006). *Lactobacillus* and *Propionibacterium* also removed **trichothecenes** (DON, 3-AcDON, NIV, FX, DAS, T-2) from liquid media (El-Nezami *et al.*, 2002a).

A trial conducted by El-Nezami *et al.* (2000) indicated that probiotic lactic acid bacteria are capable of binding AFB₁ under *in vivo* conditions. They observed that in the presence of *Lb. rhamnosus* GG 74% of the reduction in the uptake of AFB₁ by the intestinal tissue takes place within 60 min. Also, probiotic *Lb. rhamnosus* GG administration in rats demonstrated that fecal AFB₁ excretion in GG-treated rats was increased via bacterial AFB₁ binding and that AFB₁-associated growth faltering and liver injury were alleviated with GG treatment (Gratz *et al.*, 2006).

f) Other organic adsorbents

Other organic adsorbents such as humic acid, divinylbenzene-styrene, chlorophyllin, alfalfa and fibers were studied. Van Rensburg (2006) investigated oxihumate (pure, high quality humic acid)

which slightly differs chemically from humic acids obtained from other sources. Efficient results were obtained in broiler chickens fed aflatoxin-contaminated diets and it was proven that oxihumate is highly effective in the amelioration of aflatoxicosis in broilers. Furthermore, divinylbenzene-styrene showed to reduce the absorption and the toxic effects of ZEA (Guerre, 2000) and T-2 toxin in rats (Carson and Smith, 1983) when using anion-exchange and not cation-exchange resins. This anion exchange resin has been shown to prevent zearalenone toxicosis by binding the toxin in the digestive tract to prevent absorption (Smith, 1980). Little information is available concerning chlorophyllin (CHL) binding capacity. Breinholt *et al.* (1995) supported that CHL is a potent dose-responsive inhibitor of aflatoxin I, DNA adduction and hepatocarcinogenesis in the rainbow trout.

Furthermore, Jouany (2007) indicated that alfalfa and oat fibers are capable of reducing the estrogenic effect of ZEA on rats and bleaching clays that had been used to process canola oil were found to lessen the effects of T-2 (CAST, 2003).

A trial conducted with rats and swine to determine the potential of dietary alfalfa as a treatment for zearalenone toxicosis showed that alfalfa inclusion in diet reduced the inhibitory effects of ZEA on growth and feed consumption, minimized ZEA-induced liver enlargement, increased hepatic 3 α -HSD activity, reduced concentrations of residual ZEA liver and decreased uterine enlargement. These studies proved that dietary alfalfa promotes ZEA metabolism in rats and that this feedstuff may also be useful for treating ZEA toxicosis in livestock (James and Smith, 1982).

Besides micronized wheat fibers (MWF) were evaluated to decrease the levels of ochratoxin A in plasma, kidney and liver of piglets fed a naturally contaminated diet. Results demonstrated a significantly protective effect against usual increased kidneys and liver weights caused by OTA-contaminated diets. MWF significantly protected against high OTA concentration in plasma (45.6% decrease), kidney (40.8% decrease) and liver weights (26.5% decrease). Thus, these results suggest that the addition of MWF is effective in decreasing the bioavailability of OTA from contaminated diets in piglets (Aoudia *et al.*, 2009).

II. BIOLOGICAL DETOXIFICATION

Enzymatic or microbial degradation of mycotoxins (“biotransformation”) leading to less- or even non-toxic metabolites has already been a subject of research for more than 40 years as this strategy is a quite attractive method for the decontamination of crops. Microorganisms including yeast, moulds and bacteria have been screened for their ability to modify or inactivate different mycotoxins. Only a few microorganisms with respective activity were isolated, the first was *Flavobacterium aurantiacum* with the ability to detoxify **aflatoxins** (Ciegler *et al.*, 1966). This organism has since then been studied extensively for possible degradation products (Bata and Lasztity, 1999). Apart from *F. aurantiacum*, a number of bacterial and especially fungal species have been found to detoxify aflatoxins (Karlovsky, 1999). *Rhizopus sp.* has been claimed to be particularly suitable for large-scale detoxification of aflatoxin-contaminated feeds by solid-state fermentation (Knol *et al.*, 1990, as cited by Karlovsky, 1999).

Ochratoxin A is rapidly degraded by micro-organisms in the rumen to ochratoxin α (OT α) and phenylalanine (Hult *et al.*, 1976; Kiessling *et al.*, 1984). Wegst and Lingens (1983) proved degradation of OTA by the aerobic bacterium *Phenyllobacterium immobile*. Cheng-An and Draughon (1994) have screened bacteria, yeast and moulds for their ability to detoxify ochratoxin A and found *Acinetobacter calcoaceticus* to be able to degrade OTA in an ethanol-containing medium. Different strains of *Lactobacillus*, *Bacillus* and *Saccharomyces* have also been shown to degrade ochratoxin A *in vitro* to varying degrees up to 94 % (Böhm *et al.*, 2000). The same strains were also tested for degradation of trichothecenes, but with less success. Styriak *et al.* (2001) showed partial degradation of ochratoxin A, nivalenol, deoxynivalenol, zearalenone and fumonisins by yeast strains.

A yeast strain isolated from the hindgut of a termite and identified as a member of the genus *Trichosporon* showed a potential deactivation of both, OTA and ZEA, in animal feeds (Molnar *et al.*, 2004; Schatzmayr *et al.*, 2006). Due to the yeast’s main property to degrade mycotoxins this strain was named *T. mycotoxinivorans* (lat. vorare = degrade). The yeast detoxifies OTA by cleavage of the phenylalanine moiety from the isocoumarin derivate ochratoxin α (OT α). This metabolite has been described to be nontoxic or at least 500 times less toxic than the parent compound (Bruinink *et al.*, 1999; Schatzmayr *et al.*, 2003). Feeding trials with the aim to test the efficacy of *T. mycotoxinivorans* to suppress ochratoxicosis proved that the dietary inclusion of this yeast blocks ochratoxin-induced immune suppression in broiler chicks (Politis *et al.*, 2005; Binder, 2007). More recently, a feeding trial with broiler chicks was performed in order to evaluate the toxic effects of OTA and attenuating effects of a commercial toxin deactivator containing the yeast *Trichosporon mycotoxinivorans* on performance parameters, serum enzymes and clinic-pathomorphology of internal organs.

Improvement in FCR and less pronounced histological changes in kidneys, liver, bursa and spleen were verified in animals fed the toxin deactivator. Thus in the presence of this feed additive, the harmful effects in the pathomorphological and histological changes in internal organs were attenuated (Hanif *et al.*, 2008).

Zearalenone has no acute toxicity, but it mimics the reproduction hormone estrogen, and therefore causes substantial fertility problems. The metabolisation of this toxin by *T. mycotoxinivorans* leads to a compound that is no longer estrogenic. This has been proven in an *in vitro* assay with breast cancer cells (Schatzmayr *et al.*, 2003).

Reduction of zearalenone to α - and β -zearalenols has been shown in ruminal fluid and for many mixed and pure cultures of bacteria, yeast and fungi. However, this transformation cannot be considered as a detoxification as zearalenols still show significant estrogenic activity. Non-estrogenic ZEA-metabolites were obtained from degradation by e.g. *Thamnidium elegans*, *Mucor baineri*, *Rhizopus sp.*, *Streptomyces rimosus*, *Cunninghamella baineri* and *Gliocladium roseum* (Kamimura, 1986; El-Sharkawy and Abul-Hajj, 1987; 1988). The latter strain detoxified zearalenone by ring opening with subsequent decarboxylation in yields ranging between 80 and 90% (El-Sharkawy and Abul-Hajj, 1988).

A great deal of literature is available concerning the biotransformation of **trichothecenes**, which are among the world's most important agricultural toxins. Their toxicity can mainly be attributed to their 12,13-epoxide ring. Thus, reductive de-epoxidation by ruminal and intestinal flora of pigs, hens and rats (King *et al.*, 1984; Kollarczik *et al.*, 1994; Swanson *et al.*, 1987, 1988; Yoshizawa *et al.*, 1983) or by a new strain of *Eubacterium* isolated from bovine rumen contents (Binder *et al.*, 2000) results in a significant loss of toxicity. The latter bacterium referred to as BBSH 797 is actually the first bacterial strain cultured, produced and stabilized in order to be applied as feed additive to counteract the negative effects of trichothecenes by biotransformation. Fuchs *et al.* (2000; 2002) identified non-toxic metabolites after the microbial degradation of type A and type B trichothecenes by BBSH 797 and showed that de-acetylation occurs simultaneously to de-epoxidation when the strain was applied. The *in vivo* efficacy of the additive has been tested in trials with pigs and chickens. Significant feed gain and improved feed conversion ratios were determined in piglets fed 2.5 mg/l DON and the chickens fed 10.5 mg/l DON showed reduced mortality and a positive influence on weight development (Binder *et al.*, 2001; Plank *et al.*, 2009). Moreover, a BBSH 797-containing feed additive was capable of counteracting the adverse effects on performance of growing broiler chickens caused by the dietary administration of 2 ppm T-2 toxin (Diaz *et al.*, 2005).

Other investigations concerning microbial detoxification of trichothecenes concentrated on the use of aerobic soil bacteria. He *et al.* (1992) incubated soil with DON and measured a significant

decrease in toxin-concentration during incubation. Attempts to isolate a pure culture failed. In 1983, Ueno *et al.* isolated the *Curtobacterium sp.* (strain 114-2), a soil bacterium capable of deacetylating T-2 toxin to the less toxic metabolites HT-2 toxin and T-2 triol. Finally, in 1997, Shima *et al.* were successful in isolating a soil bacterium, belonging to the *Agrobacterium-Rhizobium* group, capable of biotransforming DON to the less-toxic metabolite 3-keto-DON. Protective effect of a feed additive against the toxic effects of 4,15-diacetoxisciepenol (DAS) in broiler chickens was also investigated. Results suggested that feed additive supplementation in the diet protected against the adverse effect of DAS on feed intake and body weight at different levels of inclusion.

Enzymes capable of degrading **fumonisin**s have been isolated from a filamentous saprophytic fungus growing on maize (Blackwell *et al.*, 1999; Duvik, 2001). Recently scientists isolated and characterized new fumonisin-metabolizing bacterial strains. Some of these isolates were found to be active in the gastrointestinal tract of animals. One of the strains with the highest technological potential belongs to the family of *Sphingomonadaceae*. It degrades fumonisins by first cleaving off tricarballic acid side chains and subsequently catabolising the rest of the molecule into non-toxic products (Moll *et al.*, 2009).

Moreover, biological detoxification was also tested in multi-mycotoxin contaminated feeds. Cheng *et al.* (2006) investigated the combined effects of **deoxynivalenol and zearalenone** on growth performance, blood biochemistry and immune response of weaning piglets and the alleviating effects of a mycotoxin degrading enzyme (MDE) on the toxicity of these *Fusarium* mycotoxins. Based on the results, it was suggested that the combination of DON and ZEA confers a chemical multi-organ toxicity in pigs and MDE provides a partial or complete toxic sparing effect of mycotoxins.

Biotechnology in animal feed production has become stronger and more important during the last decades. The uses of microbiological (enzymes and microbes) in feed have dramatically increased and are prime examples for the necessity of new approaches in animal production.

Isolation and characterization of microorganisms or enzymes that are able to biotransform mycotoxins could possibly be the breakthrough for the practical application of biotechnology in specific decontamination processes taking place directly in the intestinal tract of animals. This biological decontamination may become a technology of choice, as enzymatic reactions offer a specific, efficient and environmentally friendly way of detoxification.

Ensiling of mycotoxin-contaminated crops for detoxification has been proposed as an interesting and possible method for elimination or reduction of mycotoxins. Normal ensiling has, however, only rarely been studied for its mycotoxin degrading potential. A study by Rotter *et al.* (1990) showed that ensiling of ochratoxin-contaminated barley could reduce the toxin by approximately 68%. However,

in feeding studies with chicken, no improvement in performance or mortality could, be found compared with the non-ensiled diets. Yeasts in grass silage have been found to degrade patulin in silage inoculated with *Paecilomyces sp.* to induce patulin production (Dutton *et al.*, 1984). Both, bacteria and yeasts from maize silage have been shown to be able to degrade fumonisins (Camilo *et al.*, 2000). Stimulation of mycotoxin degradation by naturally occurring micro-organisms in silage or the addition of yeasts or bacteria with known mycotoxin degradation ability to silage may in the future become practical means to detoxify mycotoxins in certain crops.

CONCLUSION

Prevention and reduction of mycotoxin contamination during crop and feed production has become more important, good agricultural practices and HACCP in the production process has become more a requirement and the need of feed additives to prevent absorption and toxic effects of mycotoxins in farm animals has increased significantly.

Actually, there is already an excellent potential for organic and inorganic binders to help manage the mycotoxin problem in a safe, economic and easy way. Adsorbents can be used as a complement, acting in the animal's organism, to physical and chemical methods used mainly in the decontamination of raw materials.

Since feedstuffs are commonly contaminated with more than one mycotoxin, it is important to take into account that a good mycotoxin inactivator has to be as effective as possible against several mycotoxin contaminations, incorporated in a small amount in the complete diet, have a high binding capacity and be free of impurities and odors.

It is now known that adsorbents belonging to the same category have different physical and chemical properties, thus their efficacy can vary markedly. To assure efficiency and safety, all additives have to be carefully tested before coming into the market, being the *in vitro* tests mostly important for their scientific development and improvement. Moreover, sensitive parameters have to be measured such as biochemistry, gross pathology, histopathology and immune parameters.

As adsorption is not a viable option regarding trichothecenes, zearalenone and ochratoxins, mycotoxin inactivation by biotransformation is a very promising strategy to detoxify these mycotoxins. Indeed, biotransformation is currently the most recent and challenging process of mycotoxin deactivation being under development and therefore, requiring further investigations.

Another important target for the future is the development of products that are effective against multi-mycotoxin contaminations.

There are many factors that negatively influence and complicate the setting of safe levels for the different mycotoxins in different countries, however regulations exist worldwide and maximum levels for certain contaminants in foodstuffs were already set by EU legislation.

Currently, a large part of the feed industry has already applied for production standards, which are often stricter than required by legislation. The HACCP analysis of the production practices that is required to comply with the EU Feed Hygiene legislation, and the quality systems it triggered such as Fami-QS, is an obvious qualifier to operate in the European feed industry.

Mycotoxin contamination, however, still occurs despite the most strenuous efforts of prevention, therefore there is an obvious need for mycotoxin inactivating products to complement it in the

perspective of HACCP analysis conclusions. It is believed that mycotoxin inactivating products may reduce or even close the gaps left open by the introduction of maximum limits, preventive actions and analytical control.

TRAVAIL EXPERIMENTAL

OBJECTIFS DE LA THESE

Les mycotoxines présentent autant de propriétés toxiques qu'elles divergent structurellement. Néanmoins, ces substances ont quasiment toutes la capacité de moduler la réponse immunitaire de l'organisme, et ce, même lors de l'exposition à de faibles doses. Les mycotoxines peuvent agir sur l'expression et la fonction de différents médiateurs et cellules du système immunitaire, impliqués dans le système de défense de l'hôte, à savoir l'inflammation, la réponse à médiation cellulaire et la réponse à médiation humorale. En conséquence, il a été montré que ces effets immunotoxiques peuvent sensibiliser les animaux aux infections, diminuer l'efficacité vaccinale et thérapeutique, ou encore réactiver des infections.

Dans ce contexte, l'équipe d'immuno-mycotoxicologie au sein du pôle ToxAlim de l'INRA de Toulouse, s'intéresse à ces effets immunomodulateurs lors d'exposition à faible doses, et plus particulièrement après un challenge antigénique. La thématique de l'équipe s'est également orientée depuis peu sur l'effet des mycotoxines sur le tractus gastro-intestinal. En effet, la voie principale d'exposition aux mycotoxines étant l'ingestion d'aliments contaminés, l'intestin est un organe cible des mycotoxines, et peut être exposé à de fortes concentrations en toxines. Considérant les différentes fonctions du système digestif, telles que l'immunité, la perméabilité intestinale ou l'absorption des nutriments, nos recherches ont porté une attention particulière à certains de ces critères.

L'activité de la société BIOMIN est basée sur le développement de stratégies de lutte contre les mycotoxines majeures présentes dans les aliments. Contrairement à de nombreuses sociétés, qui élaborent des produits à base d'argile ou de levures permettant l'adsorption des mycotoxines, BIOMIN axe ses travaux sur l'élimination des toxines par voie enzymatique. Un long travail en amont d'isolement et de screening de micro-organismes, capable de détoxifier les mycotoxines, leur permet de proposer des solutions innovantes, et ont l'avantage de cibler spécifiquement chaque mycotoxine, considérant leur grande diversité structurelle. Leur intérêt dans ce partenariat résidait dans leur souhait d'avoir une expertise plus approfondie de l'efficacité de leurs produits, et ce, sur des paramètres plus fins que des paramètres zootechniques. De plus, une de leurs dernières approches, encore non-commercialisée, cible spécifiquement les fumonisines, mycotoxines difficilement éliminées par les méthodes physiques, chimiques et d'adsorption.

Ainsi, la thèse a comporté différents objectifs. Le premier a été de déterminer la toxicité individuelle et combinée de faibles doses de déoxynivalénol et de fumonisines (fumonisines B1 + B2) chez le porcelet. Pour cela, différents critères ont été évalués. L'impact des mycotoxines sur la

toxicité générale, telle que la performance, l'hématologie et la biochimie, et les organes cibles (foie, rein, poumon), a été analysé. L'impact sur la mise en place d'une réponse immunitaire spécifique ou acquise, suite à un challenge antigénique a été étudié. Nous avons pour cela intégré dans notre étude un protocole de vaccination avec un antigène de laboratoire, l'ovalbumine ; l'utilisation de cet antigène ayant déjà fait ses preuves dans des expériences antérieures. Et finalement, l'impact sur le compartiment intestinal, avec des analyses histopathologiques et immunologiques, a également été déterminé.

Le second objectif a été de comparer la toxicité de la fumonisine B1 (FB1), la mycotoxine prédominante des fumonisines, et de son dérivé hydrolysé (HFB1). En effet, BIOMIN a purifié une enzyme, une carboxylestérase issue d'une bactérie isolée de compost, douée de propriétés d'hydrolyse et permettant ainsi l'élimination des deux chaînes latérales d'acide tricarballyle de la FB1. Après hydrolyse totale de la FB1 par cette approche, le métabolite obtenu HFB1 a été confronté à la molécule mère FB1 (à une dose aigüe en comparaison de celle utilisée dans la première expérience) lors d'une expérience *in vivo* menée chez le porcelet. Les deux substances ont été administrées oralement aux animaux, et la toxicité a été déterminée au niveau hépatique et intestinal.

Enfin, l'efficacité d'un produit commercial développé par BIOMIN et combiné avec la carboxylestérase récemment purifié, a été évaluée lors de leurs incorporations dans des aliments contaminés en déoxynivalénol et fumonisines, et ingérés par des porcelets. Les mêmes paramètres que dans l'expérience de co-contamination décrite ci-dessus ont été étudiés.

Le porc a été notre modèle d'étude expérimental du fait de sa sensibilité, de son exposition naturelle aux mycotoxines via son régime alimentaire, et aussi de son degré de similitude avec les systèmes biologiques chez l'homme, notamment immunitaire et digestif.

CHAPITRE 1

**Toxicité *in vivo* du Déoxynivalénol et
de la Fumonisine, seuls ou en
combinaison chez le porcelet**

RESUME DES ETUDES

L'objectif de cette première partie du travail expérimental a été de déterminer le risque et le type d'interaction suite à l'exposition simultanée à deux mycotoxines majeures, le déoxynivalénol (DON) et la fumonisine (FB). Des porcelets ont ainsi ingéré durant cinq semaines des aliments contaminés à des doses qui n'entraînent pas de manifestations cliniques. Cette exposition mime donc une situation de terrain, avec des concentrations en toxines proches des valeurs individuelles fixées par la commission européenne pour un aliment complet chez le porc.

Afin d'évaluer au mieux ce risque, nous avons analysé les effets des régimes contaminés sur de nombreux paramètres, avec une attention particulière sur le développement de la réponse immunitaire suite à l'immunisation des animaux, et aussi sur le tractus gastro-intestinal. Ces deux champs d'investigations sont présentés séparément ci-après.

1. Toxicité *in vivo* du déoxynivalénol et de la fumonisine, seuls ou en combinaison sur la réponse immunitaire

Vingt quatre porcelets ont été répartis aléatoirement dans quatre groupes et ont reçu pendant 35 jours des régimes différents. Le premier a servi de régime contrôle, le second était contaminé avec 3,0 mg DON/kg d'aliment, le troisième avec 6,0 mg FB/kg d'aliment, et le dernier avec la présence des deux toxines aux concentrations respectives des régimes individuels. Pour stimuler la réponse immunitaire, les animaux ont reçu à 12 jours d'intervalle deux injections sous-cutanées d'ovalbumine.

Les régimes mono et co-contaminés n'ont pas modifié la croissance des animaux et n'ont eu qu'un effet mineur sur les paramètres hématologiques et biochimiques. En revanche, l'ingestion des mycotoxines a provoqué des lésions hépatiques, rénales et pulmonaires, avec une interaction de type additive sur les lésions du foie lorsque les toxines étaient en association.

En ce qui concerne les effets sur le système immunitaire, aucune altération de la réponse immunitaire totale et non-spécifique n'a été observée. A l'inverse, nos résultats sur la prolifération des lymphocytes après stimulation *in vitro* avec de l'ovalbumine et les titres en IgG anti-ovalbumine nous laissent penser que les toxines sont capable d'affecter la réponse immunitaire spécifique, cellulaire et humorale. De plus, le dosage par RT-PCT temps réel des ARNs de la rate, codant pour des cytokines, a révélé une baisse de l'expression de certains médiateurs impliqués dans le recrutement

des cellules présentatrices d'antigène et dans l'activation et la prolifération des lymphocytes. Ces observations couplées aux données de la littérature impliquent que l'établissement de la réponse immunitaire suite à la sensibilisation par notre antigène a été significativement affecté par l'ingestion des mycotoxines, et en particulier avec le régime co-contaminé.

RESEARCH ARTICLE

Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets

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Scope: Deoxynivalenol (DON) and fumonisins (FB) are the most frequently encountered mycotoxins produced by *Fusarium* species and most commonly co-occur in animal diets. These mycotoxins were studied for their toxicity in piglets on several parameters including plasma biochemistry, organ histopathology and immune response.

Methods and results: Twenty-four 5-wk-old animals were randomly assigned to four different groups, receiving separate diets for 5 wk, a control diet, a diet contaminated with either DON (3 mg/kg) or FB (6 mg/kg) or both toxins. At days 4 and 16 of the trial, the animals were subcutaneously immunized with ovalbumin to assess their specific immune response. The different diets did not affect animal performance and had minimal effect on hematological and biochemical blood parameters. By contrast, DON and FB induced histopathological lesions in the liver, the lungs and the kidneys of exposed animals. The liver was significantly more affected when the two mycotoxins were present simultaneously. The contaminated diets also altered the specific immune response upon vaccination as measured by reduced anti-ovalbumin IgG level in the plasma and reduced lymphocyte proliferation upon antigenic stimulation. Because cytokines play a key role in immunity, the expression levels of IL-8, IL-1 β , IL-6 and macrophage inflammatory protein-1 β were measured by RT-PCR at the end of the experiment. The expression of these four cytokines was significantly decreased in the spleen of piglets exposed to multi-contaminated diet.

Conclusion: Taken together, our data indicate that ingestion of multi-contaminated diet induces greater histopathological lesions and higher immune suppression than ingestion of mono-contaminated diets.

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Keywords:

Co-contamination / Deoxynivalenol / Fumonisins / Immunity / Subclinical doses

1 Introduction

Mycotoxins are secondary metabolites of fungi that may contaminate animal feeds and human foods. They are frequently detected in grains, but also in fruits, vegetables, nuts and silages. The Food and Agricultural Organization

estimated that as much as 25% of the world's agricultural commodities are contaminated with mycotoxins and the economic losses due to mycotoxin contamination are estimated in billions of dollars annually worldwide [1]. Clinical signs caused by mycotoxins range from mortality to slow growth and reduced reproductive efficiency. Consumption

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Abbreviations: APC, antigen-presenting cells; BALT, bronchiole-associated lymphoid tissue; DON, deoxynivalenol; FB, fumonisins; HE, hematoxylin-eosin; MAPK, mitogen-activated protein kinase; MIP-1 β , macrophage inflammatory protein-1 β ; OVA, ovalbumin

of mycotoxins may also result in impaired immunity and decreased resistance to infectious diseases [2].

Worldwide surveys on the occurrence and contamination levels of mycotoxins in raw materials indicate that toxins produced by *Fusarium* mold species are of concern [3–5]. Among the fusariotoxins, deoxynivalenol (DON) and fumonisins (FB) are frequently detected with concentrations up to 927 mg DON/kg and 300 mg FB/kg [4]. Among cereal samples collected from European countries, 54% were co-contaminated with DON and FB [6]. Similarly, in France, 65% of the maize kernels harvested during 2004–2006 were co-contaminated with DON and FB (Arvalis-Institut du végétal, unpublished data). These two mycotoxins are of major concern not only in terms of their ubiquitous distribution but also because of their effects on human and animal health.

At high concentrations, FB cause equine leukoencephalomalacia and porcine pulmonary edema, and it is nephro- and hepatotoxic and carcinogenic in rats and mice. FB1 has been classified as a potential human carcinogen (class 2B) by the International Agency for Research on Cancer. In humans, consumption of FB-contaminated food has been linked with human esophageal cancer and neural tube defects [7]. Disruption of sphingolipid biosynthesis appears to be one mechanism involved in FB toxicity, with inhibition of ceramide synthase [7] leading to accumulation of sphingoid bases (sphinganine and sphingosine). The effects of ingestion of low doses of FB are less documented but revealed pathological alterations of the lungs and an increase in intestinal colonization by opportunistic pathogenic bacteria in piglets [8–10].

Acute exposure to high doses of DON induces diarrhea, vomiting, leukocytosis and gastrointestinal hemorrhage. Anorexia, growth retardation and immunotoxicity occur in rodents and pigs following chronic DON ingestion [11]. At the cellular level, DON interferes with the active site of peptidyl transferase on ribosomes, and inhibits protein synthesis [11]. Further, binding of DON to the ribosome in eukaryotic cells triggers a “ribotoxic stress response”, which involves phosphorylation of the mitogen-activated protein kinases (MAPKs) [12]. MAPK activation modulates the expression of genes associated with the immune response, chemotaxis, inflammation and apoptosis. The cellular and molecular mechanisms of the immunomodulating action of DON have been described in numerous studies using mice and murine cell lines [13]. Depending on the dose and frequency of exposure, DON can be either immunosuppressive or immunostimulatory [11, 14]. Prolonged ingestion of DON produces elevation of immunoglobulin A in plasma [13–15] while increasing the susceptibility to infectious diseases [11].

The toxicity of combinations of mycotoxins cannot always be predicted based upon their individual toxicities [1]. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive or synergistic. The data on combined toxic effects of mycotoxins are limited and, therefore, the actual combined health risk from exposure to mycotoxins is unknown. Assessment of the interaction of *Fusarium* myco-

toxins has been investigated *in vitro* on immune cells and intestinal epithelial cells [16, 17]. *In vivo* experiments have also been done on mice, pigs and poultry using high doses of toxins in which the authors mainly looked for differences in animal performance. Among them, few studies were concerned with the interaction between DON and FB [18, 19].

The purpose of this study was to compare the effects of low doses of DON and FB in pigs when fed individually and in combination with particular emphasis on their effects on the immune response. The experimental design was a factorial assay including control feed and feed contaminated with 3 and 6 mg/kg DON and FB individually and in combination, respectively. These contamination levels correspond to levels that frequently occur naturally in cereals [1]. Results have been reported in terms of both general toxicological parameters including weight gain, hematology, plasma biochemistry and organ histology as well as specific parameters describing immune system responses (total and specific antibody, lymphocyte proliferation, cytokine expression).

2 Materials and methods

2.1 Animals

All animal experimentation procedures were carried out in accordance with the European Guidelines for the Care and Use of Animals for Research Purposes (Directive 86/609/EEC). Twenty-four 4-wk-old weaned castrated male pigs (Pietrain/Duroc/Large-white) were obtained locally. Male pigs were used in this protocol as it was previously demonstrated that a greater effect of DON and FB occurs in male pigs compared to female pigs [20]. Animals were acclimatized for 1 wk in the animal facility of the INRA Laboratory of Pharmacology and Toxicology (Toulouse, France), prior to being used in experimental protocols. Six pigs were allocated to each treatment on the basis of body weight. During the 35-day experimental period each treatment group was given free access to water and the assigned diet. The pigs were observed daily and weighed weekly.

2.2 Experimental diets

Diets were manufactured at INRA facilities in Rennes (France), and formulated according to the energy and amino acid requirements for piglets. Feed composition is detailed in Table 1. Four different batches were prepared, one control batch and three batches artificially contaminated with the mycotoxins. Two strains of *Fusarium*, *F. graminearum* DSM-4528 and *F. verticillioides* M-3125 were used to produce DON and FB, respectively. These strains were grown separately on rice. FB were produced as previously described [21]. DON was extracted with ethyl acetate, and the extract dried on silica gel 60 (Merck, Darmstadt, Germany). The homogenized extracts contained 24 and

Table 1. Composition of the experimental diet

Ingredient (%)	
Wheat	47.50
Soybean meal	24.30
Barley	22.90
Calcium phosphate	1.12
Calcium carbonate	1.00
Vitamin and mineral premix ^{a)}	0.50
Vegetable oil	1.40
Sodium chloride	0.40
Phytase	0.01
Lysine	0.465
Methionine	0.165
Threonine	0.195
Tryptophan	0.045
Composition ^{b)}	
Starch (g)	476.8
Crude protein (g)	218.3
Crude fiber (g)	37.5
Ca (g)	10.5
P (g)	6.5
K (g)	8.7
Net energy (MJ)	15.6

a) Vitamin A, 2 000 000 IU/kg; vitamin D3, 400 000 IU/kg; vitamin E, 4000 mg/kg; vitamin C, 8000 mg/kg; vitamin B1, 400 mg/kg; vitamin K3, 400 mg/kg; iron, 20 000 mg/kg; copper, 4000 mg/kg; zinc, 20 000 mg/kg; manganese, 8000 mg/kg.

b) Corresponding to 1000 g dry matter/kg.

21 g/kg DON and FB, respectively. The extracts containing the mycotoxins were mixed into the vitamin mineral supplements and then incorporated into the cereal mixture before granulation.

The feed was analysed for mycotoxin content by Quantas Analytik (Tulln, Austria) and by using a multi-mycotoxin method [22]. DON, zearalenone and enniatin were found to be naturally present in the cereals used, resulting in concentrations of 500, 50 and 100 µg/kg of feed, respectively. All other mycotoxins, including aflatoxins, T-2 toxin, HT-2 toxin and ochratoxin A were below the limit of detection. The mono-contaminated diets contained 2.8 mg of DON/kg of feed and 5.9 mg of FB/kg of feed (4.1 mg FB1/kg + 1.8 mg FB2/kg of feed) while the co-contaminated diet contained 3.1 mg of DON and 6.5 mg of FB/kg of feed (4.5 mg FB1/kg + 2.0 mg FB2/kg of feed).

2.3 Experimental design and sample collection

On the 4th and 16th day of the experiment, all piglets were immunized by subcutaneous inoculation with 1 and 2 mg of ovalbumin (OVA), 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. Plasma samples were obtained after centrifugation of heparinized blood and stored at –20°C for later analysis. After 35 days of dietary exposure to mycotoxins, immediately after electrical stunning, pigs were killed by exsanguination. Samples of lungs, liver and kidneys were collected from all groups and fixed in 10% buffered formalin for histopathological analysis. In addition, a portion of the spleen was collected from euthanatized animals, flash-frozen in liquid nitrogen and stored at –80°C until processed for measurements of cytokine mRNA.

2.4 Hematology and biochemistry

Hematological analysis was carried out using the impedance coulter LH500 (Beckman Coulter, Villepinte, France). Subpopulations of white blood cells (lymphocytes, monocytes, neutrophils, eosinophils and basophils) were also studied and made manually on 100 leukocytes on May-Grünwald Giemsa stained smears.

Plasma concentrations of total proteins, albumin, urea, creatinin, cholesterol, triglycerides and activity of γ -glutamyl transferase were determined by a Vitros 250 analyzer (Ortho Clinical Diagnostics, Issy les Moulineaux, France) at the Veterinary School of Toulouse (France).

2.5 Histology

The tissue pieces were dehydrated through graded alcohols and embedded in paraffin wax. Sections of 3 µm were stained with hematoxylin–eosin (HE) for histopathological evaluation. For each organ, three slides *per* animal were prepared for analysis, and an area of 2000–2500 µm² *per* slide was observed. As displayed in Table 2, microscopic observations led to the identification of different lesions in the different organs, and allowed for establishing a lesion score *per* animal. Based on a recent method published [23], we calculated the lesion score by taking into account the degree of severity (severity factor) and the extent of each lesion (according to intensity or observed frequency, scored from 0 to 3). For each lesion, the score of the extent was multiplied by the severity factor. For each tissue, the minimal scores were 0 and the maximal scores were 21, 33 and 15 for liver, lung and kidney, respectively (Table 2).

2.6 Measurement of hepatocyte proliferation

The cellular proliferation activity was assessed by counting Ki-67-positive nuclei on formalin-fixed embedded liver sections as already described [24]. Briefly, the sections were incubated with the primary antibody (Zymed (South San Francisco, CA, USA) Ki-67 Clone 7B11 – diluted 1:50) at 4°C overnight in a humidity chamber; then the secondary

antibody (Kit Super Picture™ Zymed) was applied and followed by the addition of a chromogen (3,3'-diaminobenzidine). Finally, the tissue sections were counterstained with hematoxylin and mounted under coverslips using a permanent mounting medium.

The number of Ki-67-positive nuclei among the total of 100 nuclei was counted on the sections under light microscopy at 40 × magnification. The proliferative index was calculated by Ki-67-positive cells/total cells × 100.

Table 2. Establishment of a lesion score – endpoints used to assess histological lesions^{a)}

Tissue	Type of lesions (severity factor)	Maximal score
Liver	Disorganization of hepatic cords (1)	21
	Hepatic cell vacuolization (1)	
	Apoptosis (2)	
	Megalocytosis (2)	
	Nuclear vacuolation (1)	
Lung	Alveolar edema (2)	33
	Interstitial pneumonia (2)	
	BALT depletion (2)	
	Hypertrophy muscle cell (2)	
	Hemorrhage (2)	
	Vascular congestion (1)	
Kidney	Nuclear change (1)	15
	Mitosis (1)	
	Cytoplasmic vacuolization (1)	
	Tubular casts (1)	
	Congestion (1)	

a) The score for each lesion was obtained by multiplying the severity factor with the extent of the lesion. The organ score was then obtained by the sum of each lesion score. Severity factor (or degree of severity), 1 = mild lesions, 2 = moderate lesions; the extent of each lesion (intensity or observed frequency) was evaluated and scored as 0 = no lesion, 1 = low extent, 2 = intermediate extent, 3 = large extent.

Table 3. Nucleotide sequences of primers for real-time PCR^{a)}

Gene	Primer sequence	Genbank no.	References
RPL32	F (300 nM) TGCTCTCAGACCCCTTGTGAAG R (300 nM) TTTCCGCCAGTTCGCTTA	NM_001001636	[46]
β2-microglobulin	F (900 nM) TTCTACCTTCTGGTCCACACTGA R (300 nM) TCATCCAACCCAGATGCA	NM_213978	[27]
IL-12p40	F (300 nM) GGTTTCAGACCCGACGAACTCT R (900 nM) CATATGCCACAATGGGAGATG	NM_214013	[27]
IL-8	F (300 nM) GCTCTCTGTGAGGCTGCAGTTC R (900 nM) AAGGTGTGGAATGCGTATTTATGC	NM_213867	Present study
IL-1β	F (300 nM) GAGCTGAAGGCTCTCCACCTC R (300 nM) ATCGCTGTCTATCTCCTTGAC	NM_001005149	[27]
MIP-1β	F (300 nM) AGCGCTCTCAGCACCAATG R (300 nM) AGCTCCGCACGGGTGTATG	AJ311717	Present study
IL-6	F (300 nM) GGCAAAAGGGAAAGAATCCAG R (300 nM) CGTTCTGTGACTGCAGCTTATCC	NM_214399	Present study

a) RPL32, ribosomal protein L32.

2.7 Measurement of total and specific immunoglobulin subsets

The total concentration of the immunoglobulin subsets was measured by ELISA as already described [25]. Briefly, the different isotypes were detected with the appropriate peroxidase anti-pig IgA or IgG (Bethyl, Interchim, Montluçon, France) and were quantified by reference to standard curves constructed with known amounts of pig immunoglobulin classes. Titers of specific antibody anti-OVA were also measured by ELISA [14]. Briefly, the anti-OVA antibodies were detected with peroxidase-labeled anti-pig IgG or IgA (Bethyl). Absorbance was read at 450 nm using an ELISA plate reader (Spectra thermo, Tecan, NC, USA) and the Biolise 2.0 data management software.

2.8 Determination of lymphocyte proliferative index

Lymphocyte proliferation was measured on blood samples collected at different times of the experimental period. The quantification was performed in 96-well plates as already described [15, 26]. The results were expressed as stimulating index of lymphocyte proliferation calculated as counts *per* minute in stimulated culture/cpm in control non-stimulated culture.

2.9 Determination of the expression of mRNA encoding for cytokines by real-time PCR

Tissue RNA was processed in 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). Concentrations, integrity and quality of RNA were determined spectrophotometrically (OD₂₆₀) using Nanodrop ND1000 (Labtech International, Paris,

France). Besides this inspection, a 200 ng sample of RNA was analyzed by electrophoresis. The reverse transcription and real-time PCR steps were performed as already described [26]. RNA non-reverse transcribed was used as the non-template control for verification of a no genomic DNA amplification signal. Specificity of PCR products was checked out at the end of the reaction by analyzing the curve of dissociation. In addition, the size of amplicons was verified by electrophoresis. The sequences of the primers used are detailed in Table 3. Primers for macrophage inflammatory protein-1 β (MIP-1 β), IL-8 and IL-6 detection were designed using Primer Express[®] software (Applied Biosystems, Courtaboeuf, France). Primers were purchased from Invitrogen (Cergy Pontoise, France). Amplification efficiency and initial fluorescence were determined by Data Analysis for Real Time-PCR method; then values obtained were normalized by both house-keeping genes, β 2-microglobulin and ribosomal protein L32, and finally, gene expression was expressed relative to the control group as already described [27].

2.10 Statistics

Following the Fisher test on equality of variances, one-way ANOVA was used to analyze the differences between the different groups of animals at each time point. *p*-Values of 0.05 were considered significant.

Table 4. Individual or combined effects of DON and FB on weight gain^{a)}

Body weight gain/day (kg)	Animal diets			
	Control	DON	FB	DON+FB
Days 1–14	0.36 ± 0.05 ^a	0.35 ± 0.03 ^a	0.43 ± 0.05 ^a	0.32 ± 0.07 ^a
Days 14–35	0.76 ± 0.05 ^a	0.65 ± 0.03 ^a	0.74 ± 0.06 ^a	0.68 ± 0.03 ^a

a) Results are expressed as mean ± SEM for five animals.

Table 5. Individual or combined effects of DON and FB on hematological parameters^{a)}

Hematological parameters	Animal diets (wk 6)			
	Control	DON	FB	DON ± FB
White blood cells (thousands/ μ L)	21.2 ± 1.9 ^a	19.6 ± 2.3 ^a	20.3 ± 2.8 ^a	18.2 ± 1.6 ^a
Lymphocytes (thousands/ μ L)	12.4 ± 1.9 ^a	11.4 ± 1.4 ^a	14.7 ± 2.1 ^a	12.6 ± 1.0 ^a
Neutrophils (thousands/ μ L)	7.3 ± 1.1 ^a	7.0 ± 1.1 ^{a,b}	4.5 ± 0.9 ^b	4.6 ± 0.6 ^b
Red blood cells (thousands/ μ L)	6.2 ± 0.3 ^a	5.7 ± 0.2 ^a	6.1 ± 0.4 ^a	5.9 ± 0.4 ^a
Mean corpuscular volume (fL)	47.6 ± 0.8 ^a	47.1 ± 0.7 ^a	46.2 ± 0.5 ^a	50.4 ± 1.9 ^a
Hematocrit (%)	29.8 ± 1.6 ^a	27.0 ± 0.5 ^a	28.0 ± 1.9 ^a	29.5 ± 1.6 ^a
Hemoglobin (g/dL)	9.6 ± 0.5 ^a	9.0 ± 0.2 ^a	9.4 ± 0.5 ^a	9.7 ± 0.5 ^a
Mean corpuscular hemoglobin (pg)	15.4 ± 0.2 ^a	15.6 ± 0.2 ^a	15.6 ± 0.2 ^a	16.5 ± 0.8 ^a
Mean corpuscular hemoglobin concentration (%)	32.4 ± 0.4 ^a	33.2 ± 0.3 ^a	33.8 ± 0.6 ^a	32.8 ± 0.4 ^a

a) Results are expressed as mean ± SEM for six animals. Values in rows with different letters are significantly different.

3 Results

3.1 Individual or combined effects of DON and FB on weight gain, hematological and biochemical parameters

During the experiment, piglets were weighed weekly and as reported in Table 4, ingestion of individual or combined DON- and FB-contaminated diets did not significantly impair animal growth.

At the end of the experiment, blood samples were taken from all piglets to investigate the effects of mycotoxins on hematological and biochemical variables (Tables 5 and 6). Piglets fed either FB- or FB+DON-contaminated diets displayed a significant decrease in neutrophil number (Table 5). An increase in creatinin concentration ($p = 0.047$) and a decrease in albumin concentration ($p = 0.015$) was also observed in the animal groups fed with FB- or DON-contaminated diets, respectively. These alterations were not observed in animals fed the diet contaminated with both toxins (Table 6).

3.2 Individual or combined effects of DON and FB on organ histopathology

Liver, lungs and kidneys were collected at the end of the trial for histopathological analysis. The lesions observed in these three organs were mild to moderate for animal fed any of the three contaminated diets (DON, FB, DON+FB) (Fig. 1).

The main histological lesions observed in the livers were a disorganization of hepatic cords, cytoplasmatic and nuclear vacuolization of hepatocytes and megalocytosis (Figs. 1A and B). Piglets fed either DON- or FB-contaminated diets displayed significant liver lesions when compared to animals fed the control diet. The lesion score was further increased for animals fed diet contaminated with both toxins. The proliferation of hepatocytes was assessed by counting Ki-67-positive cells in liver sections.

Table 6. Individual or combined effects of DON and FB on biochemical parameters^{a)}

Biochemical parameters	Animal diets (wk 6)			
	Control	DON	FB	DON+FB
Urea (mmol/L)	3.8±0.4 ^a	3.3±0.4 ^a	4.2±0.3 ^a	4.0±0.4 ^a
Creatinin (µmol/L)	102.5±5.3 ^a	98.0±4.1 ^a	120.5±5.6 ^b	101.6±5.5 ^a
Cholesterol (mmol/L)	2.6±0.2 ^a	2.4±0.2 ^a	2.3±0.1 ^a	2.3±0.1 ^a
Triglycerides (mmol/L)	0.51±0.07 ^a	0.34±0.04 ^a	0.39±0.06 ^a	0.41±0.06 ^a
Total proteins (g/L)	59.8±1.0 ^a	57.1±2.1 ^a	59.9±2.5 ^a	57.6±2.5 ^a
Albumin (g/L)	34.3±0.7 ^a	29.2±1.5 ^b	35.1±2.1 ^a	32.8±2.1 ^{a,b}
GGT (IU/L)	65.4±8.6 ^a	88.6±14.4 ^a	79.4±15.0 ^a	77.0±11.5 ^a

a) GGT, γ -glutamyl transferase. Results are expressed as mean±SEM for five animals. Values in rows with different letters are significantly different.

The mean proliferation indexes were 16.4±1.5 in the control group, 18.8±3.3 in the DON-treated group, 22.8±1.7 in the FB-treated group and 39.4±12.8 in the DON+FB-treated group ($p < 0.001$, $p < 0.01$ and $p < 0.05$, for comparison between DON+FB and control, DON or FB groups, respectively).

In the lungs, depletion of bronchiole-associated lymphoid tissue (BALT) and vascular disorders (peribronchiolar, alveolar hemorrhage and congestion) were the most frequent observed lesions (Fig. 1C). Of note, BALT structures were checked and were present in all individual pigs, evaluated in a comparable size and area between experimental groups. Alveolar edema showed a focal presentation (Fig. 1D). As demonstrated by the lesion scores, lung lesions were only observed in animals receiving FB- or FB+DON-contaminated diets. In this latter group, a medial hypertrophy of pulmonary arterioles was observed in half of the animals.

Lesions in the kidneys were mild as indicated by low lesion scores. The main observed lesions were degenerative changes in tubular epithelial cells (vacuolization of the cytoplasm and nucleus, Figs. 1E and F) and interstitial infiltration of lymphocytes with a focal or multifocal pattern. These lesions were observed in animals receiving diets contaminated with DON, FB and both toxins.

3.3 Individual or combined effects of DON and FB on the immune response

The main objective of this study was to assess the individual and combined effects of DON and FB on the immune response in piglets. Ingestion of diets contaminated with individual or combined mycotoxins neither altered the total plasmatic concentration of IgG and IgA nor modulated the lymphocyte proliferation upon concanavalin A stimulation (data not shown).

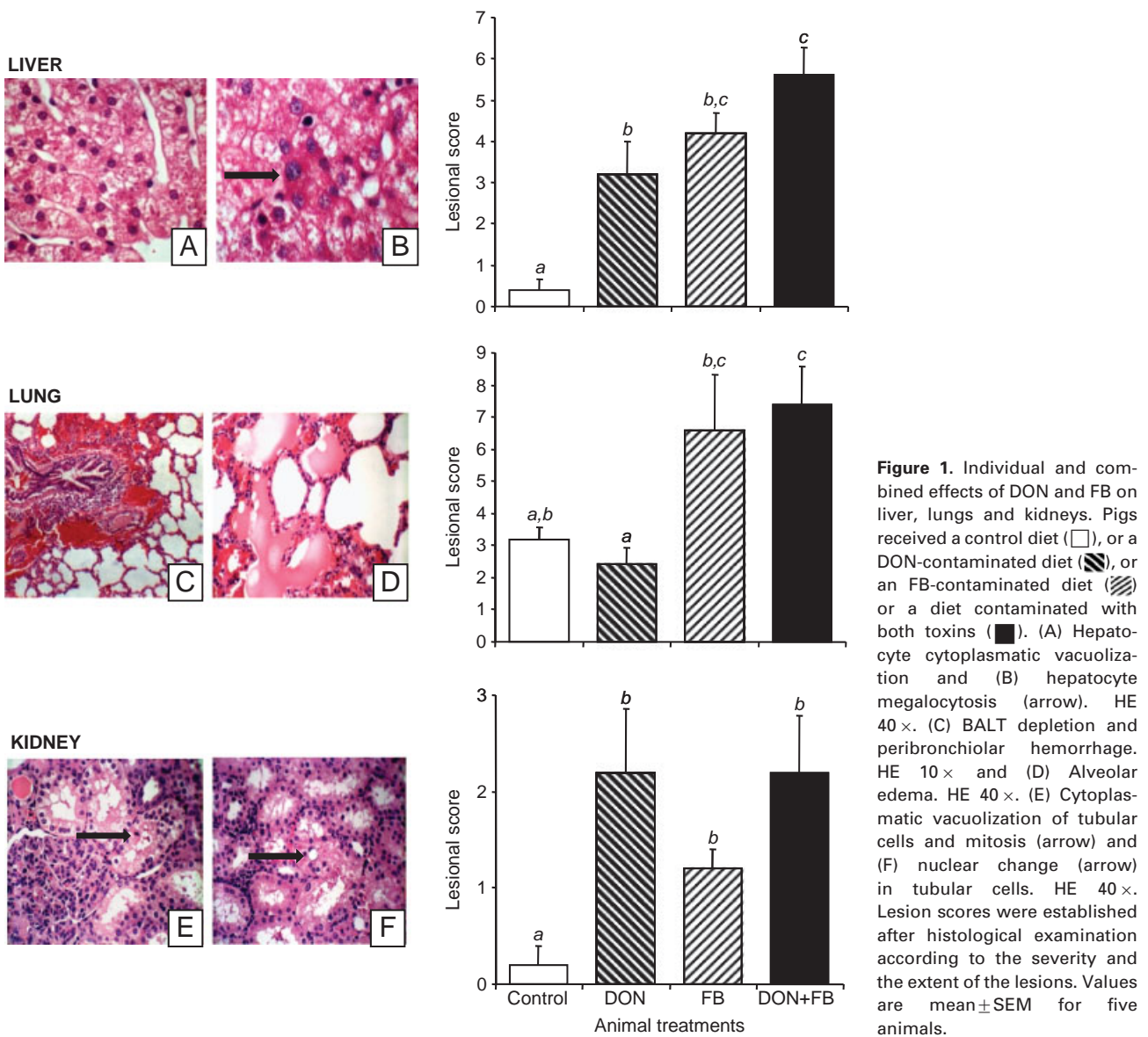
The immunization protocol with OVA allowed us to investigate the effects of mycotoxins on antigen-specific immunity [14, 26]. The ingestion of diets contaminated with DON or FB individually or in combination significantly altered the production of immunoglobulins after OVA

vaccination (Fig. 2). Animals fed mycotoxin-contaminated diets displayed a reduced anti-OVA IgG concentration in their plasma. However, because of high individual variability, the decrease was only significant for animals receiving FB-contaminated feed. This decrease was further pronounced for animals fed the diet containing both toxins. Concerning the effect of mycotoxins on the specific IgA concentration, as expected, we observed a significant increase of this immunoglobulin isotype in piglets fed the DON-contaminated diet. However, when DON was fed in combination with FB, the increase of plasmatic-specific IgA concentration was not observed (Fig. 2).

As already observed [14, 26], piglets receiving the control diet displayed a significant increase in the lymphocyte proliferation upon OVA stimulation after the second immunization (1.4-fold increase, $p = 0.191$; 3.3-fold increase, $p = 0.012$ and 2.8-fold increase, $p = 0.020$ at days 21, 28 and 35 of the experiment, respectively). By contrast, the lymphocyte proliferation upon OVA stimulation in the animals receiving any of the three contaminated diets (DON, FB and DON+FB) remained as low as in control unstimulated lymphocytes (Fig. 3).

3.4 Individual or combined effects of DON and FB on the expression of cytokines

Cytokines play a key role in regulating both humoral and cell-mediated immunity. The mRNA expression of five cytokines (IL-12p40, IL-8, IL-1 β , IL-6 and MIP-1 β) was measured by real-time RT-PCR in spleen samples collected at the end of the experiment (Fig. 4). Animals fed the diet containing both DON and FB demonstrated a significant decrease in mRNA for all tested cytokines when compared to the control pigs ($p = 0.009$ for IL-8; $p = 0.035$ for IL-1 β ; $p = 0.004$ for IL-6; $p = 0.031$ for IL-12p40; $p = 0.006$ for MIP-1 β). Animals fed the diet contaminated with DON demonstrated a significant decrease in mRNA encoding for IL-8, whereas animals fed the diet contaminated with FB demonstrated a significant decrease in mRNA encoding for IL-1 β and IL-6.



4 Discussion

In the present 5-wk study, piglets were exposed to low doses of two major *Fusarium* mycotoxins, DON and FB, at levels commonly found in crops. Most of the current data concerning the effects of DON or FB on animals, including rodents, have been obtained using highly mono-contaminated feeds [9, 12, 13, 28]. It was thus of interest to determine the effect of ingestion of feeds contaminated with low level of these toxins, present alone or in combination, on zootechnical, hematological, biochemical, histopathological and immune parameters of piglets.

We did not observe any effect of mycotoxin-contaminated diets (DON, FB, DON+FB) on the body weight gain of the animals. Considering the low contamination levels we used, these results are not surprising. Indeed, no

effect on body weight gain has been reported in pigs and in poultry fed with up to 70 mg FB/kg feed [18, 29]. The effects of DON on body weight gain are more controversial, especially in pigs. Some studies indicate that dietary concentrations of DON above 1–2 mg/kg have an effect on weight gain, whereas in other studies no effect is observed for up to 4.5 mg DON/kg feed [30]. A weight gain reduction has also been described when DON and FB were given together to growing barrows [18]. However, in this study, the dose of FB was ten-fold higher than the one used in the present experiment.

Exposure of piglets to low doses of either DON or FB did not have a major impact on the hematological and biochemical parameters investigated. For blood hematology, only a reduction in neutrophil numbers was noticed in FB-exposed piglets. This observation is in relation with the

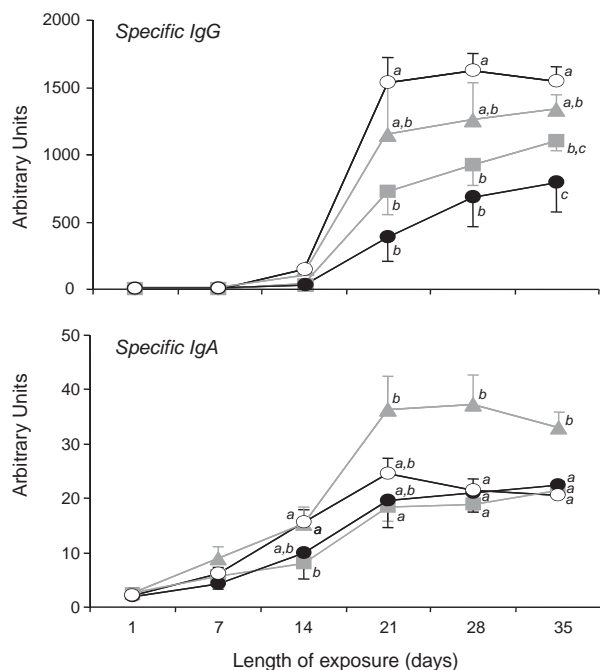


Figure 2. Individual and combined effects of DON and FB on plasma concentrations of specific immunoglobulin (IgA and IgG) anti-OVA. Pigs received a control diet (○), or a DON-contaminated diet (△), or an FB-contaminated diet (■) or a contaminated diet with both toxins (●). At days 4 and 16 of the trial, animals receiving either control or contaminated feeds were subcutaneously immunized with OVA. Plasma samples were collected weekly and the levels of IgA and IgG specific for OVA were determined by ELISA and normalized against a standardized reference plasma. Values are mean \pm SEM for five animals. Statistics are mentioned when significant changes were observed.

reduced viability measured in human neutrophils exposed *in vitro* to this toxin [31]. For blood biochemistry, there was a decrease in albumin concentration in DON-exposed animals and an increase in creatinin concentration in FB-exposed piglets in accordance with previously published studies [18, 20, 32, 33]. Ingestion of diets co-contaminated with DON and FB had less effect on hematology and biochemistry parameters than did mono-contaminated diets. Some studies have already reported a weaker effect on plasma biochemical parameters for piglets fed multi-contaminated diets than for piglets receiving mono-contaminated feeds [18, 19], which suggests an opposite effect of the two mycotoxins.

Despite the absence of effects on zootechnical, hematological and biochemical parameters, ingestion of feeds contaminated with low concentrations of either DON or FB induced histopathological lesions in liver, lungs and kidneys. Toxic effects of FB on liver have been reported in several papers using highly contaminated materials [9, 28]. The effects included a disorganization of hepatic cords, hepatocellular vacuolation, megalocytosis, apoptosis,

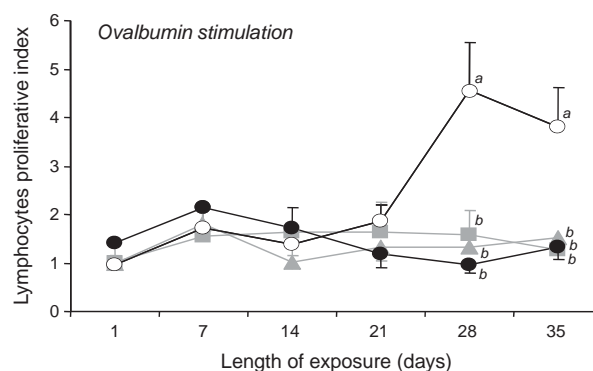


Figure 3. Individual and combined effects of DON and FB on lymphocyte-specific (OVA) proliferation. Pigs received a control diet (○), or a DON-contaminated diet (△), or an FB-contaminated diet (■) or a contaminated diet with both toxins (●). At days 4 and 16 of the trial, animals were subcutaneously immunized with OVA. Blood samples were taken weekly to measure the lymphocyte proliferation. Results are expressed as stimulating index of lymphocyte proliferation calculated as counts per minute in stimulated culture/cpm in control non-stimulated culture. Values are mean \pm SEM for five animals. Statistics are mentioned when significant changes were observed.

necrosis and cell proliferation. In the present study, it was observed that even when present at a lower dose, FB induced similar liver histopathological lesions. Liver lesions, such as hepatic cell vacuolation, were also observed in piglets fed the DON-contaminated diet [34]. These lesions were not associated with major biochemical alterations. The biological meaning of the hepatic lesions remains to be determined. Histopathological analysis of lung confirmed that this is a target organ for FB. At high doses (≥ 92 mg/kg of feed for 4–7 days), FB induce lethal pulmonary edema in swine [9]. In the present study, the low dose of FB also induced pulmonary damages, mainly BALT depletion and vascular disorders. By contrast, when present at a low dose in the diet, DON did not induce any lesion in the lung.

For the three organs investigated, the damages elicited from the ingestion of the diet co-contaminated with DON and FB were equal to or higher than the ones elicited by the ingestion of a single mycotoxin. Very few publications analyzed the effects of mixed mycotoxins on histopathological parameters, especially at low doses [35, 36]. The histopathological lesions observed in the lungs of co-exposed piglets were slightly more pronounced than the ones observed in the lungs of FB-exposed animals. In the liver, ingestion of the co-contaminated diet induced significantly higher lesions than ingestion of either of the mono-contaminated feeds as demonstrated by the lesion score and the hepatocyte proliferation. One explanation for the high liver toxicity of DON and FB when present simultaneously could be the higher absorption of FB in the presence of DON. Indeed, DON has recently been shown to decrease the barrier function of the intestine [37]. Thus, ingestion of

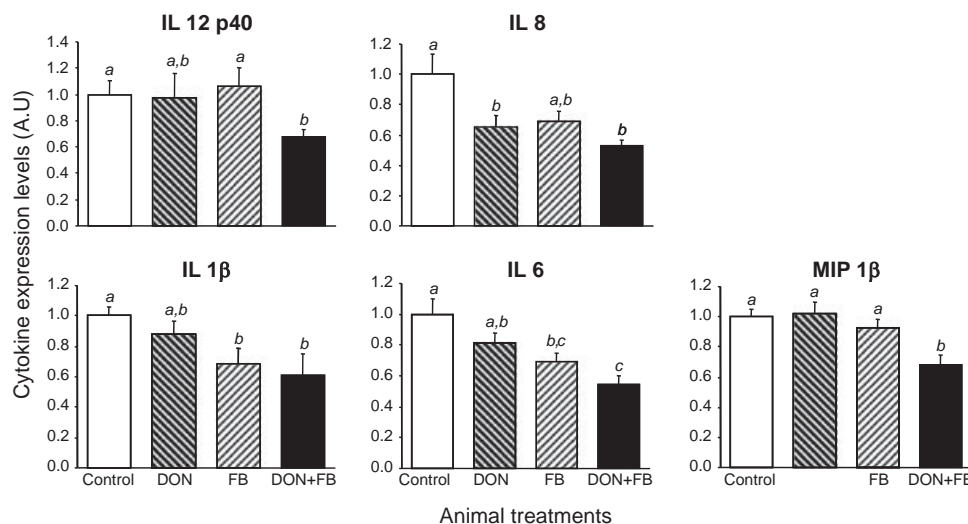


Figure 4. Individual and combined effects of DON and FB on splenic mRNA expression of cytokines. Pigs received a control diet (□), or a DON-contaminated diet (▨), or an FB-contaminated diet (▧) or a diet contaminated with both toxins (■). Quantification of the relative cytokine mRNA level for each sample is expressed in arbitrary units (A.U). Values are mean ± SEM for five animals.

DON may increase the absorption of FB, mycotoxins already known to be poorly absorbed [7, 9].

The main objective of this study was to investigate the effect of low doses of DON or FB ingested separately or in combination on the immune response of piglets. As in previous experiments, it was observed that at low doses, mycotoxins have little or no effect on the total non-specific immune responses as measured by lymphocyte proliferation upon mitogenic stimulation and the plasmatic concentration of immunoglobulin classes. Immunization protocols, as already described, were needed to observe an effect of low doses of mycotoxins, fed either alone or in combination on the immune responses [14, 26, 38].

A very low proliferative index, close to the one observed in unstimulated cells, was obtained in cells isolated from animals fed either DON-, FB- or DON+FB-contaminated diets. This alteration of lymphocyte proliferation might be due to an effect of these toxins on antigen-presenting cells (APC) as suggested by recent *in vitro* studies on monocyte-derived APC treated with DON [39, 40] or *in vivo* studies with piglets acutely exposed to FB [27].

Interestingly, the diet co-contaminated with DON and FB appeared to be able to counteract the increased level of specific IgA observed in the animal receiving only the DON-contaminated diet. Indeed, consumption of the DON-contaminated diet increased the level of specific IgA in the plasma [11, 14] whereas ingestion of diet contaminated with both DON and FB did not alter the plasma level of this immunoglobulin isotype. We can hypothesize that FB interfere with the DON-induced IgA elevation at the intestinal level through its action on sphingolipids. Indeed, FB are known to disrupt the sphingolipid metabolism leading to depletion of ceramide and all ceramide-derived complex sphingolipids, such as sphingomyelin [41, 42]. This latter compound has been recently reported to control the amount of IgA in the large intestine [43].

Depending on the mycotoxin, DON or FB significantly impaired the specific IgG concentration and the level of

cytokine expression. Nonetheless, the diet co-contaminated with DON and FB led to a strong decrease of specific IgG concentration, greater than the one observed in animals receiving only one toxin. Similar effects were observed for the five cytokines investigated, where the impact of the co-contaminated diet was higher than either of the mono-contaminated diets. Several studies investigated cytokine expression during chronic exposure to mycotoxins [14, 15, 25, 27], but none of them concern the co-contamination. Cytokines are important mediators in the immune response. Expressions of IL-8 and MIP-1β, which are involved in cell chemotaxis, were significantly inhibited in animals fed the co-contaminated diet, and it can be anticipated that in these animals, recruitment and migration of APC to peripheral lymphoid tissue were reduced. Similarly, the decreased mRNA levels of IL-1β and IL-6 mRNA in piglets receiving the co-contaminated diet may lead to a defective antigen presentation and an impaired activation of lymphocytes and may explain the decreased IgG response observed in this study.

Find a mechanism that explains the observed effects after the combination of both toxins is not easy, but at the cellular level, it might be hypothesized that MAPK activation could be involved. Indeed, both DON and FB have been shown to activate MAPKs [12, 44], and these kinases are well known to modulate numerous physiological processes, such as cell growth, apoptosis or immune response [45].

In conclusion, chronic exposure to low doses of DON or FB, either alone or in combination did not elicit important clinical signs (body weight gain, hematology, biochemistry), but induced microscopic lesions and altered the immune response, especially when the mycotoxins were fed in combination. The modulation of the immune response was only observed when the immune system was activated. Considering (i) that vaccination or infection by pathogens is a common situation encountered in animal husbandry and (ii) the natural occurrence of these mycotoxins in feedstuffs, the present experiment suggests a significant disruption in

the establishment of an appropriate specific response in animals receiving mycotoxin-contaminated diets. This study also highlights the complexity of mycotoxin interactions; some effects are not enhanced by the combination of toxins (biochemistry, lung and kidney lesions, specific IgA content), while others are (specific IgG content, cytokines expression, liver lesions). These results may have some impact on the current regulation/recommendation that only takes into account individual mycotoxins and not multi-mycotoxin contamination.

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2. Toxicité *in vivo* du déoxynivalénol et des fumonisines, seuls ou en combinaison sur la morphologie et la réponse locale de l'intestin

L'expérimentation animale présentée dans la première étude a également fait l'objet de recherches sur l'effet des quatre régimes sur l'intestin. Des échantillons d'intestin grêle ont été prélevés lors de l'autopsie des animaux, et des analyses histologiques et immunologiques ont été réalisées.

Les résultats histologiques ont montré une augmentation du nombre de lésions après l'ingestion des mycotoxines, au niveau du jéjunum et de l'iléon. L'effet observé sur les cellules en mitose pourrait expliquer l'aplatissement des villosités dans la partie jéjunale. Néanmoins, ces conclusions sur les analyses histologiques ont été principalement confirmées pour le régime mono-contaminé avec le DON. Et il semblerait que la présence de FB ait un effet antagoniste sur l'effet du DON dans le régime co-contaminé. Ce même type d'interaction a été observé sur la diminution du nombre de cellules caliciformes dans le jéjunum.

L'analyse de l'expression des cytokines par RT-PCR temps réel a montré de manière intéressante, une augmentation du niveau des ARN dans le jéjunum et l'iléon. L'augmentation de l'IL-6 causée par le DON pourrait être corrélée à l'élévation du titre en IgA spécifiques observée dans la première étude. L'augmentation générale des ARNs codant pour diverses cytokines tout au long de l'intestin grêle, suggère un état inflammatoire chronique suite à l'exposition continue aux régimes contaminés pendant les cinq semaines. Dans ce sens, il a été reporté dans la littérature que lors d'inflammations digestives chroniques, l'expression exacerbée de certaines cytokines (IL-1 β , TNF- α et IFN- γ) induisait une plus grande perméabilité intestinale. Basé sur ces observations et sur les récents travaux de notre équipe sur l'altération de l'expression de protéines de jonctions par le DON, nous avons ainsi montré qu'*in vivo*, les régimes contaminés diminuaient l'expression protéique de l'E-cadhérine et de l'occludine au niveau de l'iléon; un effet additif étant obtenu pour le régime co-contaminé.

Chronic ingestion of deoxynivalenol and fumonisin B1 induce morphological and immunological changes in the intestine of piglets

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Abbreviations: AJ, adherens junctions ; DON, deoxynivalenol ; FB, fumonisins ; HE, hematoxylin-eosin ; TJ, tight junctions ; WB, western blotting

Keywords: co-contamination, deoxynivalenol, fumonisins, intestinal tract, morphology, immunology

ABSTRACT

Although low levels of multiple mycotoxins are frequently encountered in commodities, there is a lack of data on the potential risk of mycotoxin multi-contamination, especially at low doses. Deoxynivalenol (DON) and Fumonisin (FB) are both produced by *Fusarium* species and naturally co-occur in animal diets. Individually, these mycotoxins exert numerous toxicological effects according to doses ingested, and are therefore of concern in terms of animal health. Because the gastrointestinal tract represents the first barrier met by exogenous food/feed compounds, the purpose of this study was to investigate the effects of DON and FB, alone and in combination on some intestinal parameters, including morphology, expression of tight junction or cytokines expression. Twenty-four 5-wk-old piglets were randomly assigned to four different groups, receiving separate diets for 5 weeks, a control diet, a diet contaminated with either DON (3mg/kg) or FB (6mg/kg) or both toxins. Chronic ingestion of these contaminated diets induced histological changes, as shown by the decreased villi height and cell proliferation in jejunum, and by the reduced number of goblet cells in both jejunum and ileum. The low doses used potentiated the cytokines secretion all along the intestine, especially IL-1 β and TNF- α . Upregulation of these two cytokines could be the cause of the defective expression observed in the ileal segment for two junction proteins, occludin and E-cadherin. This alteration in cell adhesion suggests that consumption of DON and FB could predispose animals to enteric infection through impairment of the intestinal barrier function. To conclude, the intestinal response observed for the co-contaminated diet was similar to the response to mono-contaminated diets for the majority of parameters evaluated, indicating no synergistic effect.

INTRODUCTION

Mycotoxins are secondary metabolites of various fungi commonly found in feed and foodstuffs. Based on their known and suspected effects on human and animal health, aflatoxin, fumonisin, deoxynivalenol, ochratoxin A and zearalenone are recognized as the five most important agricultural mycotoxins (Shephard, 2008). The toxic effects of *Fusarium* mycotoxins in animals include reduced growth, feed refusal, immunosuppression, gastrointestinal lesions, and neurological and reproductive disorders (Rocha *et al.*, 2005).

Recent surveys demonstrated regular occurrence of low levels of multiple mycotoxins in cereals (Roscoe *et al.*, 2008; Tabuc *et al.*, 2009; Scudamore and Patel, 2009). Also, mycotoxins may be present in grains in conjugated chemical forms that escape detection through conventional analytical methods (Zhou *et al.*, 2007), resulting in an underestimation of the total amount of mycotoxins and feed toxicity. *Fusarium* mycotoxins in combination can exert more pronounced effects in animals than individual mycotoxins (Smith *et al.*, 1997; Grenier *et al.*, 2011).

The intestinal tract is the first barrier against ingested antigens, including mycotoxins and pathogenic bacteria. Following ingestion of mycotoxin-contaminated food, enterocytes may be exposed to high concentrations of toxins (Bouhet and Oswald, 2005). Studies focusing on the influence of food-derived antigens on intestinal morphology as an indicator of animal health are common; meanwhile, there are few publications on the effects of chronic exposure to a mycotoxin co-contaminated diet.

Fumonisin (FB) are toxic and carcinogenic mycotoxins produced by *Fusarium verticillioides*, a common contaminant of maize. Fumonisin B1 (FB1) causes porcine pulmonary edema and equine leukoencephalomalacia. An association between human esophageal cancer and FB1 exposure in developing countries has been reported (Zhang *et al.*, 1997). Fumonisins are structurally related to sphingoid bases and cause inhibition of ceramide synthase, leading to accumulation of corresponding free sphingoid bases, sphingoid base metabolites, and depletion of more complex sphingolipids (Wang *et al.*, 1991). *In vitro*, FB1 induces apoptosis, necrosis, and inhibition of proliferation in pig renal epithelial (LLC-PK1) cells and human colonic cells (HT29) (Gopee *et al.*, 2003; Schmelz *et al.*, 1998). FB1 can impair the intestinal absorption of nutrients. This can be explained by the villous fusion and atrophy observed in the intestines of pigs treated with 30 mg FB1/kg of feed and/or by sphingolipid disturbances (Taranu *et al.*, 2005).

Deoxynivalenol (DON) causes toxic and immunotoxic effects in a variety of cell systems and animal species. DON is produced by *F. graminearum* and *F. culmorum* mainly in wheat, barley and maize (Pestka and Smolinski, 2005). Swine is more sensitive to DON than other species, in part

because of differences in the metabolism of DON. Chronic low dietary concentrations induce anorexia, decreased weight gain, and immune alterations, while acute higher doses induce vomiting, hemorrhagic diarrhea and circulatory shock (Rotter *et al.*, 1996a). At the cellular level, the main effect is inhibition of protein synthesis via binding to the ribosomes. Low exposure to DON was shown to upregulate expression of cytokines and inflammatory genes with concurrent immune stimulation, whereas high exposure promoted leukocyte apoptosis associated with immune suppression (Pestka *et al.*, 2004).

The purpose of this study was to compare the effects of low doses of DON and FB in pigs when fed individually and in combination with particular emphasis on their effects on the intestine. The experimental design was a factorial assay including control feed and feed contaminated with 3 and 6 mg/kg DON and FB individually and in combination, respectively. These contamination levels correspond to levels that frequently occur naturally in cereals. We investigated the effect of DON and FB1 on intestine morphology, on the expression of tight junction as well as on the intestinal expression of cytokines.

MATERIAL & METHODS

1) Animals and diets

A total of 24 crossbred weaned piglets were used in this study. Animals were kept in batch pens for 35 days. Pigs were acclimatized for 1 week in the animal facility of the INRA Laboratory of Pharmacology and Toxicology (Toulouse, France) prior to being used in experimental protocols. Feed and water were provided *ad libitum* throughout the experimental period. The animals were submitted to one of four dietary treatments for 35 days: control non-contaminated diet, diet containing 2.8 mg of deoxynivalenol/kg (DON) of feed, diet containing 5.9 mg of fumonisins (4.1 mg FB1 + 1.8 mg FB2)/kg of feed and diet containing 3.1 mg of DON plus 6.5 mg FB (4.5 mg FB1 + 2.0 mg FB2)/kg of feed. The diets were artificially contaminated with the mycotoxins. The diet formulations and nutrient contents were described elsewhere (Grenier *et al.*, 2011).

The experimental design used in this study was entirely randomized with six repetitions (each animal represented one repetition). At the end of the experiment, pigs were submitted to electrical stunning and euthanized by exsanguination. The institutional Ethics Committee for Animal Experimentation approved the study.

2) Histological assessment

At postmortem examination, samples from the jejunum and ileum were collected from all groups and fixed in 10% buffered formalin. The tissue pieces were then dehydrated through graded alcohols and embedded in paraffin wax. Sections of 3 µm were stained with hematoxylin-eosin (HE) for histopathological evaluation. To evaluate mucus production, sections of intestine were stained with alcian blue. Positively stained goblet cells were counted randomly in five fields per sample at 40x magnification, and the means were submitted to statistical analysis.

Villi height and crypt depth were measured randomly on thirty villi using a MOTIC Image Plus 2.0 ML[®] image analysis system. The numbers of lymphocytes, plasma cells, and eosinophils were counted randomly on three fields per sample at 40x magnification. The number of mitotic figures was counted in 20 fields per slide using 40x magnification. The means of intestinal morphometry, number of goblet cells, inflammatory infiltrate and mitosis were utilized for statistical analysis.

3) Immunohistochemical assessment

E-cadherin expression was analyzed on formalin-fixed embedded intestinal sections to evaluate intestinal cell adherens junctions. Tissue sections were deparaffinized with xylene and dehydrated

Table 10 : Origins and dilutions of primary antibodies used for detecting tight junction proteins and β -actin by Western blotting

Protein	Origin	Dilution
E-Cadherin (24E10) Rabbit mAb	Cell Signaling Technology® - Massachusetts, USA (ref. 3195)	1:500
Rabbit anti-Occludin (672381A)	Invitrogen Corporation® - California, USA (ref. 71-1500)	1:500
β -actin mAB MOUSE (8H10D10)	Cell Signaling Technology® - Massachusetts, USA (ref. 3700)	1:1000

Table 11 : Nucleotide sequences of primers for real-time PCR

GENE	PRIMER SEQUENCE	GENBANK NO.	REFERENCES
RPL32	F (300 nM) TGCTCTCAGACCCCTTGTGAAG R (300 nM) TTTCCGCCAGTTCCGCTTA	NM_001001636	Pinton <i>et al.</i> (2010)
β 2- μ globulin	F (900 nM) TTCTACCTTCTGGTCCACACTGA R (300 nM) TCATCCAACCCAGATGCA	NM_213978	Devriendt <i>et al.</i> (2009)
IL-12p40	F (300 nM) GGTTTCAGACCCGACGAACCTCT R (900 nM) CATATGGCCACAATGGGAGATG	NM_214013	Devriendt <i>et al.</i> (2009)
IL-8	F (300 nM) GCTCTCTGTGAGGCTGCAGTTC R (900 nM) AAGGTGTGGAATGCGTATTTATGC	NM_213867	Grenier <i>et al.</i> (2011)
IL-1 β	F (300 nM) GAGCTGAAGGCTCTCCACCTC R (300 nM) ATCGCTGTCATCTCCTTGAC	NM_001005149	Devriendt <i>et al.</i> (2009)
MIP-1 β	F (300 nM) AGCGCTCTCAGCACCAATG R (300 nM) AGCTTCCGCACGGTGTATG	AJ311717	Grenier <i>et al.</i> (2011)
IL-6	F (300 nM) GGCAAAGGGAAAGAATCCAG R (300 nM) CGTTCTGTGACTGCAGCTTATCC	NM_214399	Grenier <i>et al.</i> (2011)
IFN- γ	F (300 nM) TGGTAGCTCTGGGAAACTGAATG R (300 nM) GGCTTTGCGCTGGATCTG	NM_213948	Royae <i>et al.</i> (2004)
TNF- α	F (300 nM) ACTGCACTTCGAGTTATCGG R (300 nM) GGCGACGGGCTTATCTGA	NM_214022	Meissonnier <i>et al.</i> (2008)
IL-2	F (300 nM) GCCATTGCTGCTGGATTAC R (300 nM) CCCTCCAGAGCTTTGAGTTC	AY294018	Meurens <i>et al.</i> (2008)
IL-10	F (300 nM) GGCCAGTGAAGAGTTTCTTTC R (300 nM) CAACAAGTCGCCCATCTGGT	NM_214041	Present study

through a graded ethanol series. The sections were placed in a microwave-resistant container and immersed in EDTA buffer (pH 9.0). Endogenous peroxidase activity was blocked by incubation in methanol/H₂O₂ solution. After adding the primary antibody, the secondary antibody (Kit Super Picture™ Zymed) was applied. The sections were incubated with the primary antibody (Zymed anti-E-cadherin Clone 4A2C7 - diluted 1:50) at 4°C overnight, followed by the addition of a chromogen (3,3'-diaminobenzidine). Finally, the tissue sections were counterstained with hematoxylin and mounted on coverslips using a permanent mounting medium. Tissue sections were examined, and the proportion of samples expressing E-cadherin was estimated. Each sample was assessed as showing either normal or reduced staining. Normal staining was considered when a homogeneous and strong basolateral membrane staining of enterocytes was detected. Heterogeneous and weak staining was considered to indicate reduced expression.

4) Western Blotting

Proteins were extracted from ileum and assayed as described previously (Schaffner and Weissmann, 1973). Briefly, the extraction was carried out on ice in extraction buffer. The protease inhibitors (antipain, pepstatin, benzamidine, AEBSF, aprotinine and leupeptin) were added to the extraction buffer just before use. Extracts tissue proteins were then separated by SDS-PAGE electrophoresis. Equal amounts of proteins were loaded on a 12,5% acrylamide gel. Migration was conducted in a 250 mM Tris buffer (pH 7.6) containing 1% SDS and 1,92 M Glycine. After separation, proteins were transferred onto Optitran BA-S 83 membrane (Whatman®, Germany). The primary antibodies against E-Cadherin, Occludin and β -actin used in this study are presented in **Table 10**. Expression of β -actin was used for checking the equal protein load across gel tracks. Band densities were obtained by scanning the membranes using Odyssey® Infrared Imaging System (LI-COR Biosciences, USA). Density data were standardized within membranes by expressing the density of each band of interest relative to that of β -actin in same lane.

5) Determination of the expression of mRNA encoding for cytokines by real-time PCR

Tissue RNA was processed in 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, integrity and quality of RNA were determined spectrophotometrically (O.D.₂₆₀) using Nanodrop ND1000 (Labtech International, Paris, France). In addition to this inspection, 200 ng of RNA was analyzed by electrophoresis. The reverse transcription of 2 μ g of total RNA was performed using M-MLV reverse-transcriptase, Rnasin® plus (Promega,

Charbonnière, France) and random primers (Invitrogen, Cergy Pontoise, France) (5 min at 37°C, 1 hour at 42°C, 15 min at 70°C) as already described (Oswald *et al.*, 2001). Real-time PCR assays were performed on 8 ng of cDNA (RNA equivalent) in a 25- μ l volume reaction per well using Power SYBR® Green PCR Master Mix as the reporter dye and the automated photometric detector ABI Prism 7000 Sequence Detection System for data acquisition (Applied Biosystems, Courtaboeuf, France). The amplification conditions were as follows: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. RNA non-reverse transcript was used as a non-template control (NTC) for verification that no genomic DNA amplification signal existed. Specificity of PCR products was checked at the end of the reaction by analyzing the curve of dissociation. In addition, the size of amplicons was verified by electrophoresis. The sequences of the primers used are detailed in **Table 11**. Primers for MIP-1 β , IL-8 and IL-6 detection were designed using Primer Express® software (Applied Biosystems). Primers were purchased from Invitrogen (Cergy Pontoise, France). Amplification efficiency and initial fluorescence were determined by the DART-PCR method (Peirson *et al.*, 2003), and the values obtained were then normalized by two housekeeping genes, beta2- μ globulin and ribosomal protein L32 (RPL32); and finally, gene expression was calculated relative to the control group as already described (Le Gall *et al.*, 2009).

6) Statistical analysis

Data were analyzed with Statview software, version 5.0 (SAS Institute Inc, Cary, NC), using ANOVA, Tukey and PLSD Fisher test. *P* values < 0.05 were considered significant.

Figure 5

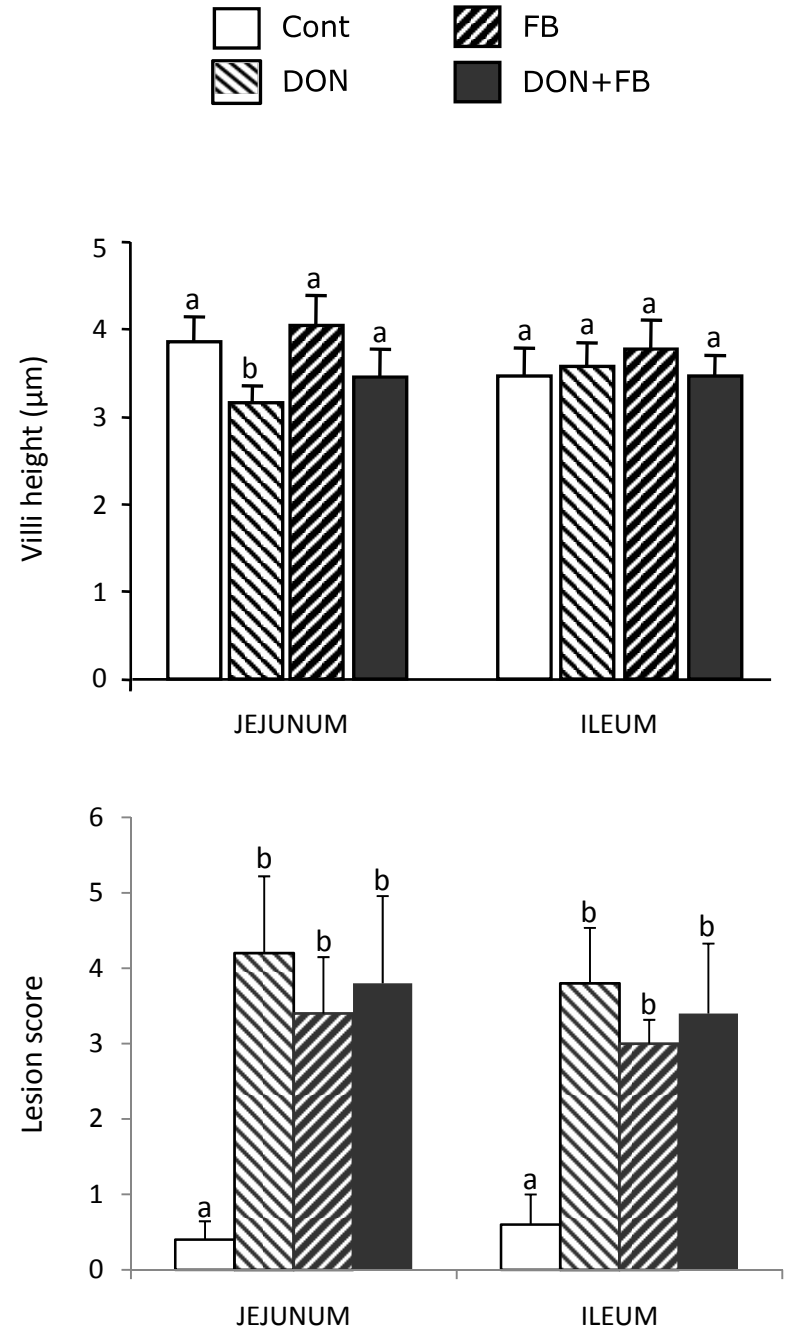
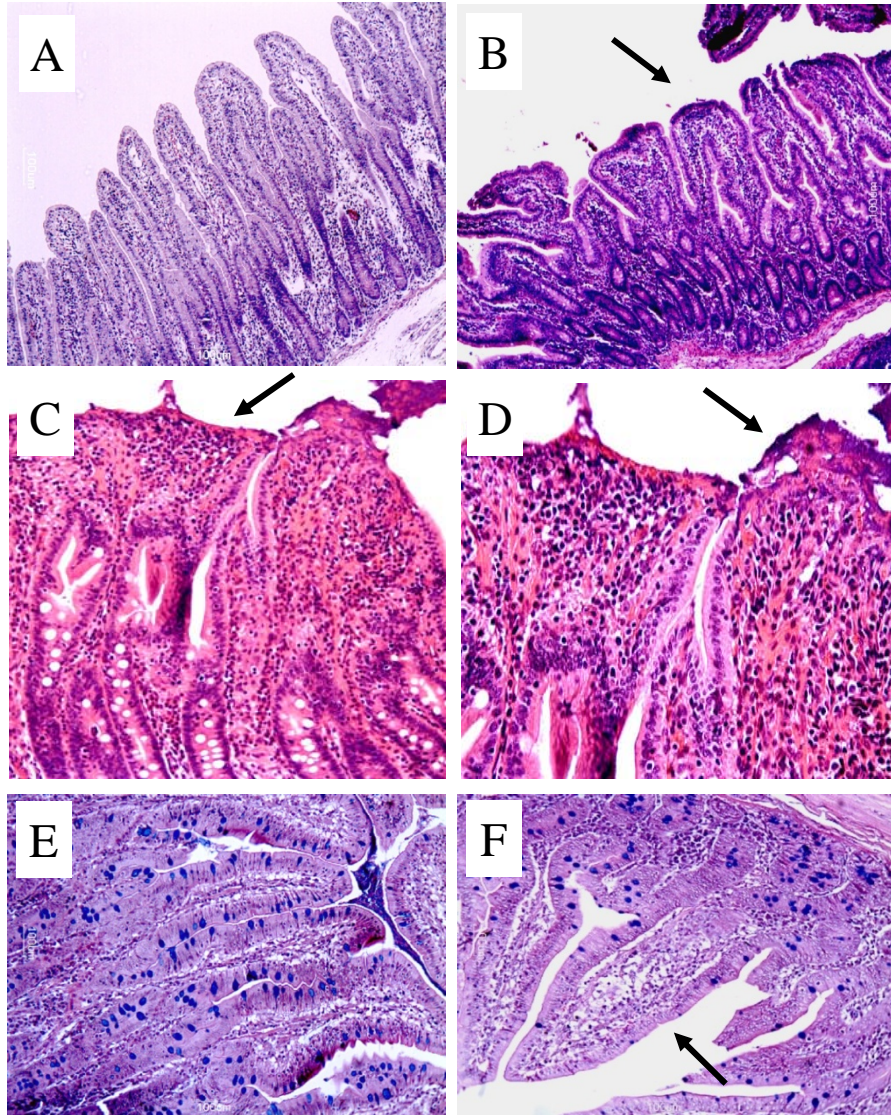
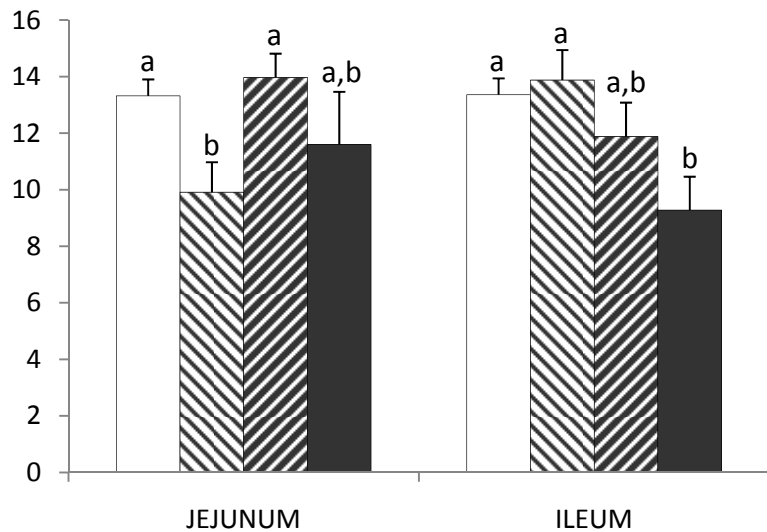


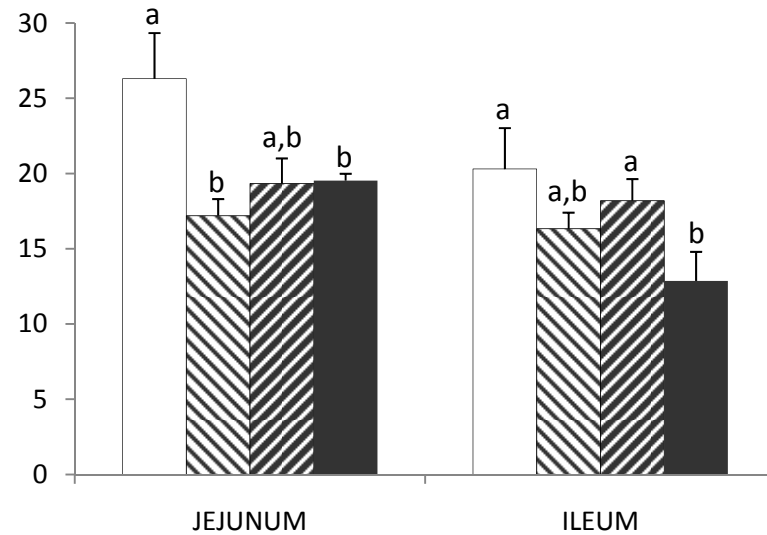
Figure 6

Control DON FB DON+FB

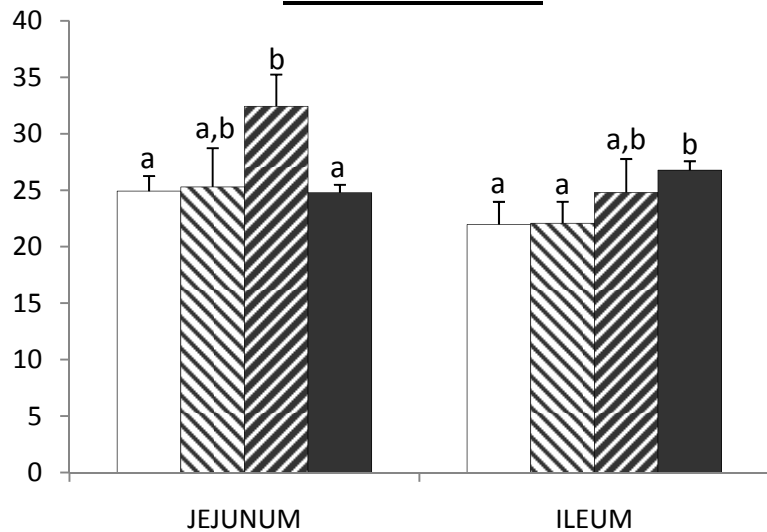
GOBLET CELLS



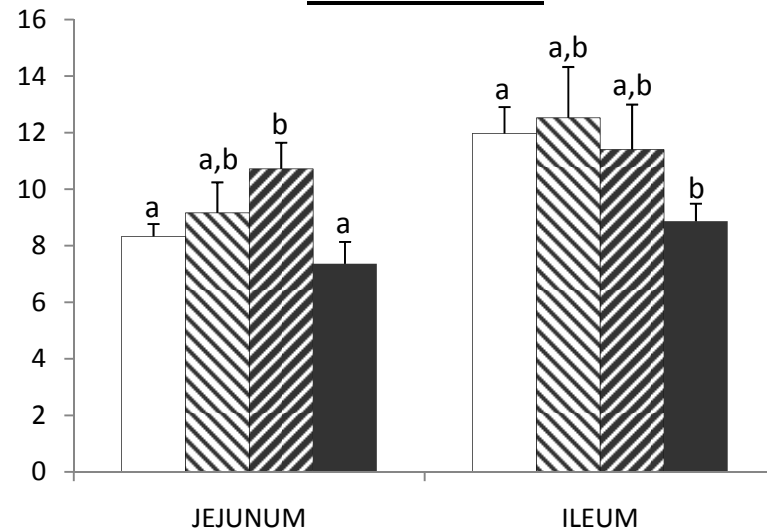
LYMPHOCYTES



PLASMA CELLS



EOSINOPHILS



RESULTS

1) Histomorphometrical analysis

Samples of jejunum and ileum were collected for histomorphometrical analysis. Piglets fed diets contaminated with mycotoxins showed mild to moderate intestinal lesions. The main histological changes observed were lymphatic vessel dilation, mild eosinophil granulation within the cytoplasm of enterocytes, and prominent lymphoid follicles. The lesional score increased for animals fed contaminated diets (**Figure 5**).

Changes in villous height and crypt depth are indicative of enterocyte loss and impaired absorption of nutrients. As shown in **Figure 5**, villi height decreased significantly in the jejunum of the animals that received DON and DON+FB when compared with controls. Focal apical necrosis of villi (**Figures 5C and 5D**) was also observed in the intestine of piglets fed mycotoxins diets. No change in crypt depth was observed in any intestinal region. Goblet cells synthesize and secrete mucin, which is involved in gut physiology. The number of goblet cells decreased (**Figures 5F and 6**) significantly in the DON- and FB+DON-treated animals in the jejunum and ileum.

Inflammatory infiltrate of lymphocytes, plasma cells and eosinophils was observed in all regions of the intestine. In the groups treated with mycotoxins, a reduction in lymphocytic infiltrate was observed in all regions of the intestine. However, only the jejunum was significantly affected (**Figure 6**). The number of eosinophils in the lamina propria decreased significantly in the ileum of the group that received DON+FB. The mean number of inflammatory cells per field in each region of the intestine is summarized in **Figure 6**.

2) Cell proliferation

Cell proliferation was estimated by counting the number of mitosis figures in enterocytes on HE slides. The mean number of mitosis figures in the jejunum were 2.36 ± 1.64 in the control group, 1.73 ± 1.35 in the DON treated group, 1.66 ± 1.11 in the FB treated group and 1.91 ± 1.19 in the DON+FB treated group. In the ileum the mean number were 1.75 ± 1.26 , 1.78 ± 1.46 , 1.62 ± 1.17 and 1.89 ± 1.11 for the control group, DON treated group, FB treated group and DON+FB treated group, respectively. A significant decrease ($p < 0.05$) was verified in the jejunum of the groups fed mono-contaminated diets.

Figure 7

JEJUNUM

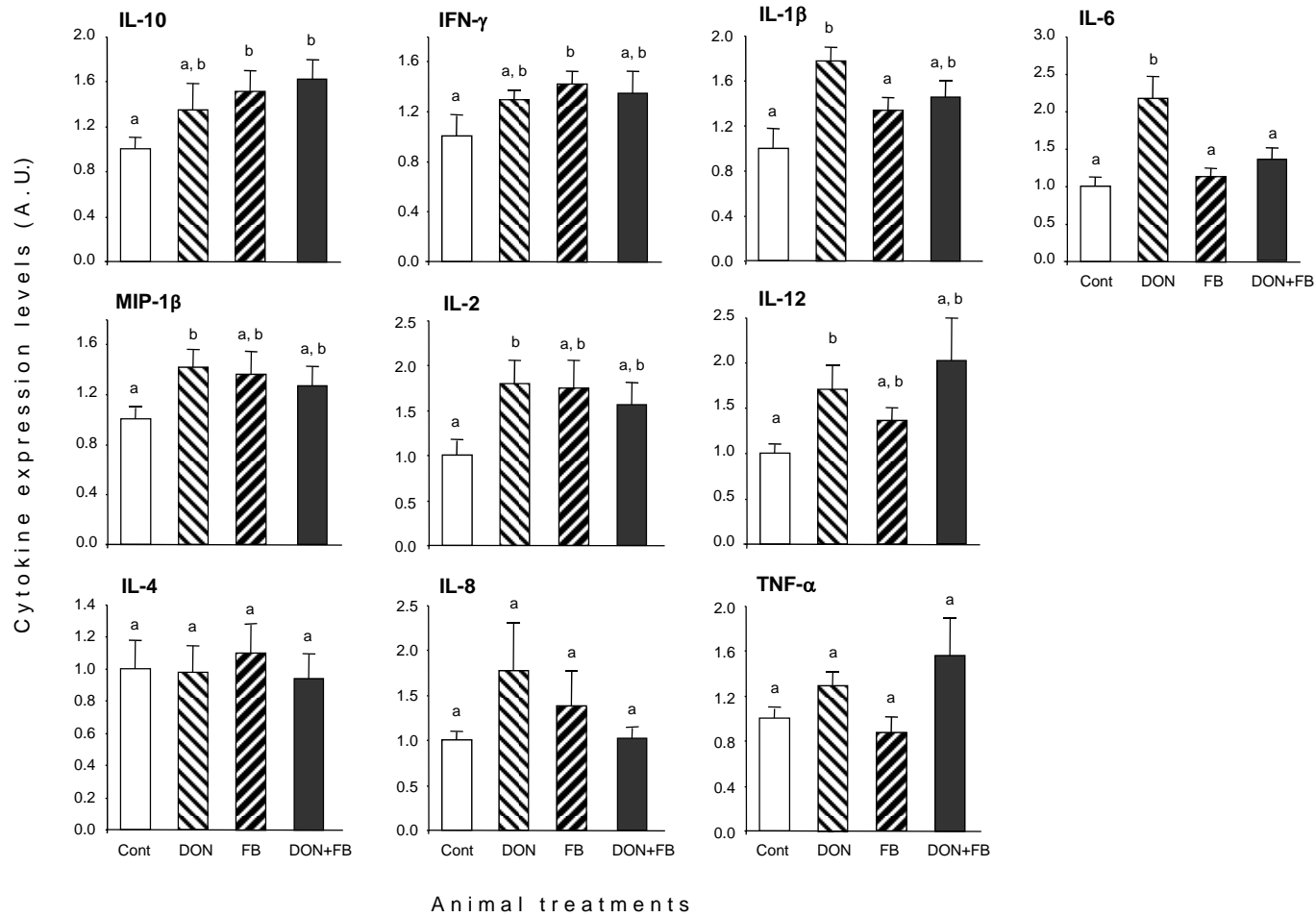


Figure 8

ILEUM

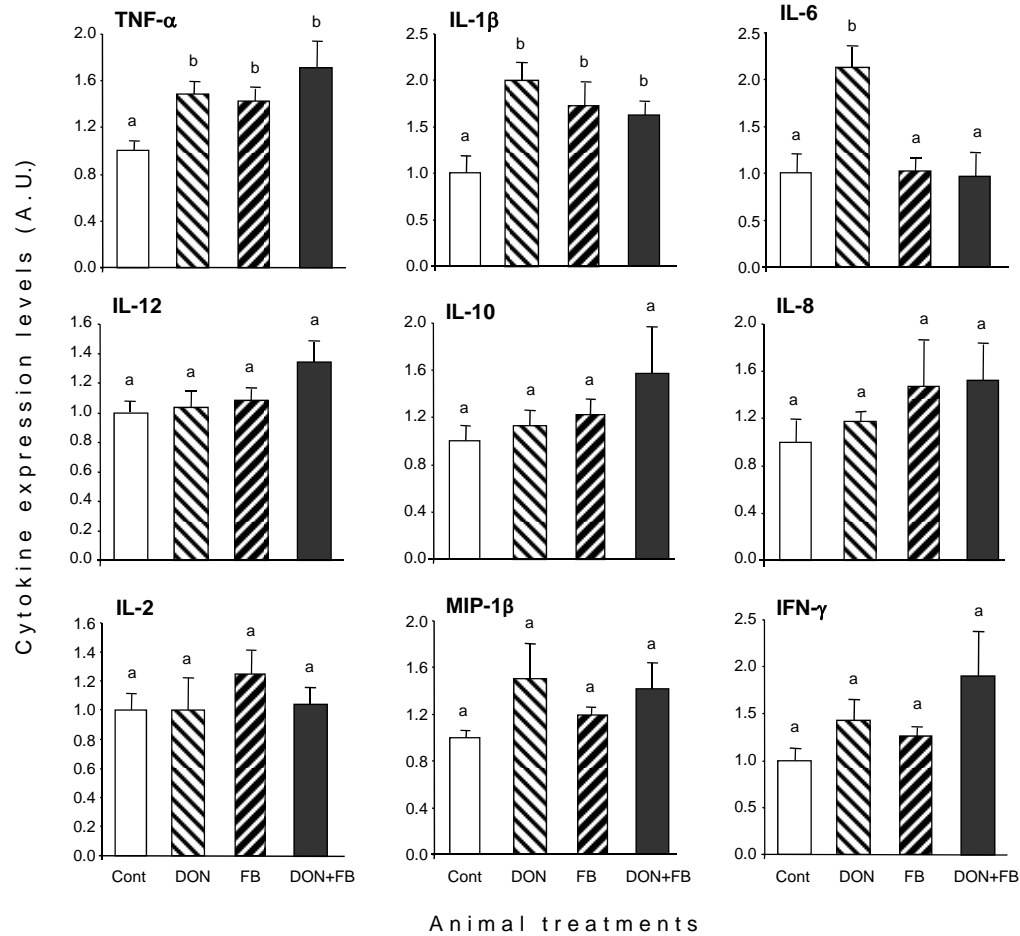
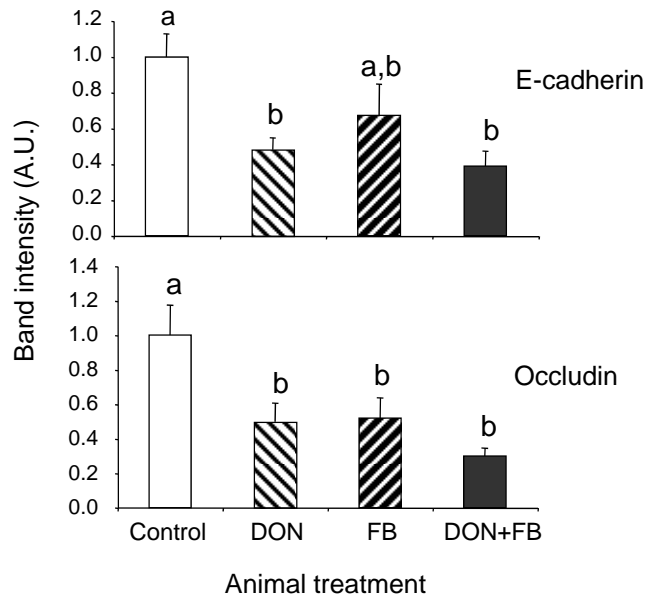
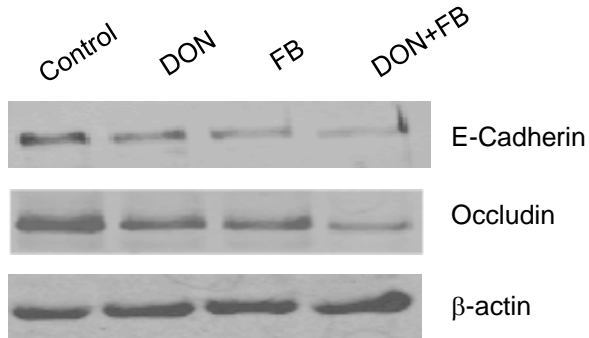
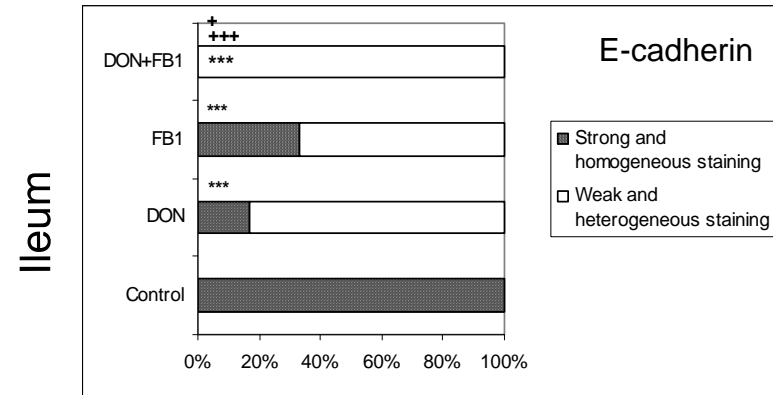
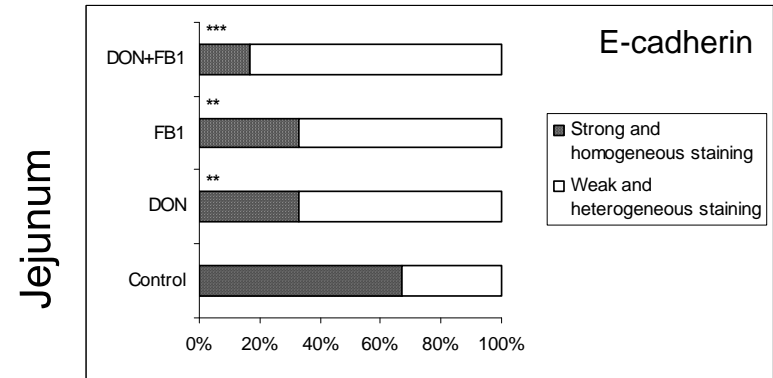
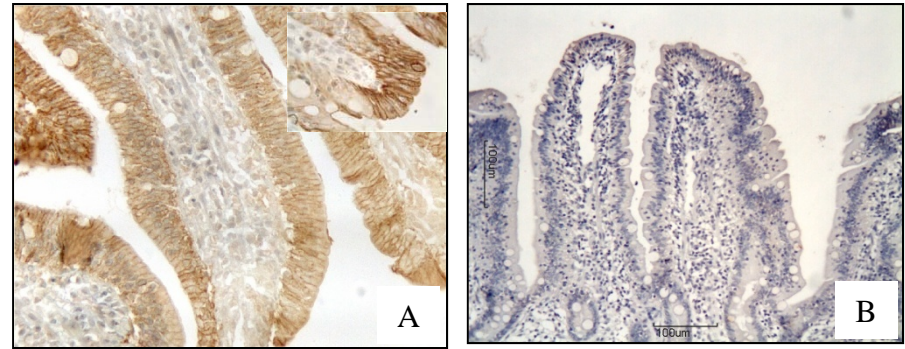


Figure 9

WESTERN BLOT ANALYSIS



IMMUNOHISTOCHEMICAL ANALYSIS



3) Intestinal immune response

To evaluate the mechanisms of porcine intestinal defense against multi-mycotoxin exposure, we quantified the expression of genes coding for pro-inflammatory cytokines after chronic ingestion of mycotoxin co-contaminated feed. Statistically significant changes in gene expression in different regions relative to control and treated animals are shown in **Figures 7 and 8**.

In general, the jejunum showed greater induction of cytokines as more cytokines were expressed in this region after mycotoxin exposition. DON treated group demonstrated significant increase in mRNA encoding for IL-1 β , IL-6, MIP-1 β , IL-2, and IL-12p40 in the jejunum, whereas FB induced a significant increase in IL-10 and IFN- γ . In the ileum, all treatments induced significant increases in TNF- α and IL-1 β .

4) Expression of adherens junction proteins

The expression of E-cadherin and occludin, two junction proteins involved in the adherence and permeability of enterocytes, was analyzed in the ileum of animals by western blotting (WB). After normalization by the housekeeping gene β -actin, the data revealed a significant decrease of expression of both proteins compared to the control animals. In animals exposed to the co-contaminated diet, the defective expression of E-cadherin and occludin was more pronounced (**Figure 9**).

As WB indicated a significant decrease in the total amount of E-cadherin expression in the ileum, we decided to evaluate the expression of this protein in enterocytes, using an immunohistochemical assay. The expression of E-cadherin in the jejunum and ileum was significantly reduced in the groups that received mono-contaminated or co-contaminated diets (**Figure 9**). The reduction was most pronounced in the ileum of the DON+FB group, in which all animals showed weak and irregular staining.

DISCUSSION

Co-contamination of grains and feed is frequently reported by analytical laboratories around the world. In fact, the occurrence of single-mycotoxin contamination seems to be rare (Kubena *et al.*, 1997). However, most studies in pigs have employed mono-contaminated diets. Fumonisin (FB) and deoxynivalenol (DON) exert effects on different mechanisms in the intestinal tract. FB blocks sphingolipid synthesis, which is essential for the formation of cell membranes, while DON inhibits protein synthesis via ribosome binding (Scott, 1993). Enterocytes have a continuous replicating cycle, making the intestine a main target for the toxins' action (Bouhet *et al.*, 2004). Considering the co-contamination of FB and DON in feed and the intestine as a main target for toxin action, it is important to know whether these mycotoxins have additive, synergistic or antagonistic effects in the intestines of pigs.

The main histological findings observed were villi flattening and a reduction in the number of goblet cells. Decreased villi height was significant only in the jejunum with the diet mono-contaminated with DON. Similar changes were observed in *in vivo* and *ex vivo* studies with DON exposition (Awad *et al.*, 2006; Obremski *et al.*, 2008; Kolf-Clauw *et al.*, 2009). The mode of toxic action of DON is inhibition of protein synthesis, thus primarily affecting rapidly dividing cells such as those of the gastrointestinal tract and immune system (Leeson *et al.*, 1995). The villi flattening in the jejunum is likely due to impairment of cell proliferation, as could be observed by the decrease in the number of mitotic figures in the same region.

Swine are considered as possessing an intermediate level of sensitivity to fumonisins, and levels under 10 mg/kg in the total ration are considered safe (USFDA, 2001). There were no differences in villi height or crypt depth between the FB group and the control group. The absence of effects in the intestine of the FB group is probably related to the low dose (6 mg/kg) used, which is associated with poor transport of this mycotoxin across the epithelium of the intestine (Bouhet and Oswald, 2007). Villous fusion and atrophy were observed in the intestines of pigs treated with diets containing high levels of FB1 (Dilkin *et al.*, 2004; Piva *et al.*, 2005).

Mucin production can be estimated by the number of goblet cells in the intestinal wall. Considering that goblet cells originate from intestinal stem cells, the decrease observed here could be explained by the inhibitory effect of DON on protein synthesis in cells under proliferation. In domestic animals, hyperplasia of intestinal goblet cells has been observed in piglets receiving 10 to 30 mg FB1/kg of feed for 4 weeks and in broiler chicks receiving 300 mg FB1/kg of feed for 2 weeks (Dilkin *et al.*, 2004; Brown *et al.*, 1992). In the present study, a decrease in the number of goblet cells was shown in the DON-contaminated and co-contaminated diets in the jejunum, while in the FB-

treated group there was no difference. Although not significant, it seems that an antagonistic interaction between FB and DON affects the number of goblet cells as FB interfered with DON's effect of decreasing the number of goblet cells.

Controversial results have been reported with respect to intestinal proliferation in *in vivo* and *in vitro* studies of mycotoxicosis. In this study, we have evaluated cell proliferation by counting mitotic figures in intestinal crypts, which showed a significant decrease in the jejunum of the FB and DON-treated groups. Theumer *et al.* (2002) observed that subchronic experimental FB1 mycotoxicosis increased the number of mitotic figures in rat intestinal crypts, while Obremski *et al.* (2008) observed numerous dividing cells within the glandular epithelium in pigs fed a diet containing DON, zearalenone and T-2 toxin. Using *in vitro* models, Bouhet *et al.* (2004) verified a decrease in the proliferation of undifferentiated porcine epithelial cells treated with FB1 due to a blockade in the G0/G1 cell cycle phase. Because DON acts as a protein synthesis inhibitor, it is expected that cells with high turnover such as crypt enterocytes will be a target for the toxin's action. Recently, it was shown in Caco-2 cells that DON causes a concentration-dependent decrease in total protein content associated with a reduction in the incorporation of [3H]-leucine, demonstrating its inhibitory effect on protein synthesis (Van de Walle *et al.*, 2010). The modes of action of FB and DON to inhibit cell proliferation are quite different. FB lead to accumulation of free sphingoid bases that are pro-apoptotic, cytotoxic and growth cell inhibitors (Lalles *et al.*, 2010), while DON acts as a protein synthesis inhibitor.

Mononuclear and eosinophil inflammatory infiltrate has been reported in FB mycotoxicosis in several species. In addition, proliferation of lymphoid nodules in the ileum and cecum was also observed (Theumer *et al.*, 2002; Piva *et al.*, 2005). We have shown that the number of lymphocytes in the lamina propria decreased significantly in the jejunum of the treated groups, mainly in the DON treatment. Studies of macrophages and lymphocytes have shown that the trichothecene-mediated immunosuppressive effect was associated with induction of apoptosis (Rocha *et al.*, 2005). DON initiates its toxic effects by inducing ribotoxic stress, which activates c-jun terminal kinase and p38 mitogen-activated protein kinase, stimulating apoptosis. DON was particularly potent in this respect, elevating caspase levels more than twelvefold (Shifrin and Anderson, 1999). Because lymphoid cells are constantly renewing, lymphocytes could be particularly sensitive to DON. It is interesting that our results show that even low doses of both mycotoxins induced a decrease in lymphocyte infiltration. This effect also occurred in the jejunum along with other histological alterations, supporting the finding that this region is a main target for toxin action.

In the present study, the chronic treatment of piglets with FB, DON or both by the oral route induced activation of the proinflammatory cytokine network in the intestine. Increases in the mRNA

levels of the nine cytokines evaluated (TNF- α , MIP-1 β , IFN- γ , IL-1 β , IL-2, IL-6, IL-8, IL-10, IL-12p40) were observed in the jejunum and ileum. A systemic intestinal cytokine mRNA profile indicative of macrophage and TH1 activation has been reported after a single oral dose of DON in mice (Azconaolivera *et al.*, 1995). It has been established that the intestine has its own immune network, which can cause localized induction of various cytokines and chemokines (Stadnyk, 2002). Low-dose exposure to trichothecenes generally results in stimulatory effects, causing increased resistance to pathogens, elevated serum IgA levels (mediated by IL-6) and up-regulated expression of genes encoding for cytokines and chemokines; on the other hand, high-dose exposure causes immunosuppression, characterized by decreased resistance to pathogens, reduced IgM and IgG levels and impaired and delayed hypersensitivity responses (Pestka *et al.*, 2004; Shifrin and Anderson, 1999; Rocha *et al.*, 2005). This diverse spectrum of effects is likely to result from differences in the intensity and duration of kinase signaling and the resultant gene expression (Pestka, 2010).

Although all of the cytokines showed elevated expression, only TNF- α and IL-1 β showed increases in all treatments. A dose-dependent increase in TNF- α mRNA levels was observed in fumonisin B1-treated murine peritoneal macrophages and in the liver (Dugyala *et al.*, 1998; Bhandari *et al.*, 2002). TNF- α and IL-1 β are known to stimulate the production of IL-8 and other cytokines (Fiers, 1991; Azconaolivera *et al.*, 1995), but also to induce apoptosis via the receptor-ligand-mediated mechanism (Van Cruchten and Van den Broeck, 2002). We hypothesize that, besides the known apoptotic mechanisms, FB and DON could induce TNF-mediated lymphocyte apoptosis in the intestine, which could explain the decrease in the number of these cells observed in exposed pigs. A relationship between clinically relevant concentrations of TNF- α (1–10 ng/ml) and IL-1 β and an increase in intestinal tight junctions (TJ) permeability has been demonstrated in Caco-2 cells, mediated by an increase in myosin light chain kinase (MLCK) protein expression (Ye *et al.*, 2006; Al-Sadi *et al.*, 2008). With regard to this association, we can consider that the increased levels of TNF- α and IL-1 β verified after the ingestion of DON and FB1 could also contribute to TJ intestinal barrier defects.

In a previous study, we observed alterations in enterocyte TJ in *in vitro* and *in vivo* DON exposure due to reduced expression of claudins (Pinton *et al.*, 2009). Considering that paracellular permeability of the intestinal epithelium is regulated by intercellular TJ and adherens junctions (AJ), we wish to know if other transmembrane proteins, as occludin and E-cadherin were also affected by mycotoxins exposition. The AJ, which is immediately subjacent to the TJ, comprises the transmembrane protein E-cadherin and the associated cytoplasmic proteins, the catenins, and plays an important role in the formation of TJs.

The potential effects of DON, FB and both mycotoxins on AJ function were analyzed using western blotting and immunohistochemical assays. DON and DON+FB treatments altered total cellular amount of E-cadherin, whereas all treatments induced changes in the amount of occludin. A reduction of E-cadherin and occludin, therefore, suggests a loss of enterocytes' adhesive properties that permits increased intestinal permeation by toxic luminal antigens, promoting intestinal inflammation. However, to the best of the author's knowledge, no data have been reported concerning reduced expression of E-cadherin in the intestinal tract after ingestion of a mycotoxin mono- or co-contaminated diet. It has been suggested that derangement of the apical junction complex of the intestinal epithelium may be involved in the generation of an aberrant immune response due, for example, to a loss of epithelial cell polarity or an abnormal delivery of antigens via a paracellular pathway (Hershberg and Mayer, 2000; Soderholm *et al.*, 2002).

Considering that the intestine is the first tissue involved in the absorption of mycotoxins, a pro-inflammatory effect, as we observed in this study, is expected as a consequence of TJ barrier dysfunction. Increased intestinal permeability could allow luminal antigens to enter into the mucosa, inducing proinflammatory cytokine expression. Considering that TNF- α , IFN- γ and IL-1 β caused decreases in ZO-1 and occludin protein expression in Caco-2 cells (Han *et al.*, 2003; Cui *et al.*, 2010), we can hypothesize that increased levels of these cytokines also affect E-cadherin expression.

Multi-contamination with low doses of mycotoxins is more likely to occur in natural contamination, but there are few data indicating the effects of co-contaminated mycotoxin diets in pigs. Taken together, the present data provide strong evidence that chronic ingestion of low doses of mycotoxins induces histological changes in an *in vivo* model, potentiates cytokines secretion, and affects the expression of proteins involved in cell adhesion, suggesting that ingestion of DON, FB and both toxins could predispose animals to infections by enteric pathogens through alteration of intestinal barrier function. Moreover, the response observed for the co-contaminated diet was similar to the response to mono-contaminated diets for the majority of the parameters evaluated, indicating no synergistic effect in the intestine.

FIGURES LEGEND

Figure 5 : Effect of individual and combined DON and FB exposure on jejunum and ileum histology.

Pigs received a control diet (□), or a DON-contaminated diet (▣), or a FB-contaminated diet (▤), or a contaminated diet with both toxins (■).

(A) Jejunum of a control piglet and (B) DON treated piglet. Villi flattening (arrow). HE. 10x. (C) Villi apical necrosis (arrow). HE. 10x and (D) Bacterial adhesion in the area with necrosis (arrow). HE. 40x. (E) Globet cells in a control piglet and (F) Decrease in the number of globet cells (arrow). Alcian-Blue. 20x.

Morphometry of villi height in the jejunum and ileum. Data are mean height (μm) \pm SEM for 6 pigs. Lesional score after histological examination according to the occurrence and the severity of lesions. Values are mean scores for 6 pigs. Statistics are mentioned when significant changes were observed.

Figure 6 : Effect of individual and combined DON and FB exposure on the number of inflammatory cells and globet cells in jejunum and ileum.

Pigs received a control diet (□), or a DON-contaminated diet (▣), or a FB-contaminated diet (▤), or a contaminated diet with both toxins (■).

Values are mean number of inflammatory and globet cells \pm SEM for 6 pigs. Statistics are mentioned when significant changes were observed.

Figure 7 : Effect of individual and combined DON and FB exposure on jejunum mRNA expression of cytokines.

Pigs received a control diet (□), or a DON-contaminated diet (▣), or a FB-contaminated diet (▤), or a contaminated diet with both toxins (■).

Quantification of the relative cytokine mRNA level for each sample is expressed in arbitrary units (A.U). Values are mean \pm SEM for 5 pigs. Statistics are mentioned when significant changes were observed.

Figure 8 : Effect of individual and combined DON and FB exposure on ileum mRNA expression of cytokines.

Pigs received a control diet (□), or a DON-contaminated diet (▣), or a FB-contaminated diet (▤), or a contaminated diet with both toxins (■).

Quantification of the relative cytokine mRNA level for each sample is expressed in arbitrary units (A.U). Values are mean \pm SEM for 5 pigs. Statistics are mentioned when significant changes were observed.

Figure 9 : Effect of individual and combined DON and FB exposure on intestinal expression of E-cadherin and occludin.

Pigs received a control diet (□), or a DON-contaminated diet (▣), or a FB-contaminated diet (▤), or a contaminated diet with both toxins (■).

Quantification of the relative protein level for each sample is expressed in arbitrary units (A.U). Values are mean \pm SEM for 5 pigs.

(A) Jejunum of a control piglet. Strong and homogeneous immunoreactivity to E-cadherin. Immunoperoxidase, 20x. (B) Jejunum of a piglet fed DON-contaminated diet. Weak immunoreactivity to E-cadherin. Immunoperoxidase, 10x.

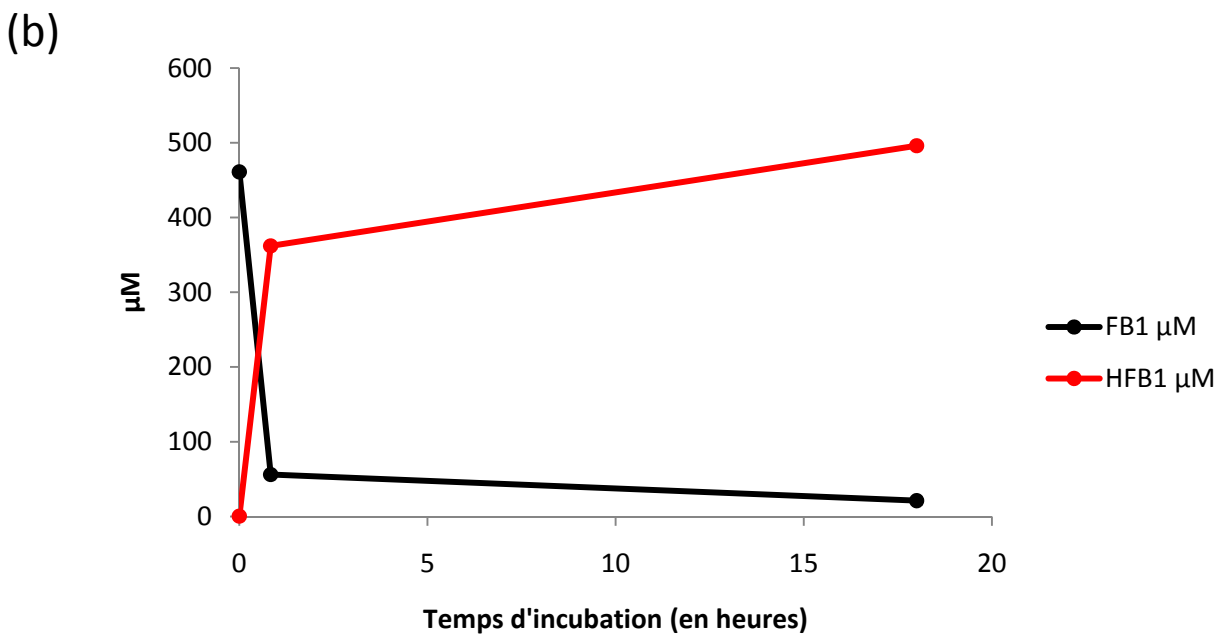
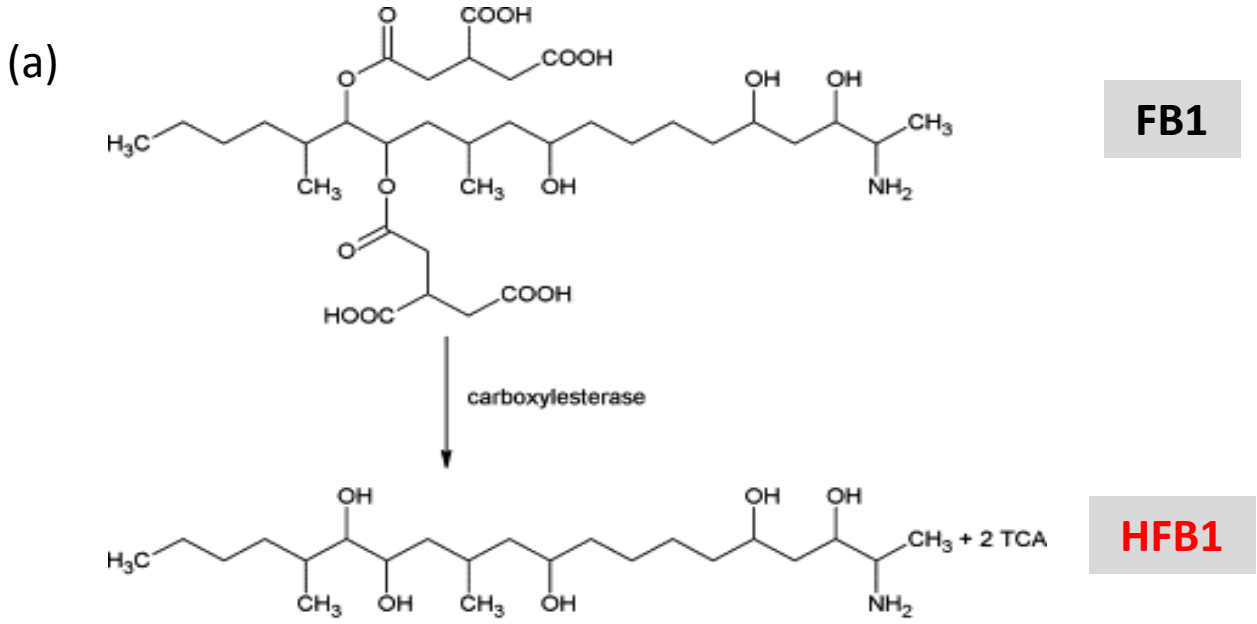
Quantification of the expression of immunoreactivity to E-cadherin (%) in intestinal slides. Statistics are mentioned when significant changes were observed.

CHAPITRE 2

Évaluation de la toxicité du produit d'hydrolyse de la Fumonisine B1 chez le porcelet

Figure 10 :

- (a) Réaction de deestérification par la carboxylestérase sur la Fumonisine B1 (FB1), résultant en Fumonisine B1 totalement hydrolysée (HFB1)
- (b) Cinétique *in vitro* d'hydrolyse de la Fumonisine B1 par la carboxylestérase



RESUME DE L'ETUDE

Comme déjà souligné dans le présent manuscrit, les fumonisines et plus particulièrement la fumonisine B1 (FB1) sont des mycotoxines relativement stables et difficiles à éliminer par des traitements physiques et chimiques, et des phénomènes d'adsorption.

Ainsi, notre partenaire industriel BIOMIN a développé au cours des dernières années une approche de détoxification de la fumonisine B1 par voie enzymatique. Cette réaction de biotransformation a été rendue possible après le screening et l'identification d'une souche bactérienne capable de dégrader complètement la FB1. Cette souche a initialement été isolée à partir de compost et s'est révélé appartenir au genre *Sphingopyxis*. L'analyse du cluster de gènes de cette souche a permis d'isoler un gène encodant pour une carboxylestérase, et responsable du catabolisme de la FB1. Ainsi, après expression de ce gène en système hétérologue, une dégradation complète et rapide de la FB1 a été obtenue *in vitro*, résultant en la production de fumonisine B1 totalement hydrolysée (**Figure 10**). De plus, ce processus de détoxification enzymatique semble agir indépendamment de la présence d'oxygène, tels que dans des conditions limitées en oxygène, à savoir les fourrages ou le tractus intestinal des animaux.

Afin d'évaluer la toxicité du produit d'hydrolyse de la FB1 (HFB1) et étant donné qu'aucune étude n'avait reporté l'effet de la HFB1 sur des animaux domestiques, nous avons traité oralement des porcelets durant deux semaines avec une solution tampon contrôle, une solution de HFB1 et une solution avec la molécule mère FB1. La solution HFB1 a été obtenue après hydrolyse totale de la FB1, avec la carboxylestérase produite par BIOMIN. Afin de comparer la toxicité entre FB1 et HFB1, nous avons traité les animaux avec des doses élevées. En effet, la FB1 a été administrée à 2 mg/kg pv/jour, ce qui correspond dans nos conditions à une alimentation contaminée approximativement avec 40 mg FB1/kg d'aliment. La HFB1 a quant à elle été administrée de façon équimolaire à 1,1 mg/kg pv/jour.

La toxicité a principalement été évaluée sur le foie et sur le tractus gastro-intestinal. Peu de données ont été rapportées sur l'effet de la FB1, et encore moins sur l'effet de la HFB1, sur l'intestin. Ainsi, cette étude apporte également de nouveaux éléments sur la toxicité intestinale aiguë de ces substances. De manière générale, nos données confirment chez le porc que l'hydrolyse de la FB1 réduit fortement sa toxicité. Tout d'abord, le ratio des bases sphingoides Sa/So (Sa, sphinganine et So, sphingosine), considéré comme un bon marqueur de toxicité, était similaire à celui des animaux du groupe contrôle, contrairement à ceux des animaux traités avec la FB1, et ce, quelque soit la matrice biologique (plasma, foie, reins et poumons). Au niveau hépatique, les analyses n'ont pas

révélé de lésions microscopiques, et très peu d'effets sur des marqueurs de fonction et d'inflammation lorsque les porcelets avaient ingéré la solution de HFB1, à l'inverse de ceux ayant ingéré la solution de FB1. De plus, l'intestin ne présentait pas ou très peu de lésions et d'altérations des villosités, contrairement aux intestins des animaux du groupe FB1. De la même manière, l'immunosuppression reflétée par une diminution de l'expression des cytokines, n'a pas été observée le long de l'intestin grêle après administration de la HFB1.

Hydrolysis of fumonisin B1 strongly reduced the toxicity for piglets at both intestinal and hepatic levels

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Abbreviations: FB1, fumonisin B1 ; HFB1, hydrolyzed fumonisin B1 ; Sa, sphinganine ; So, sphingosine

Keywords: fumonisin, hydrolyzed fumonisin, sphingoid bases, liver, digestive tract

ABSTRACT

Fumonisin, produced by *Fusarium verticillioides*, are of major worldwide concern in terms of ubiquitous and frequency distribution, and toxicity. The need to restrict in feeds the levels of fumonisins, and thereby to ensure animal health and productivity, is a continuous challenge. Biotechnological approaches through microorganisms or enzymes can provide an alternative solution. In this study, the comparative toxicity of fumonisin B1 (FB1), the predominant fumonisin in this family, and its hydrolyzed form (HFB1) was investigated in swine over a short-term trial. Eighteen 5-week-old animals were orally and daily treated for two weeks with three different solutions, a control buffer solution, a FB1 solution (2 mg/kg body weight/day) and a HFB1 solution (equimolar to 2 mg FB1/kg body weight/day). The HFB1 solution was initially obtained after a deesterification reaction on FB1 solution, through the use of a carboxylesterase. Results were reported in terms of liver and intestinal toxicity. Besides, exposure was assessed by determining the sphingoid bases content in plasma and liver samples. On contrary to FB1, HFB1 did not trigger hepatotoxicity as revealed by biochemical compounds, inflammatory markers and microscopical lesions. Similarly, HFB1 did not impair the intestinal integrity and intestinal immune system, as evaluated in different segments of gastro-intestinal tract by villi and lesions characterization and cytokines expression. These results are supported by the ratio of sphinganine and sphingosine in biological samples, which were not affected at intermediate and end points of the *in vivo* experiment. It can be concluded that hydrolysis of FB1 strongly reduced toxicity in piglets.

INTRODUCTION

Fumonisin is a family of mycotoxins produced by *Fusarium verticillioides* (formerly *F. moniliforme*), which are common fungal contaminants of corn and some other grains (Marasas, 2001). Fumonisin exerts complex biological effects. The toxic effects of fumonisin range from hepatotoxicity and renal toxicity to species-specific effects such as pulmonary edema in pigs and leukoencephalomalacia in horses (Voss *et al.*, 2007). In humans, exposure to fumonisin has been linked to esophageal cancer and neural tube defects (Voss *et al.*, 2007; Waes *et al.*, 2005). The effect of low doses has been less investigated but revealed reduced performance, pathological alterations of the lungs and an increase in intestinal colonization by opportunistic pathogenic bacteria in piglets (Rotter *et al.*, 1996b; Halloy *et al.*, 2005; Oswald *et al.*, 2003; Haschek *et al.*, 2001).

Fumonisin is structurally similar to sphingoid bases, and was determined to be a potent inhibitor of sphinganine *N*-acyl transferase (ceramide synthase) (Wang *et al.*, 1991). Research since this discovery has provided substantial evidence that the toxicity and carcinogenicity of fumonisin is related to the disruption of sphingolipid metabolism that occurs as a result of inhibition of ceramide synthase (Riley *et al.*, 1993, 1994a; Voss *et al.*, 2007).

As much as 59% of the corn and corn-based products worldwide have been estimated to be contaminated with variable amounts of fumonisin B1 (FB1), the most prevalent of the fumonisin subspecies (Desai *et al.*, 2002; Haschek *et al.*, 2001), and therefore, it is not surprising that livestock farming and feed processing industries are interested in efficient and applicable methods for reducing fumonisin concentrations in animal feed. Most physical and chemical methods, such as thermal food processing, ammoniation or alkali treatment, reduce but do not completely eliminate fumonisin present in feed samples (Norred *et al.*, 1991; Sydenham *et al.*, 1995; Visconti *et al.*, 1996). Besides, most of the methods are difficult to implement into the production process. A new approach consists of finding organisms or enzymes able to metabolize fumonisin and that could lead to the formation of hydrolyzed fumonisin (HFB) (Heinl *et al.*, 2010).

Except in one case in which FB1 metabolism led to a modest amount of HFB1 in vervet monkeys (Shephard *et al.*, 1994), hydrolysis of fumonisin occurs after the well-known alkali treatment named nixtamalization. This process is widely used in regions such as Central and South America, to produce tortillas or other corn products made from corn cooked and steeped in a lime solution (Dombrink-Kurtzman *et al.*, 2000; Palencia *et al.*, 2003; Cortez-Rocha *et al.*, 2002).

As never investigated in domestic animals, we proposed in the present study to compare the toxicity of fumonisin B1 (FB1) and hydrolyzed fumonisin B1 (HFB1) in piglets orally exposed to moderate doses. In terms of toxicity assessment, we also reported effects of these treatments at the

intestinal level, the first compartment of the organism to be exposed to natural contaminants. Furthermore, the number of publications on the effects of FB1 and especially its hydrolyzed form on the gastrointestinal tract is very poor.

MATERIAL & METHODS

1) Animals

All animal experimentation procedures were carried out in accordance with the European Guidelines for the Care and Use of Animals for Research Purposes (Directive 86/609/EEC). Eighteen, 4-week-old weaned female pigs (Pietrain/Duroc/Large-white) were obtained locally. Animals were acclimatized for 1 week in the animal facility of the INRA Laboratory of Pharmacology and Toxicology (Toulouse, France), prior to being used in experimental protocols. Six pigs were allocated to each treatment on the basis of body weight. During the 14-day experimental period, animals were given free access to water and were fed with a basal diet *ad libitum*, as previously described (Grenier *et al.*, 2011).

2) Experimental treatments

Three solutions were prepared and orally administered to piglets. These solutions consisted to one control solution, one FB1 solution and one HFB1 solution. Lyophilized culture material of *Fusarium verticillioides*, containing 13.7 g FB1/kg, was used to prepare the FB1 solution (Biopure – Romer Labs Diagnostic GmbH, Tulln, Austria). Briefly, culture material was resuspended in sodium phosphate buffer, centrifuged and supernatant collected. This supernatant extract was separated into two equal volumes. From one of this extract, the HFB1 solution was obtained by hydrolysis of the FB1 with a carboxylesterase, FumD. This fumonisin carboxylesterase was first prepared by fermentation of recombinant *Pichia pastoris* as previously described (Heinl *et al.*, 2010), and the lyophilized fermentation supernatant containing the FumD enzyme was dissolved in water at 100 mg/mL, and added to one of the FB1 extract. Both extracts (FB1 and HFB1) were incubated overnight and heat-inactivated by boiling in a microwave. A portion of the FumD solution was heat-inactivated, and the same amount of enzyme that was used for preparation of HFB1 extract was added to the intact FB1 extract. Samples of both extracts were analyzed by LC-MS to confirm that the FB1 was hydrolyzed and intact, respectively, in the two extracts. The extracts were found to contain 530 mg FB1/L and HFB1 equimolar to this concentration. The control solution contained sodium phosphate buffer and the same concentration of heat-inactivated FumD as added to the two extracts.

The three solutions were administered to animals by gavage at 3.77 mL/kg body weight/day, concentration equivalent to 2 mg FB1/kg b.w/day (equals HFB1 equimolar to 2 mg FB1/kg b.w/day).

3) Experimental design and sample collection

Animals were treated daily with the different solutions and body weight of piglets was measured every two days in order to adjust the volume of treatment to administer. At weekly time intervals, blood samples were aseptically collected from the left jugular vein. Blood was collected in tubes containing sodium heparin for biochemistry, except fibrinogen collected in citrate tubes (Vacutainer®, Becton-Dickinson, USA). Plasma samples were obtained after centrifugation of heparinized blood and stored at -20°C for later analysis. Upon termination of the experiment, corresponding to 14 days of dietary exposure to treatments, immediately after electrical stunning, pigs were killed by exsanguination. Samples of liver, small intestine (proximal and distal jejunum, and ileum) and mesenteric nodes were collected from all groups and fixed in 10% buffered formalin for histopathological analysis. Besides, these tissues were flash-frozen in liquid nitrogen and stored at -80°C until processed for measurements of cytokine mRNA, and also for determination of sphingolipids concentration in liver (between 5 and 15 g of hepatic tissues for each animal).

4) Biochemistry

Plasma concentrations of creatinin, albumin, total proteins, cholesterol, triglycerides, fibrinogen and activity of gamma-glutamyl transferase were determined by a Vitros 250 analyzer (Ortho Clinical Diagnostics, Issy les Moulineaux, France) at the Veterinary School of Toulouse (France).

5) Determination of the sphinganine (Sa)/sphingosine (So) ratio

Assessment of sphingolipids metabolim, by measurement of the sphingoid bases content in plasma and liver samples, were carried out by HPLC-FLD (University of Natural Resources and Life Sciences, Center for Analytical Chemistry, Department for Agrobiotechnology IFA, Tulln, Austria). Liver samples were homogenized on ice in phosphate buffer and aliquots of the homogenates were worked up and analyzed as previously described for plasma samples (Schwartz *et al.*, 2009) with minor modifications. Concentration of Sa and So were determined and thereby, the ratio Sa/So was calculated for each animal in the biological samples.

6) Histology

The tissue pieces were dehydrated through graded alcohols and embedded in paraffin wax. Sections of 3µm were stained with hematoxylin-eosin (HE) for histopathological evaluation. Microscopic observations in liver led to the identification of some different lesions. Establishment of a lesion score per animal was determined, as previously described (Grenier *et al.*, 2011), according to

Table 12 : Nucleotide sequences of primers for real-time PCR

GENE	PRIMER SEQUENCE	GENBANK NO.	REFERENCES
RPL32	F (300 nM) TGCTCTCAGACCCCTTGTGAAG R (300 nM) TTTCCGCCAGTTCCGCTTA	NM_001001636	Pinton <i>et al.</i> (2010)
β 2- μ globulin	F (900 nM) TTCTACCTTCTGGTCCACACTGA R (300 nM) TCATCCAACCCAGATGCA	NM_213978	Devriendt <i>et al.</i> (2009)
IL-12p40	F (300 nM) GGTTTCAGACCCGACGAACTCT R (900 nM) CATATGGCCACAATGGGAGATG	NM_214013	Devriendt <i>et al.</i> (2009)
IL-8	F (300 nM) GCTCTCTGTGAGGCTGCAGTTC R (900 nM) AAGGTGTGGAATGCGTATTTATGC	NM_213867	Grenier <i>et al.</i> (2011)
IL-1 β	F (300 nM) GAGCTGAAGGCTCTCCACCTC R (300 nM) ATCGCTGTCATCTCCTTGAC	NM_001005149	Devriendt <i>et al.</i> (2009)
MIP-1 β	F (300 nM) AGCGCTCTCAGACCAATG R (300 nM) AGCTTCCGCACGGTGTATG	AJ311717	Grenier <i>et al.</i> (2011)
IL-6	F (300 nM) GGCAAAGGGAAAGAATCCAG R (300 nM) CGTTCTGTGACTGCAGCTTATCC	NM_214399	Grenier <i>et al.</i> (2011)
IFN- γ	F (300 nM) TGGTAGCTCTGGGAAACTGAATG R (300 nM) GGCTTTGCGCTGGATCTG	NM_213948	Royae <i>et al.</i> (2004)
TNF- α	F (300 nM) ACTGCACTTCGAGGTTATCGG R (300 nM) GGCGACGGGCTTATCTGA	NM_214022	Meissonnier <i>et al.</i> (2008)
IL-2	F (300 nM) GCCATTGCTGCTGGATTTAC R (300 nM) CCCTCCAGAGCTTTGAGTTC	AY294018	Meurens <i>et al.</i> (2008)
IL-10	F (300 nM) GGCCAGTGAAGAGTTTCTTTC R (300 nM) CAACAAGTCGCCATCTGGT	NM_214041	Present study

the importance degree of the lesion (severity factor) and its extent (intensity or observed frequency ; scored from 0 to 3). For hepatic tissue, the minimal score was 0 and the maximal score was 21.

The same method was applied for intestinal sections. The severity factor was equal for every lesion identified, and the lower score was 0 and 12 for the higher. As we accumulated scores from the two segments of jejunum and ileum, the maximal accumulated score was 36. Morphometry was evaluated in the different segments of intestine, by measuring the villi height randomly on thirty villi using a MOTIC Image Plus 2.0 ML[®] image analysis system, as already mentioned (Lucioli *et al.* manuscript in preparation, **cf chapitre 1**).

7) Determination of the expression of mRNA encoding for cytokines by real-time PCR

Tissue RNA was processed in 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). Concentrations, integrity and quality of RNA were determined spectrophotometrically (O.D.₂₆₀) using Nanodrop ND1000 (Labtech International, Paris, France). Besides this inspection, 200 ng of RNA was analyzed by electrophoresis. The reverse transcription of 2 µg of total RNA was performed using M-MLV reverse-transcriptase, Rnasin[®] plus (Promega, Charbonnière, France) and random primers (Invitrogen, Cergy Pontoise, France) (5 min 37°C, 1 hour 42°C, 15 min 70°C) as already described (Oswald *et al.*, 2001). Real-time PCR assays were performed on 8 ng of cDNA (RNA equivalent) in 25 µl volume reaction per well using Power SYBR[®] Green PCR Master Mix as reporter dye, and the automated photometric detector ABI Prism 7000 Sequence Detection System for data acquisition (Applied Biosystems, Courtaboeuf, France). The amplification conditions were as follows: 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. RNA non-reverse transcript was used as non-template control for verification of no genomic DNA amplification signal. Specificity of PCR products was checked out at the end of the reaction by analyzing the curve of dissociation. In addition, the size of amplicons was verified by electrophoresis. The sequences of the primers used are detailed in **Table 12**. Primers were purchased from Invitrogen (Cergy Pontoise, France). Amplification efficiency and initial fluorescence were determined by DART-PCR method (Peirson *et al.*, 2003), then values obtained were normalized by both house-keeping genes beta2-µglobulin and ribosomal protein L32 (RPL32) and finally, genes expression was expressed relative to the control group as already described (Le Gall *et al.*, 2009).

8) Statistics

Following the Fisher test on equality of variances, one way ANOVA was used to analyze the differences between the different groups of animals at each time point. *P* values of 0.05 were considered significant.

Figure 11 : Effects of FB1 and HFB1 treatments on liver lesions

Lesion scores were established after histological examination according to the severity and the extent of the lesions. Values are mean \pm SEM for five animals.

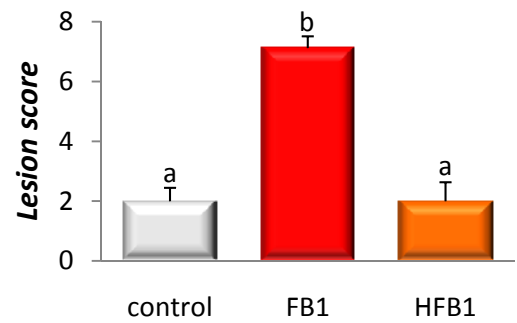


Table 13 : Effects of FB1 and HFB1 treatments on biochemical parameters

Values are mean \pm SEM for five animals. GGT, γ -glutamyl transferase.

		Control	FB1	HFB1
Creatinin ($\mu\text{mol/L}$)	Day 7	85.0 \pm 4.2 ^a	106.0 \pm 6.9 ^b	87.0 \pm 3.3 ^a
	Day 14	95.8 \pm 4.0 ^a	117.0 \pm 7.8 ^b	89.5 \pm 1.8 ^a
Albumin (g/L)	Day 7	26.2 \pm 0.8 ^a	31.3 \pm 0.7 ^b	26.1 \pm 0.9 ^a
	Day 14	26.5 \pm 1.1 ^a	31.6 \pm 1.0 ^b	25.1 \pm 0.7 ^a
Total proteins (g/L)	Day 7	49.7 \pm 0.8 ^a	58.7 \pm 1.4 ^b	49.9 \pm 1.2 ^a
	Day 14	50.4 \pm 0.7 ^a	61.4 \pm 1.2 ^b	50.7 \pm 0.6 ^a
Triglycerides (mmol/L)	Day 7	0.89 \pm 0.12 ^a	1.19 \pm 0.16 ^a	0.76 \pm 0.11 ^a
	Day 14	0.82 \pm 0.17 ^a	1.34 \pm 0.14 ^b	0.82 \pm 0.09 ^a
Cholesterol (mmol/L)	Day 7	2.70 \pm 0.22 ^a	3.78 \pm 0.21 ^b	2.16 \pm 0.34 ^a
	Day 14	2.63 \pm 0.26 ^a	4.44 \pm 0.30 ^b	2.12 \pm 0.25 ^a
Fibrinogen (g/L)	Day 7	2.16 \pm 0.13 ^a	2.91 \pm 0.21 ^b	1.89 \pm 0.27 ^a
	Day 14	1.68 \pm 0.05 ^a	2.96 \pm 0.12 ^b	1.72 \pm 0.05 ^a
GGT (U/L)	Day 7	46.0 \pm 2.4 ^a	71.2 \pm 6.2 ^b	53.2 \pm 5.3 ^{a,b}
	Day 14	51.2 \pm 1.4 ^a	146.2 \pm 32.3 ^b	71.2 \pm 12.8 ^{a,b}

Table 14 : Effects of FB1 and HFB1 treatments on cytokines expression in liver

Quantification of the relative cytokine mRNA level for each sample is expressed in arbitrary units (A.U). Values are mean \pm SEM for five animals.

	Control	FB1	HFB1
IL-1β	1.00 \pm 0.10 ^a	1.55 \pm 0.20 ^b	1.27 \pm 0.21 ^{a,b}
IL-8	1.00 \pm 0.09 ^a	1.82 \pm 0.55 ^a	1.26 \pm 0.10 ^a
IL-6	1.00 \pm 0.08 ^a	0.72 \pm 0.08 ^b	0.71 \pm 0.07 ^b
IL-10	1.00 \pm 0.15 ^a	0.49 \pm 0.06 ^b	0.98 \pm 0.08 ^a
IFN-γ	1.00 \pm 0.11 ^a	0.69 \pm 0.04 ^b	1.00 \pm 0.25 ^{a,b}
IL-18	1.00 \pm 0.01 ^a	0.59 \pm 0.06 ^b	0.86 \pm 0.05 ^a

RESULTS

1) Comparative effects of FB1 and HFB1 on the hepatic parameters

a) Histopathology results in liver

Lesions observed in the liver were considered as mild to moderate, and allowed to establish a lesion score. This score for HFB1 group did not show any important damages, and was similar to the score of the control group (**Figure 11**). By contrast, FB1 elicited significant injuries in liver, as displayed by its lesion score ($p < 0.001$ in comparison with either the control or HFB1 treatments). The main lesions observed for these animals, were nuclear vacuolization of hepatocytes, megalocytosis, and one piglet presented signs of necrosis as well.

b) Biochemical parameters reflecting liver metabolism

We investigated some biochemical compounds produced by liver cells that could be considered as good biomarker of liver dysfunction or inflammation. An increase in albumin concentration, and thereby linked to the increase observed for total proteins content, was attributed to the ingestion of FB1 at the two time points of plasma analysis (**Table 13**). Similarly, the plasmatic content of lipid compounds, such as cholesterol and triglycerides, showed a significant elevation in FB1-treated animals. The profile of inflammatory markers, fibrinogen and gamma-glutamyl transferase (GGT) was greatly altered in piglets administered with FB1. Conversely, no change was detected for animals treated with either the buffer or HFB1 solutions (**Table 13**).

c) Cytokines expression results

Liver has its own innate immune system responsible for the localized action, involving the cytokine network. Some of these cytokines may have pro- or anti-inflammatory properties. Results of mRNA expression of cytokines are reported in the **Table 14**. In the FB1-treated group, an increase of IL-1 β and IL-8 mRNA levels has been observed (+55% and +82% compared to control group, respectively). By contrast, for these animals the expression of IL-6, IL-10, IL-18 and IFN- γ decreased (-28%, -51%, -40% and -31% compared to control group, respectively). After 14 exposure days to HFB1, a decrease in IL-6 and to a lesser extent in IL-18 was obtained (-29% and -14% compared to control group, respectively).

Table 15 : Effects of FB1 and HFB1 treatments on the sphinganine/sphingosine ratio in biological samples

Values are mean \pm SEM for six animals.

	Control	FB1	HFB1
PLASMA			
✓ Day 0	0.26 \pm 0.02 ^a	0.24 \pm 0.02 ^a	0.26 \pm 0.02 ^a
✓ Day 7	0.27 \pm 0.02 ^a	2.14 \pm 0.07 ^b	0.33 \pm 0.03 ^a
✓ Day 14	0.24 \pm 0.02 ^a	2.49 \pm 0.20 ^b	0.30 \pm 0.02 ^a
LIVER	0.11 \pm 0.01 ^a	3.08 \pm 0.45 ^b	0.25 \pm 0.06 ^c

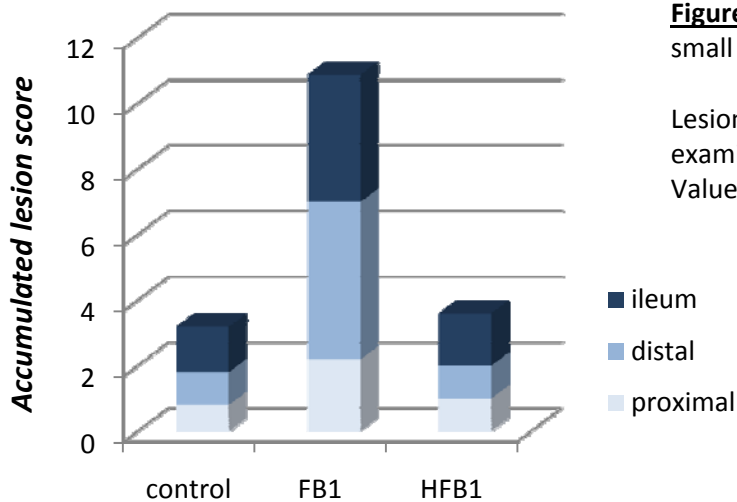


Figure 12 : Effects of FB1 and HFB1 treatments on the small intestine

Lesion scores were established after histological examination according to the extent of the lesions. Values are mean for five animals.

Table 16 : Effects of FB1 and HFB1 treatments on the villi height in the small intestine

Values are mean \pm SEM for five animals.

	Control	FB1	HFB1
Proximal Jejunum	300 \pm 16 ^a	259 \pm 17 ^a	255 \pm 19 ^a
Distal Jejunum	321 \pm 13 ^a	259 \pm 21 ^b	297 \pm 10 ^{a,b}
Ileum	265 \pm 13 ^a	182 \pm 13 ^b	241 \pm 7 ^a

2) Comparative effects of FB1 and HFB1 on the sphingolipids metabolism

Concentrations of sphinganine and sphingosine were evaluated in plasma and liver samples. Following the analysis, the Sa/So ratio was established, and results in the **Table 15** show similar ratios for the control and HFB1 groups in the biological samples. By contrast, and as expected, treatment with FB1 solution greatly affected the content in sphingoid bases, leading to an accumulation of Sa and So, both in plasma and liver ($p < 0.001$ in comparison to control and HFB1 groups).

3) Comparative effects of FB1 and HFB1 in the small intestine

a) Histopathology results

The pieces of bowel collected during slaughter corresponded to proximal and distal jejunum, and to ileum. Similarly to the liver, histopathological analysis revealed some lesions considered as mild to moderate, and a lesion score was established for each piglet. We displayed in the **Figure 12** the accumulated scores of lesions in the three parts examined in the small intestine. Ingestion of HFB1 did not elicit significant lesions, and showed comparable lesion scores to piglets from the control group. On contrary, ingestion of FB1 triggered lymphatic vessel dilation and interstitial edema in the proximal jejunum, and mostly lymphatic vessel dilation, villi atrophy and fusion, in the distal jejunum and ileum. For each segment of intestine, the lesion score for FB1-treated animals was significantly different from control animals, and at least from HFB1 animals in the distal jejunum.

In addition, the integrity of the intestine was evaluated by measuring the villi height. As reported in the **Table 16**, FB1 significantly affected the height of villi all along the intestine whereas a lesser effect was observed for HFB1 compared to control (-20% and -31% for FB1 treatment, and -7% and -9% for HFB1 treatment in the distal jejunum and ileum respectively).

b) Cytokines expression results

The digestive tract has the ability to develop immune responses, mostly through the gut-associated lymphoid tissue (GALT), such as ileum (Peyer's Patches) and mesenteric nodes that contain numerous immune cells, producers of cytokines. However, jejunum segment is also known to develop its own local defense, through intestinal epithelial cells, producers of cytokines as well (Stadnyk, 2002).

Therefore in the current study, we evaluated the expression of several cytokines in each part of the gastrointestinal tract. Overall, for FB1-treated animals, the level of cytokines mRNA was strongly reduced in all the intestinal compartments, especially mRNA encoding for IL-1 β , IL-8, IFN- γ and IL-2 (**Table 17**). Conversely, when piglets ingested the HFB1 solution, few changes in the cytokines

Table 17 : Effects of FB1 and HFB1 treatments on cytokines expression in the small intestine and the mesenteric lymph nodes

Values are mean \pm SEM for five animals.

		Control	FB1	HFB1
IL-1 β	Proximal jejunum	1.00 \pm 0.26 ^a	0.41 \pm 0.06 ^b	1.03 \pm 0.28 ^a
	Distal jejunum	1.00 \pm 0.18 ^{a,b}	0.62 \pm 0.09 ^a	1.02 \pm 0.07 ^b
	Ileum	1.00 \pm 0.26 ^a	0.44 \pm 0.13 ^b	0.66 \pm 0.07 ^{a,b}
	Mesenteric nodes	1.00 \pm 0.13 ^a	0.49 \pm 0.07 ^b	0.94 \pm 0.16 ^a
IL-8	Proximal jejunum	1.00 \pm 0.12 ^{a,b}	0.84 \pm 0.10 ^a	1.23 \pm 0.04 ^b
	Distal jejunum	1.00 \pm 0.13 ^a	0.76 \pm 0.12 ^a	1.07 \pm 0.15 ^a
	Ileum	1.00 \pm 0.13 ^a	0.58 \pm 0.08 ^b	0.87 \pm 0.12 ^{a,b}
	Mesenteric nodes	1.00 \pm 0.17 ^a	0.41 \pm 0.06 ^b	0.88 \pm 0.16 ^a
TNF- α	Proximal jejunum	1.00 \pm 0.12 ^a	0.93 \pm 0.12 ^a	1.01 \pm 0.09 ^a
	Distal jejunum	1.00 \pm 0.04 ^a	0.90 \pm 0.06 ^a	0.96 \pm 0.05 ^a
	Ileum	1.00 \pm 0.15 ^a	1.42 \pm 0.16 ^a	1.01 \pm 0.13 ^a
	Mesenteric nodes	1.00 \pm 0.04 ^a	0.86 \pm 0.04 ^b	0.74 \pm 0.02 ^c
IL-6	Proximal jejunum	1.00 \pm 0.18 ^a	0.88 \pm 0.16 ^a	1.26 \pm 0.18 ^a
	Distal jejunum	1.00 \pm 0.10 ^a	0.67 \pm 0.07 ^b	0.83 \pm 0.13 ^{a,b}
	Ileum	1.00 \pm 0.15 ^a	1.26 \pm 0.17 ^a	1.24 \pm 0.16 ^a
	Mesenteric nodes	1.00 \pm 0.07 ^a	0.73 \pm 0.04 ^b	0.82 \pm 0.05 ^b
IFN- γ	Proximal jejunum	1.00 \pm 0.18 ^a	0.54 \pm 0.05 ^b	1.00 \pm 0.21 ^a
	Distal jejunum	1.00 \pm 0.18 ^a	0.54 \pm 0.06 ^b	1.00 \pm 0.12 ^a
	Ileum	1.00 \pm 0.12 ^a	1.55 \pm 0.10 ^b	1.61 \pm 0.17 ^b
	Mesenteric nodes	1.00 \pm 0.09 ^a	0.71 \pm 0.06 ^b	1.01 \pm 0.08 ^a
IL-2	Proximal jejunum	1.00 \pm 0.04 ^a	0.60 \pm 0.06 ^b	0.86 \pm 0.11 ^a
	Distal jejunum	1.00 \pm 0.13 ^a	0.62 \pm 0.06 ^b	0.89 \pm 0.24 ^{a,b}
	Ileum	1.00 \pm 0.18 ^a	0.64 \pm 0.10 ^a	0.76 \pm 0.07 ^a
	Mesenteric nodes	1.00 \pm 0.09 ^a	0.76 \pm 0.02 ^b	1.16 \pm 0.16 ^a
IL-12p40	Proximal jejunum	1.00 \pm 0.13 ^{a,b}	0.70 \pm 0.09 ^a	1.08 \pm 0.15 ^b
	Distal jejunum	1.00 \pm 0.08 ^a	0.94 \pm 0.07 ^a	1.91 \pm 0.29 ^b
	Ileum	1.00 \pm 0.07 ^a	1.34 \pm 0.16 ^{a,b}	1.54 \pm 0.08 ^b
	Mesenteric nodes	1.00 \pm 0.21 ^a	0.55 \pm 0.06 ^b	0.77 \pm 0.08 ^a
IL-10	Proximal jejunum	1.00 \pm 0.05 ^a	0.57 \pm 0.05 ^b	1.00 \pm 0.10 ^a
	Distal jejunum	1.00 \pm 0.06 ^a	1.03 \pm 0.15 ^{a,b}	1.36 \pm 0.13 ^b
	Ileum	1.00 \pm 0.13 ^a	1.18 \pm 0.17 ^a	1.10 \pm 0.13 ^a
	Mesenteric nodes	1.00 \pm 0.12 ^a	0.73 \pm 0.08 ^a	0.92 \pm 0.05 ^a

expression were noticed according to the different tissues investigated. Expression of these mediators was quite similar to those of control group, or in some cases not so much reduced in comparison to FB1. Of note for the ileum, treatment with HFB1 seems to slightly promote the expression of two Th1 cytokines, IL-12p40 and IFN- γ .

DISCUSSION

The aim of the current study was to compare the *in vivo* toxicity of FB1 and its fully hydrolyzed form, HFB1 (also named aminopentol or AP1). Despite controversial results on this compound, we showed that the hydrolysis of FB1 strongly reduce toxicity in swine, either in liver or in gastrointestinal tract. To our knowledge, this is the first report of the HFB1 toxicity on domestic animals.

HFB1 is produced during a traditional corn treatment with calcium hydroxide and heat, also named nixtamalization, the alkaline hydrolysis process removing the tricarballylic acid side chains from FB1 contained in corn. Nixtamalization is the major way of processing corn in both Mexico and Central America, as well as in the United States, to make masa and tortillas. In addition to the occurrence of HFB1 in nixtamalized material, detoxification of FB1-contaminated foods/feeds by microorganisms may lead to the formation of HFB1 (Heinl *et al.*, 2010), and the effect of its ingestion needs to be evaluated. Considering swine shows a great sensitivity to fumonisins and because of the high percentage of corn in pig diets, these alternative methods of decontamination might be applied in husbandry. Besides, considering that many biological systems (intestinal, metabolic or immune) of pigs are very similar to that of humans, swine can be regarded as a good model that can be applied to humans (Almond, 1996; Verma *et al.*, 2011).

In this experiment, the dose of 2 mg FB1/kg bw/day allowed us to elicit toxicity and thus to compare at equimolar concentration the HFB1 effects. Based on averaged feed consumption for pigs of this age, this dose corresponds to feed contaminated with a concentration of 37-44 mg of FB1/kg.

Over the 14-day exposure, no effect was noticed on the animal growth or the feed intake (data not shown). No effect on the body weight gain has been already reported in pigs and poultry fed up to 50 mg FB1/kg feed (Harvey *et al.*, 1996; Broomhead *et al.*, 2002). The hepatotoxicity elicited by FB1 administration was not observed in HFB1-treated piglets. Indeed, biochemical changes attributed to the disturbance of some hepatic functions, such as protein and lipid metabolism, was not noticed in HFB1 group. Similarly, liver was not damaged after HFB1 ingestion as shown by the absence of microscopic lesions. Inflammation has been assessed in this short-term exposure through biochemical markers and liver cytokines as well. The plasmatic concentrations of GGT and fibrinogen were unaffected following HFB1 exposure. The level of cytokines at the end of experiment was slightly modulated in livers from animals treated with HFB1, but less than in those from FB1 group. In this latter group, levels of IL-1 β and IL-8, well known as pro-inflammatory mediators, were still elevated as already described after FB1 exposure (Bhandari *et al.*, 2002). Levels of IL-6 and IL-10, known among others as mediators with anti-inflammatory properties (de Vries, 1995; Xing *et al.*,

1998), were greatly reduced in liver and thereby, could confirm the inflammatory state in piglets exposed to FB1. By contrast, HFB1 administration did not modulate the cytokines expression as much as FB1 did, and to our knowledge this is the first report on the effect of HFB1 on cytokines in liver.

The lack of toxicity in liver after HFB1 exposure is in agreement with some authors (Collins *et al.*, 2006; Howard *et al.*, 2002; Voss *et al.*, 2009), but also in discordance with others (Hendrich *et al.*, 1993; Voss *et al.*, 1996; Voss *et al.*, 1998). Nonetheless, in these controversial studies, it is important to clarify that the toxicity was only demonstrated in animals exposed to nixtamalized culture material and not to pure HFB1. That's why, it was suggested (Kim *et al.*, 2003; Park *et al.*, 2004; Seiferlein *et al.*, 2007; Burns *et al.*, 2008; Voss *et al.*, 2009) that these effects were mediated by residual or "hidden" FB1 (matrix bound forms not detected by HPLC) remaining in the nixtamalized preparations, rather than HFB1.

We also determined the potential toxicity of these compounds on the intestine. Indeed, as the gastrointestinal tract is a primary site of mycotoxins exposure and that FB1 has been also reported to exert its toxicity, through the ceramide synthase inhibition, in the intestine (Enongene *et al.*, 2000; Loiseau *et al.*, 2007), evaluation of HFB1 effects on intestine was necessary. Following ingestion of contaminated food or feed, intestinal epithelial cells could be exposed to a high concentration of toxicants, potentially affecting intestinal functions. Some studies focused on the bowel, but most of them concern effects of HFB1 on intestinal cell lines by assessing the number of viable cells, HFB1 acylation or sphingoid bases content (Schmelz *et al.*, 1998; Humpf *et al.*, 1998; Seiferlein *et al.*, 2007).

In the current study, HFB1 ingestion did not alter the intestinal integrity in the different segments examined, as shown by the villi morphometry and the lesion scores. Among lesions, villi atrophy and fusion were evaluated. Occurrence of these lesions was considerably high in the intestines exposed to FB1, especially in the second part of the small intestine. These findings are in agreement with the villous fusion and atrophy observed in the intestine of pigs treated with 30 ppm FB1 (Piva *et al.*, 2005) and in the intestine of chicks fed with 61-546 ppm FB1 (Javed *et al.*, 2005). In addition, a report on human consumption of moldy maize (containing fumonisins) resulted in abdominal pain, borborygmi, and diarrhea (Bhat *et al.*, 1997). Therefore, it can be suggested that on contrary to FB1, HFB1 consumption would not lead to impairment of the intestinal absorption of nutrients.

The intestine is also an immune privileged site where immunoregulatory mechanisms simultaneously defend against pathogens but also preserve tissues homeostasis to avoid immune-mediated pathology in response to environmental challenges (Ramiro-Puig *et al.*, 2008). To assess the intestinal immunity following exposure to FB1 and HFB1, we focused on different sections of the jejunum, and on ileum and mesenteric lymph nodes (MLN) as well. In contrast to the jejunum, which provides a local response, the ileum containing Peyer's patches and MLN take part from the gut-

associated lymphoid tissue (GALT) and act as inductive sites for intestinal immune response. These specific tissues can be considered as specialized meeting places, where antigen-presenting cells and lymphocytes interact in presence of intestinal antigen. We investigated cytokines expression, which are important mediators in the regulation of the immune and inflammatory responses. They are produced by immune system cells (lymphocytes, macrophages, ...) but also by cells not traditionally considered as part of the immune system such as intestinal epithelial cells.

Few data are available with regard to the effects of FB1 on the intestinal immunity, and even lesser on cytokines production. FB1 decreases the expression and the synthesis of IL-8 in the porcine epithelial intestinal cell line, IPEC-1 (Bouhet *et al.*, 2006). The same study confirmed this result *in vivo*; ingestion of low doses of FB1 by piglets decreased the expression of IL-8 mRNA in the ileum. The FB1-induced IL-8 decrease may lead to a reduced recruitment of inflammatory cells in the intestine during infection and may participate in the observed increased susceptibility of contaminated piglets to intestinal infections (Oswald *et al.*, 2003). Recently, another study showed in piglets that following FB1 exposure, animals revealed a reduced intestinal expression of IL-12p40, an impaired function of intestinal antigen presenting cells (APC), with decreased upregulation of major histocompatibility complex class II molecule (MHC-II) and reduced T cell stimulatory capacity upon stimulation (Devriendt *et al.*, 2009). As outlined by the authors, these results indicate an FB1-mediated reduction of *in vivo* APC maturation. In the current study, for most of the cytokines investigated, we observed after FB1 ingestion a significant decrease of their expression in all the compartments of the intestinal tract. By contrast, the profile of cytokines after exposition to HFB1 revealed very few changes, close to the one observed in non-treated animals. These observations imply that animals consuming HFB1 would be able to defend efficiently against potential invaders. To our knowledge, this is the first study on the effects of HFB1 on some key components of the intestinal immune system.

By contrast to FB1, the lack of effects, either at the hepatic or intestinal levels, observed following HFB1 treatment might be explained by its ability to unaffected the sphingoid bases content. Indeed, FB1 acts as an inhibitor of the ceramide synthase, leading to an accumulation of sphinganine and to a lesser extent of sphingosine. The establishment of the Sa/So ratio has been widely described to be a relevant and sensible biomarker of FB1 toxicity (Soriano *et al.*, 2005, Tran *et al.*, 2006), and illustrates the degree of sphingolipid metabolism disruption. This elevation in sphinganine, a highly bioactive compound, initiates a cascade of cellular alterations that are thought to be largely responsible for the toxicity and carcinogenicity of this mycotoxin. To inhibit the ceramide synthase, aminopentol backbone of FB1 competes for binding of the sphingoid base substrate, whereas the tricarballic acids side chains (TCA) interfere with binding of the fatty acyl-CoA (Humpf *et al.*, 1998; Desai *et al.*,

2002). Removal of the tricarballylic acids, which leads to HFB1, diminishes the potency of ceramide synthase inhibition, and therefore the accumulation of sphingoid bases. In the current experiment, we may suppose that ceramide synthase inhibition did not occur, as displayed by the Sa/So ratio, either in plasma samples or in liver samples. This indicated that the TCA moieties are required for maximal inhibition of the enzyme. Our results are in agreement with studies which showed that HFB1 was at least tenfold less potent compared to FB1 (Norred *et al.*, 1992; Flynn *et al.*, 1997; van der Westhuizen *et al.*, 1998; Howard *et al.*, 2002; Collins *et al.*, 2006; Voss *et al.*, 2009). As mentioned by Voss *et al.* (1998), increased liver Sa/So ratio is directly correlated with the increased of serum chemical indicators, leading to hepatotoxicity. This observation confirms our results on FB1 and HFB1.

However, some findings showed that HFB1 becomes not only an inhibitor but also a substrate for acylation by ceramide synthase (Humpf *et al.*, 1998; Abou-Karam *et al.*, 2004; Seiferlein *et al.*, 2007). This observation was firstly reported in HT29 cells (Humpf *et al.*, 1998), where FB1 was not detectably acylated on contrary to HFB1. The author suggested at this moment that the TCA of FB1 occupy the acyl-CoA binding site and thereby block access of this co-substrate. Therefore, the removal of TCA allowed to HFB1 to be acylated, and the first acylation observed was in presence of palmitoyl-CoA which led to the formation of N-palmitoyl-AP1 (PAP1 ; hydrolyzed fumonisin B1 also named AP1 for aminopentol). As outlined by Humpf *et al.* (1998), a somewhat unexpected finding of this study was that PAP1 was highly cytotoxic for HT29 cells, and a more potent inhibitor of ceramide synthase, causing sphinganine accumulation. *In vivo* acylation was also evaluated in rats, and while formation of N-acyl-AP1 occurs and produces metabolites with fatty acids of various chain lengths, no toxicity was observed (Seiferlein *et al.*, 2007). Considering observations of Humpf and Seiferlein, we cannot exclude in our experiment that acylation did not occur, but neither increase in sphinganine concentrations nor toxicity was detected. Nonetheless, this aspect warrants further investigations, considering that there are multiple of isoforms of ceramide synthase (CerS) that are differently expressed according to tissues and differ in fatty acyl-CoA selectivity. Besides, a different outcome depending on animal species does not have to be excluded.

To conclude, we showed for the first time in domestic animals that the hydrolyzed form of FB1 is much less toxic than the parent compound, and therefore confirmed results observed in rodents exposed to pure HFB1. Furthermore, the HFB1 in this study was originally obtained after an enzymatic treatment on FB1 with a carboxylesterase. It is thus of concern to consider the potential of these biotransformation approaches in order to counteract adverse effects of FB1-contaminated feed in farms. Recently, it has also been suggested that the carboxylesterase could be combined with an enzyme, expressed from the same cluster gene of the carboxylesterase FumD (Heinl *et al.*, 2010;

Hartinger *et al.*, 2010). This enzyme possess the ability to deaminate the HFB1, following the deesterification step, leading to the removal of the C2-amino group. This chemical group has been described as responsible of the toxicity of FB1 (Voss *et al.*, 2007), and also to be acylated on the HFB1 by fatty acid (Humpf *et al.*, 1998).

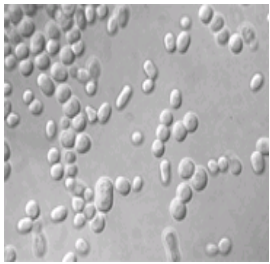
CHAPITRE 3

Évaluation des effets d'agents détoxifiants lors d'une exposition au Déoxynivalénol et à la Fumonisine chez le porcelet

Figure 13 :

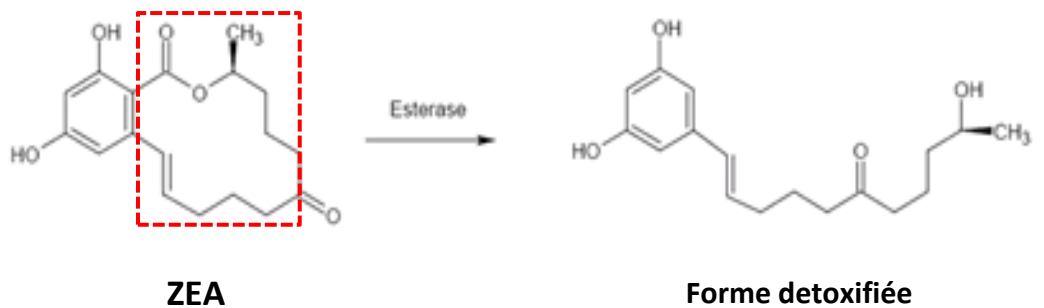
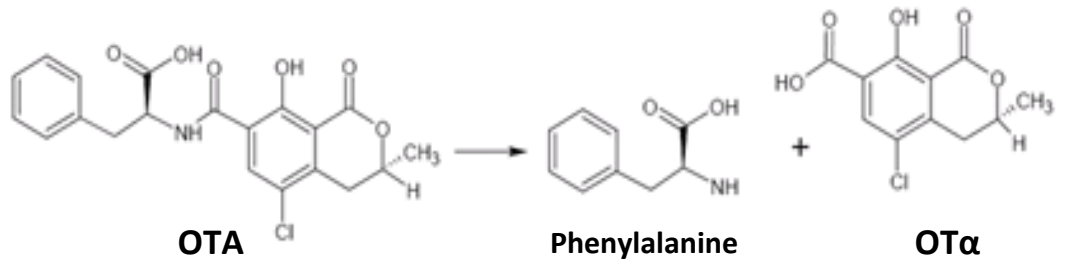
- (a) Transformation par voie enzymatique de l'ochratoxine A (OTA) et de la zéaralénone (ZEA), via l'utilisation du microorganisme *Trichosporon mycotoxinivorans*
- (b) Transformation par voie enzymatique des trichothécènes (TCT) de type A et B, via l'utilisation du microorganisme *Eubacterium BBSH 797*

(a)

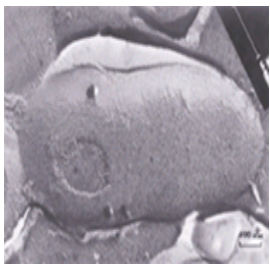


Trichosporon mycotoxinivorans

(levure isolé du tractus digestif des termites)

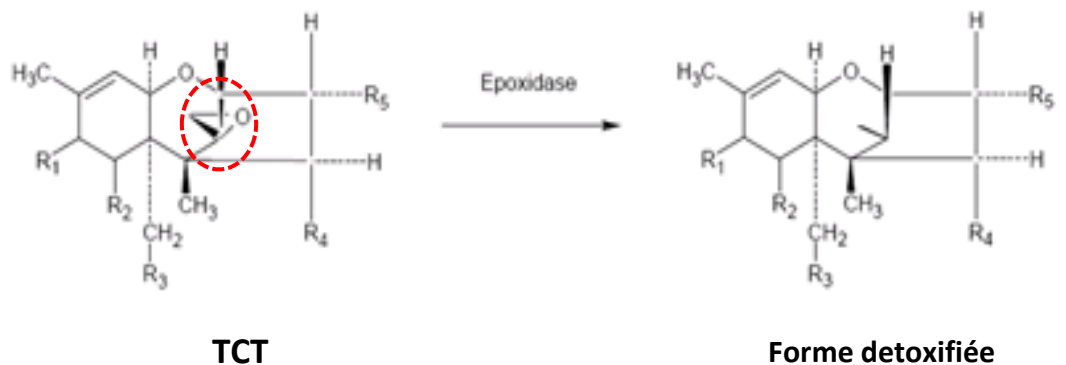


(b)



Eubacterium BBSH797

(bactérie isolée du rumen de bovins)



RESUME DE L'ETUDE

Cette étude s'inscrit dans la continuité des précédentes, avec pour objectif d'évaluer l'ajout de produits désactivateurs de mycotoxines dans des aliments contaminés. Comme mentionné précédemment, BIOMIN développe et commercialise des produits composé de micro-organismes capable de dégrader les mycotoxines. Outre l'enzyme présentée dans l'étude précédente mais encore non commercialisée, BIOMIN propose des approches essentiellement basées par voie enzymatique et qui cible spécifiquement chaque mycotoxine ou famille de mycotoxine. En effet, ces métabolites secondaires présentent une incroyable diversité structurale, et ainsi chaque approche biologique nécessite l'utilisation d'un micro-organisme/enzyme adapté à chacun. A noter que le principe de la méthode utilisée par notre partenaire pour éliminer les aflatoxines est différente des autres toxines, et repose sur des phénomènes d'adsorption par des argiles. En effet, comme résumé dans l'introduction de ce mémoire de thèse, les adsorbants inorganiques et organiques ont été largement reconnus comme efficaces vis-à-vis de leurs capacités à lier les aflatoxines et à les excréter hors de l'organisme animal. En revanche, ces produits ont essentiellement fait leurs preuves sur les aflatoxines, mais rarement sur les autres toxines majeures. Ainsi, BIOMIN a développé des approches par voie enzymatique contre l'ochratoxine A, la zéaralénone et les trichothécènes de type A et B, transformant ces métabolites toxiques en des substances non-toxiques (**Figure 13**).

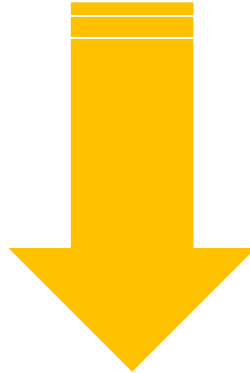
Dans la première étude sur l'exposition du déoxynivalénol et des fumonisines, seuls ou en combinaison, nous avons montré que ces toxines ont des effets toxiques sur le système immunitaire et sur différents organes cibles (foie, reins, poumons et intestin). L'objectif était donc de reproduire cette expérimentation (*menée simultanément avec celle de la première étude*) et d'évaluer l'efficacité d'agents détoxifiants (DA), par le biais d'une supplémentation de ces produits dans les aliments mono- et co-contaminés. Le premier agent désactivateur utilisé était spécifique du DON et déjà commercialisé. Il a été inclus dans l'aliment à 0,25%. Le deuxième agent désactivateur utilisé était l'enzyme expérimentale isolée récemment par BIOMIN, spécifique des FB, et présentée dans le chapitre précédent. Il a été inclus *via* sa pulvérisation directe sur l'aliment (250 mL/25 kg d'aliment).

Figure 14 :

Illustrations de la procédure d'incorporation dans l'aliment de l'agent désactivateur ciblant spécifiquement les fumonisines



**Pulvérisation du produit DA
directement sur les lots d'aliments**



**250 mL de DA sur 25 kg de granulés
étalés et formant une monocouche**



MATERIEL ET METHODES**1) Fabrication des aliments**

Sur la base d'une formule d'aliment porcelet 2^{ème} âge, 8 lots d'aliments contaminés ou non avec les mycotoxines et supplémentés ou non avec les DA ont été produits sur le site de l'INRA de Rennes (UMR SENAH). Comme détaillé dans la première étude, les extraits contenant le DON et les FB ont été mélangés au pré-mix de vitamines et minéraux, puis incorporés dans le mélange de céréales avant l'étape de granulation. Dans le cas des aliments supplémentés, le premier DA ciblant le DON (Mycofix, Biomin GmbH, Tulln, Autriche) a aussi été inclus à cette étape de la préparation à la dose de 2,5 g/kg d'aliment fini (0,25%) ; alors que le DA ciblant les FB, conditionné sous forme lyophilisée, a été repris dans de l'eau et pulvérisé sur l'aliment fini à raison de 250 mL/25 kg aliment à l'INRA de Toulouse (**Figure 14**).

Les matières premières et les aliments finis ont été contrôlés pour leurs contaminations naturelles et/ou artificielles en mycotoxines au laboratoire Quantas Analytik (Tulln, Autriche) et aussi par une méthode expérimentale d'analyse en multi-mycotoxines (Sulyok *et al.*, 2007) comme décrit dans la première étude. A noter que nous avons été contraints de formuler notre aliment sans maïs, due à une contamination naturelle importante en DON (700 µg/kg de maïs) et en FB (900 µg/kg de maïs) ; et que le DON, la zéaralénone et l'enniain ont été trouvés naturellement présents, à des concentrations de 500, 50 et 100 µg/kg dans l'aliment fini, respectivement.

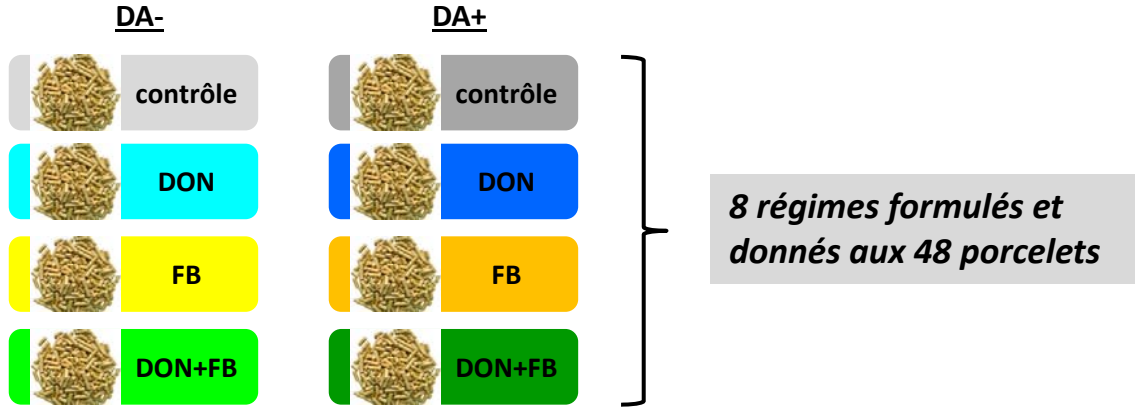
Ci-dessous, le **Tableau 18** résumant les différents régimes fabriqués avec leurs teneurs finales respectives en mycotoxines :

		<i>en µg de toxines/kg d'aliment</i>		
		DON	FB1	FB2
Sans agents désactivateurs (DA-)	A1: Contrôle	539	<LD	<LD
	A2: DON	2847	<LD	<LD
	A3: FB	421	4113	1799
	A4: DON+FB	3091	4512	2025
Avec agents désactivateurs (DA+)	A5: Contrôle	500	<LD	<LD
	A6: DON	2953	<LD	<LD
	A7: FB	476	4856	2180
	A8: DON+FB	2707	4090	1843

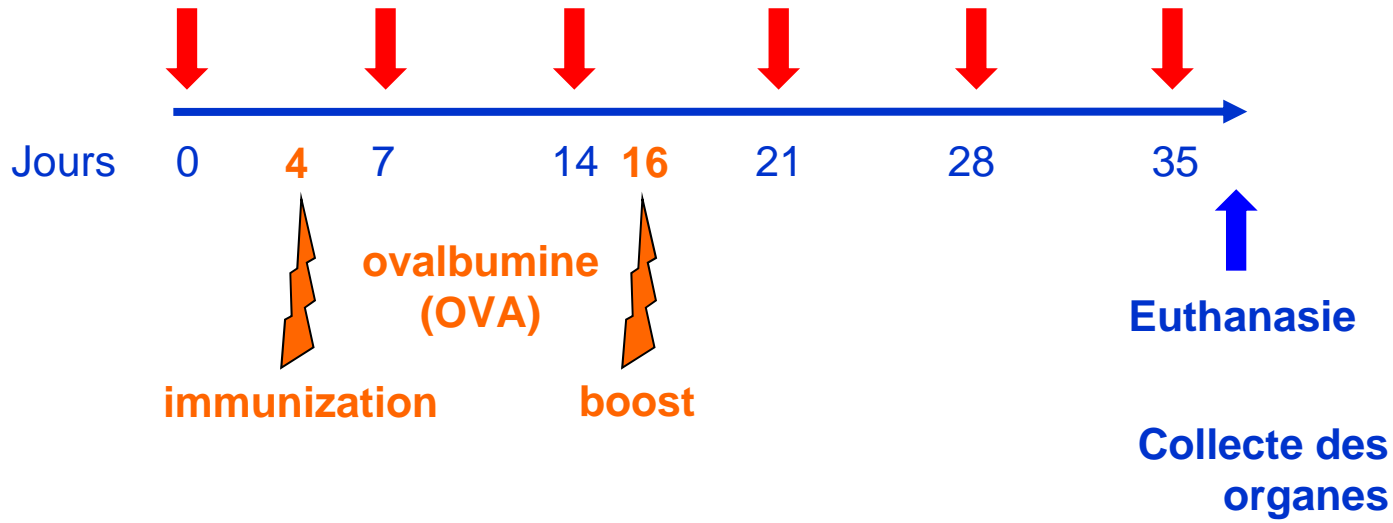
Notes : en rouge, contamination naturelle ; LD, limite de détection. Les autres mycotoxines majeures, telles que les aflatoxines, l'ochratoxine A, les toxines T-2 et HT-2 étaient sous la limite de détection

Figure 15 :

Plan expérimental de la phase animale avec le protocole de vaccination



↓ **Récupération du sang (10-15 mL)**



2) Plan expérimental de la phase animale et prélèvement des échantillons (Figure 15)

Toutes les procédures d'expérimentations animales ont été menées en accord avec les recommandations européennes au regard de l'utilisation des animaux à des fins de recherches (Directive 86/609/EEC). Des porcelets mâles castrés (Pietrain X Duroc X Large-white) et sevrés ont été obtenus dans un élevage local, et familiarisés à leur nouvel environnement les dix jours précédents le début de la phase expérimentale. Des lots homogènes basés sur le poids des animaux ont ensuite été formés. Pendant les 35 jours de l'expérience, les porcelets ont eu libre accès à l'eau et au régime alimentaire désigné. Une surveillance quotidienne, ainsi qu'une pesée hebdomadaire, ont été réalisées au cours de cette période. Les phases d'expérimentations animales avec les aliments non supplémentés et les aliments supplémentés en DA ont été menées simultanément.

Afin de stimuler et d'étudier la réponse immunitaire, deux injections contenant une solution d'ovalbumine mélangée à l'adjuvant incomplet de Freund, ont été administrées à 12 jours d'intervalle (J4 et J16 de la période expérimentale) à tous les animaux.

Des prélèvements sanguins à la veine jugulaire ont été effectués chaque semaine pour la mise en culture du sang total et pour la récupération du plasma (stocké à -20°C).

Après les 35 jours d'expositions aux différents régimes, tous les animaux ont été sacrifiés par exsanguination après électronarcose. Des échantillons de foie, poumons, rate et des sections d'intestin ont été prélevés et fixés dans un tampon de formaldéhyde à 10% pour les analyses histologiques. De plus, des échantillons de rate ont été directement congelés dans l'azote liquide, puis stockés à -70°C avant leurs traitements pour les analyses transcriptomiques.

3) Analyses hématologiques et biochimiques

L'analyse hématologique a été effectuée dans un laboratoire d'analyses médicales (Ferrandery, Tournefeuille, France) via l'utilisation d'un automate à impédance, Coulter LH500 (Beckman Coulter, Villepinte, France). La sous-population des globules blancs (lymphocytes, neutrophiles, monocytes, basophiles et éosinophiles) a été étudiée et comptée manuellement sur 100 leucocytes par la coloration de May-Grünwald Giemsa.

Les concentrations plasmatiques de l'albumine, des protéines totales, de l'urée, de la créatinine, du cholestérol, des triglycérides et de la gamma-glutamyl transférase, ont été déterminées à l'Ecole Nationale Vétérinaire de Toulouse via l'utilisation d'un automate, Vitros 250 (Ortho Clinical Diagnostics, Issy les Moulineaux, France).

4) Analyses du contenu plasmatique en bases sphingoïdes

Comme introduit dans le chapitre 2, tout au long de notre expérience les plasmas collectés hebdomadairement ont été également utilisés pour l'analyse de la concentration des bases sphinganine (Sa) et sphingosine (So), et le ratio Sa/So a ainsi pu être déterminé. Ces analyses ont été réalisées dans le laboratoire du centre de chimie analytique, du département d'agrobiotechnologie IFA à Tulln (Autriche). Brièvement, les échantillons de plasma ont été traités par hydrolyse avec une base forte (Yoo *et al.*, 1996), dérivés avec l'o-phthalaldéhyde (OPA, se lie aux groupements amines) (Riley *et al.*, 1994b) et analysés par HPLC couplée à un appareil de détection à fluorescence (Schwartz *et al.*, 2009). Les concentrations en Sa et So ont été déterminées par correction avec les pourcentages de récupération des standards internes C17-sphingosine et C20-sphinganine.

5) Analyses histopathologiques

Dans le cadre d'une collaboration avec l'École Vétérinaire de Londrina au Brésil, les analyses histologiques et immunohistochimiques des tissus ont été réalisées au laboratoire de Pathologie Animale. Les tissus fixés dans le formaldéhyde ont été inclus dans des blocs de paraffine après déshydratation par plusieurs gradients d'alcool. Des sections de 3µm ont été colorées à l'hématoxyline-éosine pour l'évaluation histologique. Comme présenté dans le chapitre 1, afin d'évaluer l'effet des toxines sur les organes, nous avons établi un score lésionnel pour chaque tissu recensant l'ensemble des lésions observées. Ce score a été calculé en fonction du degré de sévérité et de l'étendu de chaque lésion.

La prolifération cellulaire des hépatocytes a été évaluée en comptant le nombre de noyaux Ki-67 positifs sur les sections de foie. La méthode a été présentée dans le chapitre 1.

L'évaluation de la hauteur des villosités, la profondeur des cryptes, le dénombrement des cellules immunitaires et des entérocytes en prolifération a été réalisée de la même manière que dans le chapitre 1.

6) Mesure de la concentration des immunoglobulines plasmatiques totales et spécifiques

Les teneurs en immunoglobulines (Ig) totales A et G dans le plasma ont été déterminées par la technique ELISA comme décrit au chapitre 1.

Pour la mesure de réponse humorale spécifique suite à la vaccination, la technique ELISA a également été utilisée avec l'ovalbumine comme antigène de capture des anticorps plasmatiques, et des anticorps anti-IgA et IgG porcins couplés à la peroxydase HRP pour la détection (cf. chapitre 1).

7) Détermination de l'index de prolifération des lymphocytes

Comme expliqué dans le chapitre 1, la prolifération des lymphocytes a pu être déterminée après la mise en culture du sang total, et stimulé avec un mitogène et/ou un antigène (ovalbumine). La stimulation mitogénique du sang total (dilué au 1/15^{ème}) avec la concanavaleine A (10 µg/mL) nous a permis d'évaluer la réponse cellulaire non-spécifique. A l'inverse, la réponse cellulaire spécifique a été étudiée après la stimulation du sang total (dilué au 1/15^{ème}) en présence d'ovalbumine (5 et 10 µg/mL). Des cultures cellulaires contrôles, non stimulées ont aussi été inclus. Après 48h de stimulation, nous avons rajouté de la thymidine radio-marquée (tritium ³H, 0,5 µCi/puit) au milieu de culture, et laissé à incuber 24 heures supplémentaires. Les cultures cellulaires ont ensuite été stockées à -20°C. Après filtration des cultures, les niveaux de thymidine tritiée incorporée ont été mesurés à l'aide d'un compteur à scintillation (Kontron Instrument, Mila, Italie). L'index de prolifération des lymphocytes a été exprimé en cpm mesurés dans les cultures stimulées/cpm mesurés dans les cultures contrôles non stimulées.

8) Détermination de l'expression des ARNm codant des cytokines

Afin d'extraire les ARNs de la rate, les échantillons collectés à l'autopsie et congelés à -70°C, ont été broyés au FastPrep-24 (MP Biomedicals, Illkirch, France) via l'utilisation de tubes de lyse (MP Biomedicals) contenant du thiocyanate de guanidine (Extract-All®, Eurobio, les Ulis, France), et suivi d'une extraction au phénol chloroforme. Comme mentionné dans le chapitre 1, les concentrations, l'intégrité et la qualité des ARNs ont été déterminées au spectrophotomètre Nanodrop ND1000 (Labtech International, Paris, France). Les étapes de transcription inverse et de PCR temps réel ont été réalisées comme précédemment. Des ARNs non-inversement transcrits ont été utilisés comme contrôle pour vérifier que nous n'amplifions pas d'ADN génomique. En plus de cela, nos amorces étaient situées sur deux exons différents, limitant ainsi l'amplification d'ADN génomique. La spécificité des produits PCR a été contrôlée à la fin de la réaction par l'analyse de la courbe de dissociation. Basé sur la méthode de Peirson *et al.* (2003), également nommé DART-PCR (Data Analysis for Real Time-PCR), nous avons pu déterminer l'efficacité d'amplification de chaque échantillon, et ainsi la fluorescence initiale de chaque matrice d'ADNc. Cette approche rend possible l'analyse de chaque échantillon expérimental, basé sur sa propre réaction de cinétique plutôt que sur celle de standards artificiels. Enfin, les valeurs de fluorescence ont été normalisées par celle de deux gènes de ménage, la β2-µglobuline et la protéine ribosomale L32, et l'expression des gènes a été exprimée par rapport au groupe contrôle.

9) Analyses statistiques

Les données entre les groupes d'animaux à chaque temps de prélèvements ont été analysées par analyse de variance (ANOVA) et test *t* de student, après vérification de l'égalité des variances par le test de Fisher. Une valeur de $p < 0,05$ était considérée comme significative.

Tableau 20 : Effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur certains paramètres hématologiques et biochimiques à J35

	<i>Sans agents désactivateurs</i>				<i>Avec agents désactivateurs</i>			
	Contrôle	DON	FB	DON+FB	Contrôle	DON	FB	DON+FB
HEMATOLOGIE								
<i>Globules rouges (milliers/μL)</i>	6,2 \pm 0,3 ^a	5,7 \pm 0,2 ^a	6,1 \pm 0,4 ^a	5,9 \pm 0,4 ^a	6,3 \pm 0,2 ^a	5,8 \pm 0,4 ^a	6,3 \pm 0,4 ^a	6,1 \pm 0,2 ^a
<i>Globules blancs (milliers/μL)</i>	21,2 \pm 1,9 ^a	19,6 \pm 2,3 ^a	20,3 \pm 2,8 ^a	18,2 \pm 1,6 ^a	19,9 \pm 1,8 ^a	18,7 \pm 1,5 ^a	24,9 \pm 3,6 ^a	21,1 \pm 2,5 ^a
<i>Lymphocytes (milliers/μL)</i>	12,4 \pm 1,9 ^a	11,4 \pm 1,4 ^a	14,7 \pm 2,1 ^a	12,6 \pm 1,0 ^a	11,5 \pm 1,4 ^a	11,0 \pm 0,7 ^a	13,3 \pm 1,3 ^a	11,4 \pm 1,2 ^a
<i>Neutrophiles (milliers/μL)</i>	7,3 \pm 1,1 ^a	7,0 \pm 1,1 ^{a,b}	4,5 \pm 0,9^b	4,6 \pm 0,6^b	6,7 \pm 0,9 ^{a,b}	6,3 \pm 0,9 ^{a,b}	9,8 \pm 2,6 ^a	8,2 \pm 1,6 ^a
BIOCHIMIE								
<i>Urée (mmol/L)</i>	3,8 \pm 0,4 ^a	3,3 \pm 0,4 ^a	4,2 \pm 0,3 ^a	4,0 \pm 0,4 ^a	3,7 \pm 0,6 ^a	3,5 \pm 0,3 ^a	3,6 \pm 0,4 ^a	3,3 \pm 0,4 ^a
<i>Créatinine (μmol/L)</i>	102,5 \pm 5,3 ^a	98,0 \pm 4,1 ^a	120,5 \pm 5,6^b	101,6 \pm 5,5 ^a	101,7 \pm 3,7 ^a	96,5 \pm 6,9 ^a	100,0 \pm 3,1 ^a	101,6 \pm 5,1 ^a
<i>Cholestérol (mmol/L)</i>	2,6 \pm 0,2 ^a	2,4 \pm 0,2 ^a	2,3 \pm 0,1 ^a	2,3 \pm 0,1 ^a	2,6 \pm 0,2 ^a	2,1 \pm 0,2 ^a	2,3 \pm 0,2 ^a	2,3 \pm 0,2 ^a
<i>Triglycérides (mmol/L)</i>	0,51 \pm 0,07 ^{a,b}	0,34 \pm 0,04 ^a	0,39 \pm 0,06 ^a	0,41 \pm 0,06 ^{a,b}	0,42 \pm 0,07 ^{a,b}	0,45 \pm 0,06 ^{a,b}	0,43 \pm 0,04 ^{a,b}	0,58 \pm 0,07^b
<i>Protéines totales (g/L)</i>	59,8 \pm 1,0 ^a	57,1 \pm 2,1 ^a	59,9 \pm 2,5 ^a	57,6 \pm 2,5 ^a	60,8 \pm 2,2 ^a	58,8 \pm 2,4 ^a	63,4 \pm 4,5 ^a	58,8 \pm 2,2 ^a
<i>Albumine (g/L)</i>	34,3 \pm 0,7 ^a	29,2 \pm 1,5^b	35,1 \pm 2,1 ^a	32,8 \pm 2,1 ^{a,b}	34,6 \pm 1,1 ^a	33,4 \pm 2,1 ^{a,b}	35,3 \pm 2,8 ^{a,b}	30,2 \pm 1,0^b
<i>GGT (IU/L)</i>	65,4 \pm 8,6 ^a	88,6 \pm 14,4 ^a	79,4 \pm 15,0 ^a	77,0 \pm 11,5 ^a	70,6 \pm 1,8 ^a	86,4 \pm 7,2 ^a	82,8 \pm 23,7 ^a	67,8 \pm 7,4 ^a

Notes : les résultats sont présentés en moyenne \pm erreur type pour 6 animaux (Hématologie) et 5 animaux (Biochimie). ^{a,b} En ligne, les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$). GGT, γ -glutamyl transférase

RESULTATS**1) Effet des agents détoxifiants sur le gain de poids, l'hématologie et la biochimie**

Le gain de poids cumulé sur la période de 35 jours est présenté dans le **Tableau 19** ci-dessous.

Tableau 19 : Effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur le gain de poids total

<i>Sans agents désactivateurs</i>				<i>Avec agents désactivateurs</i>			
Contrôle	DON	FB	DON+FB	Contrôle	DON	FB	DON+FB
21,0 ± 1,7 ^{a,b}	18,5 ± 1,0 ^a	21,6 ± 1,5 ^{a,b,c}	18,8 ± 1,5 ^a	25,3 ± 0,9^c	22,3 ± 0,6 ^b	20,5 ± 1,9 ^{a,b}	18,8 ± 0,7 ^a

Notes : les résultats sont présentés en moyenne ± erreur type pour 5 animaux. Le gain de poids est exprimé en kilogramme.

^{a,b,c} Les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$).

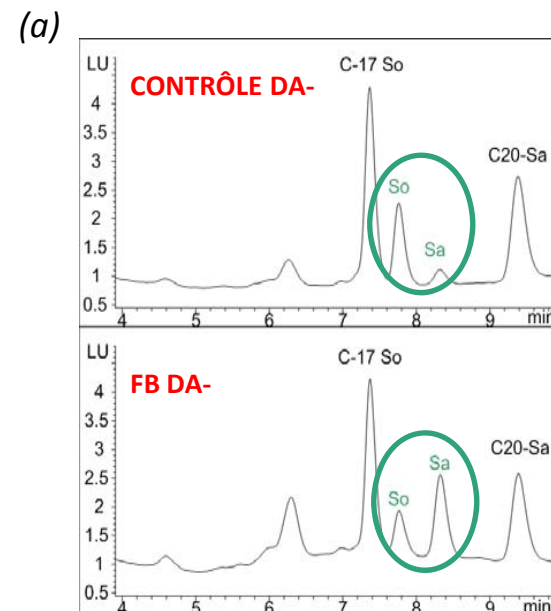
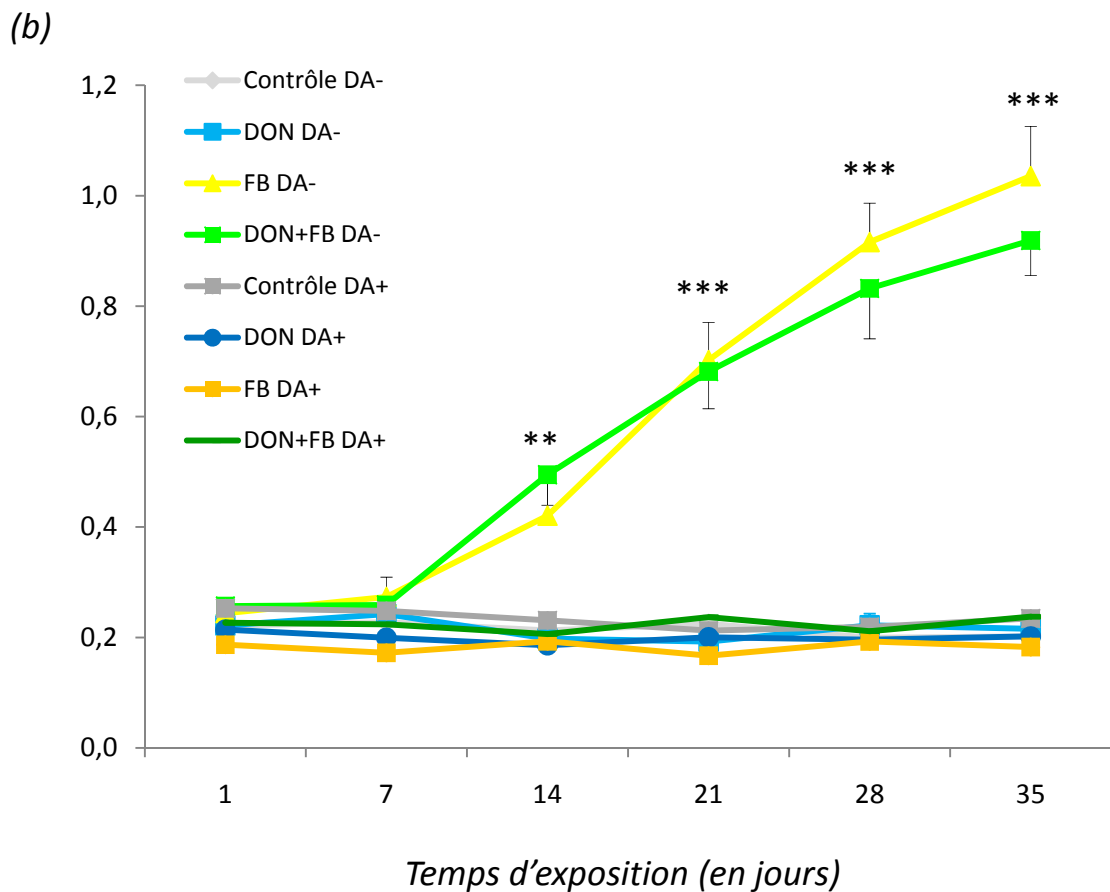
Les régimes mono- et co-contaminés n'ont pas altéré la croissance des porcelets tout au long de la période expérimentale (**Tableau 19**). En revanche, la présence d'agents détoxifiants dans les groupes contrôle et DON, a augmenté significativement les performances de croissance des animaux en comparaison respective avec les groupes non supplémentés (+21% et +20% respectivement).

Concernant les paramètres hématologiques, nous avons observé une diminution significative du nombre de neutrophiles dans le sang pour les régimes FB et DON+FB (**Tableau 20**). Cette baisse du nombre de neutrophiles n'a pas été obtenue lorsque ces régimes ont été supplémentés avec les agents désactivateurs.

L'étude de certains paramètres biochimiques a montré une augmentation de la concentration de la créatinine et une diminution de la concentration d'albumine dans les plasmas des animaux ayant ingéré les aliments mono-contaminés en FB et DON, respectivement (**Tableau 20**). Aucun effet n'a été observé pour le régime co-contaminé. Les animaux exposés aux régimes mono-contaminés et supplémentés avec les DA n'ont pas montré d'altération des valeurs biochimiques pour la créatinine et l'albumine. En revanche, une baisse significative de la concentration plasmatique d'albumine a été observée pour le régime DON+FB avec l'ajout de DA comparé aux deux groupes contrôles. A noter l'augmentation du taux de triglycérides pour le même régime (**Tableau 20**), bien que non significative par rapport aux groupes contrôles.

Figure 16 : effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur les bases sphingoides, sphinganine (Sa) et sphingosine (So)

- (a) Exemple de chromatogrammes obtenues en HPLC, C-17 So et C-20 Sa étant les standards (solutions étalons), et les pics entourés correspondant à la détection des bases Sa et So dans les échantillons.
 (b) Ratio Sa/So dans les plasmas des porcelets exposés aux différents régimes et pendant toute la période expérimentale.



Notes : DA, Agents Désactivateurs. Les résultats sont exprimés en moyenne \pm erreur type pour 6 animaux.
 *** Différence significative entre les régimes FB et DON+FB sans DA, avec les autres régimes ($p < 0,001$).

2) Effet des agents détoxifiants sur la concentration en bases sphingolipides

La concentration des bases sphingolipides, sphinganine (Sa) et sphingosine (So), a été mesurée dans les plasmas des porcelets tout au long de la période expérimentale. Afin d'évaluer l'effet sur le métabolisme des sphingolipides, les ratios Sa/So ont été calculés et sont présentés dans la **Figure 16**.

Une nette augmentation de ce ratio, caractérisé principalement par une augmentation des sphinganines, a été observée dès le 14^{ème} jour d'exposition pour les animaux exposés aux FB et aux FB associées au DON (**Figure 16**). L'élévation du ratio est dépendante du temps d'exposition et est cinq fois supérieur au contrôle pour ces deux régimes à J35. Comme attendu, le régime mono-contaminé avec le DON n'a pas induit de modification de la concentration en bases sphingolipides. De la même manière, aucun des régimes supplémentés en DA n'a montré de différences dans les ratios (**Figure 16**). Les résultats révèlent une différence largement significative entre les groupes FB et FB+DON et leurs homologues respectifs contenant les DA.

3) Effet des agents détoxifiants sur l'histopathologie des organes

➤ Sur le FOIE

La prolifération des hépatocytes a été mesurée par immunohistochimie, le noyau des cellules en prolifération ayant été marqué par un anticorps spécifique anti-Ki-67. Les résultats sont présentés ci-dessous dans le **Tableau 21**.

Tableau 21 : Effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur l'index de prolifération des hépatocytes

<i>Sans agents désactivateurs</i>				<i>Avec agents désactivateurs</i>			
Contrôle	DON	FB	DON+FB	Contrôle	DON	FB	DON+FB
16,4 ± 1,5 ^a	18,8 ± 3,3 ^{a,b}	22,8 ± 1,7 ^b	39,4 ± 12,8^c	16,7 ± 2,4 ^a	18,6 ± 2,3 ^{a,b}	21,4 ± 1,7 ^{a,b}	19,0 ± 0,9 ^{a,b}

Notes : les résultats sont présentés en moyenne ± erreur type pour 5 animaux. L'index de prolifération a été calculé selon le nombre de cellules positives Ki-67/nombre total de cellules (100). ^{a,b,c} Les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$).

L'analyse du marqueur nucléaire Ki-67 sur les sections de foie a révélé une importante prolifération des hépatocytes chez les animaux exposés au régime contenant les deux mycotoxines, DON et FB (**Tableau 21**). A l'inverse, ce même régime supplémenté en DA n'a pas montré une hausse significative de l'index de prolifération.

En plus de l'analyse immunohistochimique, l'observation des lames colorées à l'hématoxyline-éosine a permis d'identifier différents types de lésions. Comme mentionné dans la partie Matériel et Méthodes, ces lésions ont pu être évaluées numériquement afin d'établir un score lésionnel par animal. Ainsi, nous présentons ci-dessous dans la **Figure 17**, le score lésionnel en fin d'expérience pour chaque groupe :

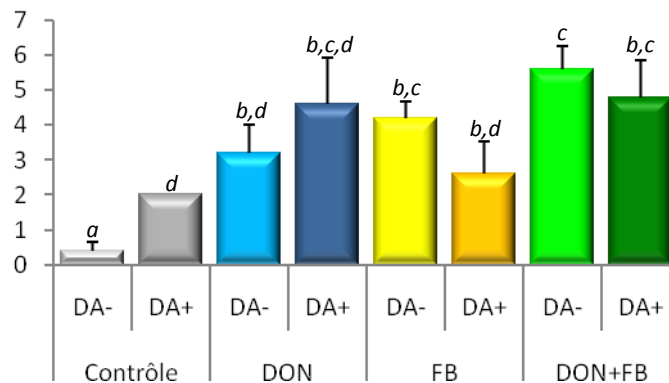


Figure 17 : Effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur le score lésionnel du foie

Notes : DA, Agents Désactiveurs. Les résultats sont présentés en moyenne \pm erreur type pour 5 animaux. ^{a,b,c,d} Les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$).

L'analyse histologique des coupes de foie a révélé différentes lésions, les principales étant la présence de vacuoles dans le noyau et le cytoplasme des hépatocytes, et des mégaloctoses. Ces lésions peuvent être considérées comme légères à modérées. Les animaux exposés aux toxines montrent un score significativement plus élevé que les animaux contrôles, en particulier pour les animaux exposés aux deux toxines simultanément (**Figure 17**). De manière surprenante, le régime contrôle supplémenté en DA présentait un score plus élevé que le même régime non supplémenté, dû à la présence de quelques lésions hépatiques. Hormis le régime FB+DA, les autres régimes contaminés (DON et DON+FB) et contenant les DA ont induit des dommages significatifs au niveau du foie, comme en témoignent les scores lésionnels.

➤ **Sur les POUMONS**

De la même manière que pour le foie, différents types de lésions ont été remarquées après coloration histologique des lames de poumons, et ont permis d'établir un score lésionnel. Ces scores sont représentés ci-dessous dans la **Figure 18** :

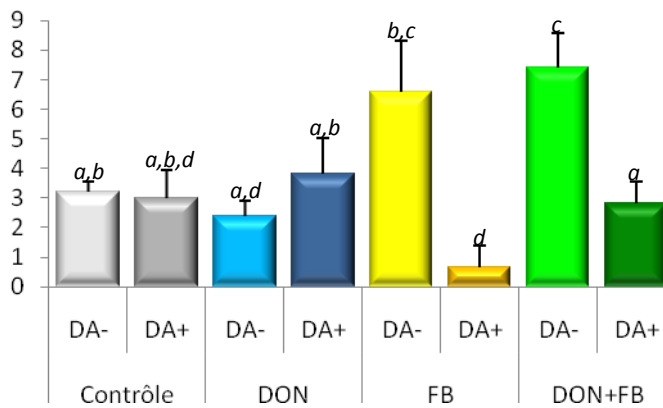


Figure 18 :

Effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur le score lésionnel des poumons

Notes : DA, Agents Désactivateurs. Les résultats sont présentés en moyenne \pm erreur type pour 5 animaux. ^{a,b,c,d} Les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$).

L'analyse histologique des coupes de poumons a révélé différentes lésions, les principales étant une déplétion du tissu lymphoïde associé aux bronchioles et des troubles vasculaires (hémorragie alvéolaire, congestion). Ces lésions peuvent être considérées comme légères à modérées. Une nette élévation du score a été obtenue pour les animaux ayant consommé les régimes contenant les FB (FB et DON+FB). Concernant les régimes supplémentés avec les DA, aucun dommage pulmonaire significatif n'a été observé (**Figure 18**). A noter que les animaux du groupe FB+DA avaient le plus petit score, même en comparaison des deux groupes contrôles.

➤ Sur l'INTESTIN

L'analyse des lésions microscopiques, telle que présentée dans le chapitre 1, est toujours en cours de réalisation et ne pourra donc être présentée dans ce manuscrit.

En revanche, des analyses histomorphométriques ont été réalisées sur le jéjunum et l'iléon des porcelets exposés aux différents régimes. Des altérations dans la hauteur des villosités et la profondeur des cryptes indiquent une possible perte d'entérocytes et une détérioration de l'absorption des nutriments. Aucun effet sur ces paramètres n'a été observé dans l'iléon des animaux. En revanche, l'analyse morphologique a révélé un effet du régime mono-contaminé en DON sur la hauteur des villosités dans le jéjunum, illustrée ci-dessous dans la **Figure 19** :

Figure 19 :

Effet de l'exposition au DON avec un régime alimentaire supplémenté ou non en agents désactivateurs sur la hauteur des villosités du jéjunum (exprimé en μm)

Notes : DA, Agents Désactivateurs. Les résultats sont présentés en moyenne \pm erreur type pour 5 animaux. ^{a,b} Les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$).

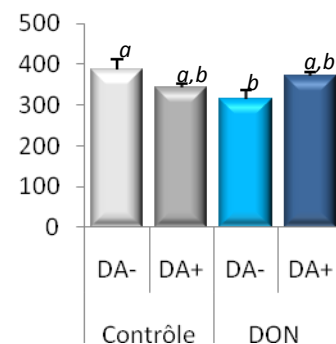


Tableau 22 : Effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur la population cellulaire et la prolifération cellulaire du jéjunum et de l'iléon.

	<i>Sans agents désactivateurs</i>				<i>Avec agents désactivateurs</i>			
	Contrôle	DON	FB	DON+FB	Contrôle	DON	FB	DON+FB
JEJUNUM								
<i>Lymphocytes</i>	26,3 ± 3,0 ^a	17,2 ± 1,1^{b,c}	19,3 ± 1,7 ^{a,b}	18,9 ± 1,0^{b,d}	21,8 ± 3,0 ^{a,b}	14,2 ± 0,8^c	18,9 ± 1,6 ^{a,b}	21,8 ± 1,3 ^{a,d}
<i>Plasmocytes</i>	24,9 ± 1,3 ^a	25,3 ± 3,4 ^{a,b}	32,4 ± 2,8^b	24,8 ± 0,8 ^a	26,5 ± 2,4 ^{a,b}	22,4 ± 1,9 ^a	24,7 ± 0,9 ^a	24,8 ± 2,5 ^{a,b}
<i>Eosinophiles</i>	8,3 ± 0,4 ^a	9,2 ± 1,1 ^{a,b}	10,7 ± 0,9^b	7,4 ± 0,8 ^a	9,0 ± 1,2 ^{a,b}	8,9 ± 0,8 ^{a,b}	8,7 ± 0,9 ^{a,b}	9,0 ± 1,2 ^{a,b}
<i>Entérocytes en mitose</i>	2,6 ± 0,3 ^a	1,5 ± 0,2^b	1,8 ± 0,2^{b,c}	1,8 ± 0,2^{b,c}	1,5 ± 0,2^b	2,3 ± 0,3 ^{a,c}	2,5 ± 0,3 ^{a,c}	2,8 ± 0,2 ^a
ILEON								
<i>Lymphocytes</i>	20,3 ± 2,7 ^a	16,3 ± 1,1 ^{a,b}	18,2 ± 1,4 ^a	12,9 ± 1,9^b	18,4 ± 1,9 ^a	17,1 ± 2,0 ^{a,b}	18,6 ± 1,9 ^a	19,1 ± 2,7 ^{a,b}
<i>Plasmocytes</i>	22,0 ± 2,0 ^a	22,7 ± 1,9 ^a	24,8 ± 3,0 ^{a,b}	26,8 ± 0,8^b	20,0 ± 1,3 ^a	22,7 ± 1,6 ^a	22,2 ± 1,7 ^a	22,4 ± 1,8 ^a
<i>Eosinophiles</i>	12,0 ± 0,9 ^a	12,5 ± 1,8 ^{a,b}	11,4 ± 1,6 ^{a,b}	8,9 ± 0,6^b	15,5 ± 2,5 ^a	11,1 ± 1,7 ^{a,b}	11,7 ± 2,4 ^{a,b}	9,9 ± 1,3 ^{a,b}
<i>Entérocytes en mitose</i>	1,9 ± 0,3 ^{a,b}	1,9 ± 0,4 ^{a,b}	1,7 ± 0,2 ^a	2,0 ± 0,1 ^a	1,9 ± 0,3 ^{a,b}	2,1 ± 0,3 ^{a,b}	1,9 ± 0,3 ^{a,b}	2,5 ± 0,2^b

Notes : les résultats sont présentés en moyenne ± erreur type pour 5 animaux. ^{a,b,c,d} En ligne, les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$).

L'exposition individuelle au DON a réduit significativement la hauteur des villosités dans le jéjunum des porcelets (**Figure 19**). La présence des DA dans le régime respectif a partiellement neutralisé l'altération induite par le DON seul. Toutefois, une différence non négligeable entre les deux groupes contrôles a été mesurée.

Le nombre de lymphocytes, de cellules plasmiques et d'éosinophiles a été évalué dans la lamina propria de l'intestin. Le dénombrement de ces cellules est reporté dans le **Tableau 22**, ainsi que la prolifération des cellules, caractérisée par le nombre d'entérocytes en mitoses.

La population lymphocytaire était significativement réduite après consommation du régime mono-contaminé en DON dans le jéjunum, et du régime co-contaminé en DON et FB dans le jéjunum et l'iléon (**Tableau 22**). Un nombre plus élevé de plasmocytes et d'éosinophiles après exposition aux FB seules a été noté dans le jéjunum des animaux. Dans l'iléon, le groupe co-contaminé a augmenté le nombre de plasmocytes et diminué le nombre d'éosinophiles. De manière intéressante, le jéjunum des porcelets était plus affecté par les régimes ne contenant qu'une mycotoxine, alors qu'au niveau de l'iléon les deux toxines en association ont montré un effet plus important. Ces effets sur la population cellulaire de la lamina propria du jéjunum et de l'iléon n'ont pas été observés en présence des DA, excepté sur les lymphocytes de la section jéjunale pour le régime mono-contaminé en DON (**Tableau 22**).

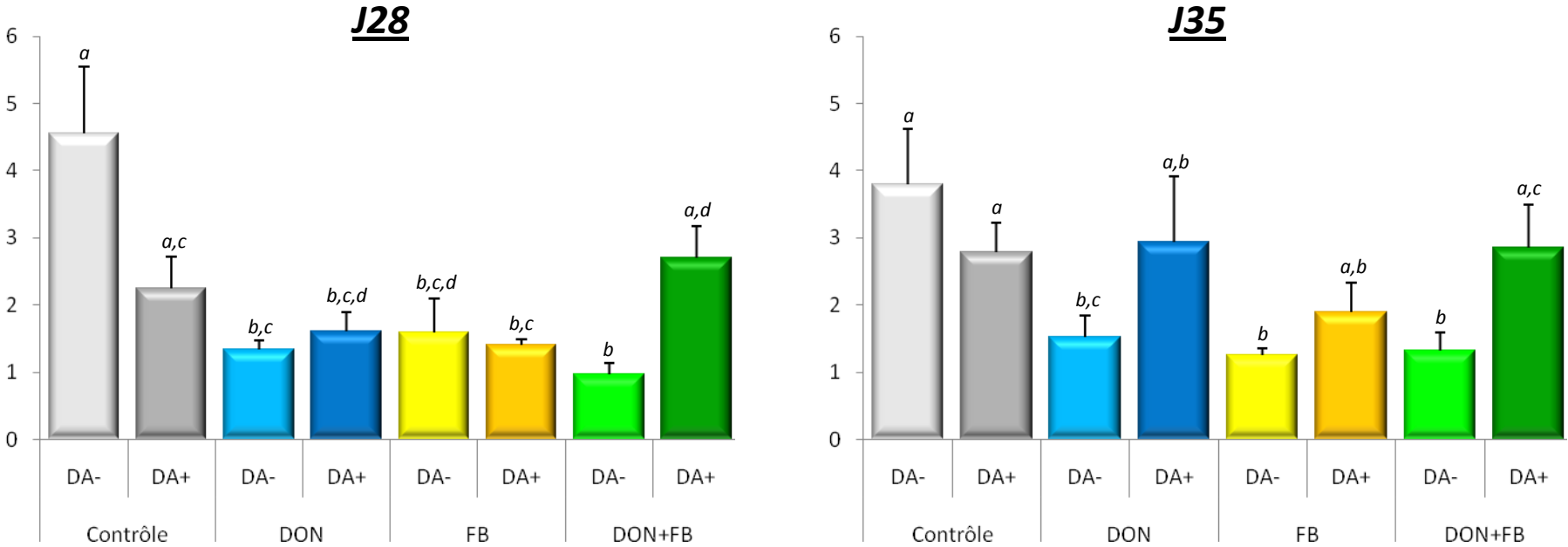
Le dénombrement des entérocytes en mitoses a montré une baisse significative de la prolifération cellulaire pour les régimes mono- et co-contaminés dans le jéjunum (**Tableau 22**). Cet effet n'a pas été observé dans la section iléale de l'intestin. L'addition des DA a contrecarré la diminution de la prolifération jéjunale observée dans les régimes homologues non supplémentés. Toutefois, le régime contrôle avec les DA présentait un nombre de cellules en mitoses aussi faible que dans les régimes mono-contaminés non-supplémentés. A noter que dans l'iléon, la prolifération des entérocytes était plus élevée pour le groupe DON+FB+DA (**Tableau 22**).

4) Effet des agents détoxifiants sur le système immunitaire

Aucun effet des mycotoxines n'a été observé sur la réponse immunitaire totale et non-spécifique. L'ingestion des régimes contaminés n'a ni altéré la synthèse des immunoglobulines (Ig) G et A totaux dans le plasma, et ni modifié la prolifération des lymphocytes après stimulation par le mitogène Concanavaline A.

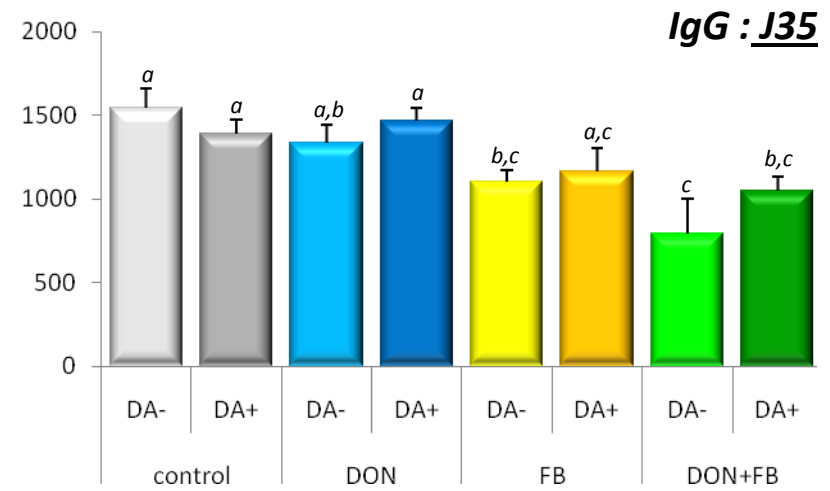
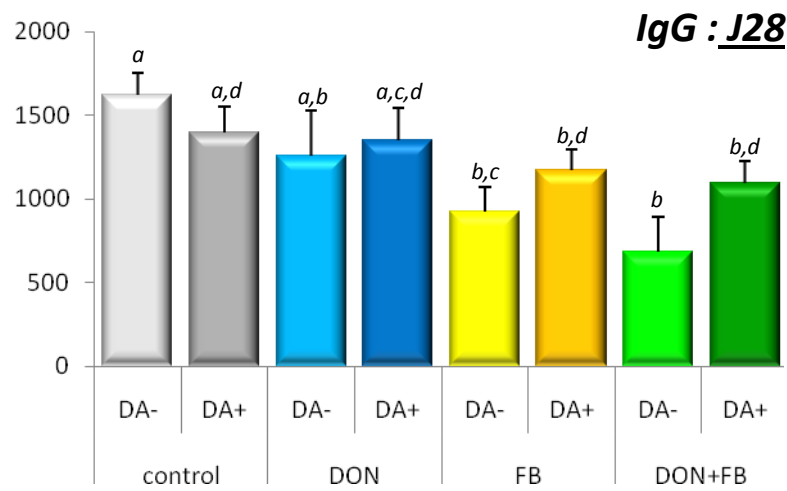
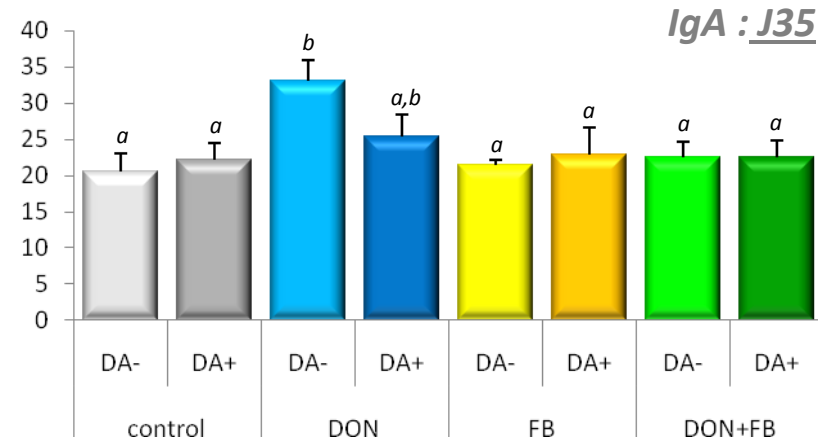
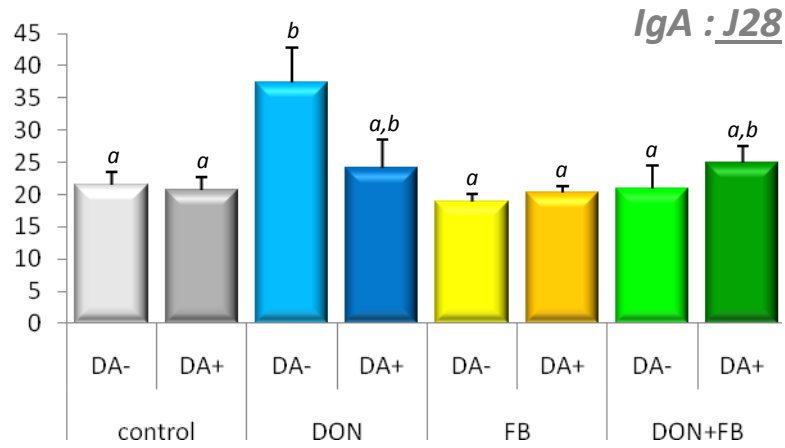
Néanmoins, le protocole d'immunisation avec l'ovalbumine nous a permis d'examiner le développement de la réponse cellulaire et humorale, spécifique de cet antigène. Les résultats sont reportés dans les sections ci-après.

Figure 20 : Effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur la prolifération des lymphocytes après stimulation antigénique, à J28 et J35



Notes : DA, Agents Désactivateurs. Les résultats sont exprimés en index de prolifération des lymphocytes (cpm dans les cultures stimulées / cpm dans les cultures contrôles non-stimulées). Les valeurs sont présentées en moyenne \pm erreur type pour 5 animaux. ^{a,b,c,d} Les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$).

Figure 21 : Effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur la concentration plasmatique des immunoglobulines A et G spécifiques de l'ovalbumine, à J28 et J35



Notes : DA, Agents Désactivateurs. Les résultats sont exprimés en unités arbitraires, normalisés avec un plasma standard de référence. Les valeurs sont présentées en moyenne \pm erreur type pour 5 animaux. ^{a,b,c,d} Les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$).

➤ **Sur la réponse spécifique CELLULAIRE**

La composante cellulaire de la réponse vaccinale a été évaluée par la mesure de la prolifération des lymphocytes, suite à la mise en culture et la stimulation des cellules du sang total avec l'antigène vaccinal, l'ovalbumine. Comme attendu, les cellules du sang n'ont pas proliféré en présence d'ovalbumine avant la vaccination des animaux (jour 1, cf. chapitre 1). Suite à l'injection d'ovalbumine et notamment après la deuxième injection (rappel), une augmentation significative de l'index de prolifération (cpm dans les cultures stimulées / cpm dans les cultures contrôles non-stimulées) a été observée pour les animaux contrôles. Dans la **Figure 20** est représentée l'index de prolifération des lymphocytes, 12 jours après la seconde injection d'ovalbumine.

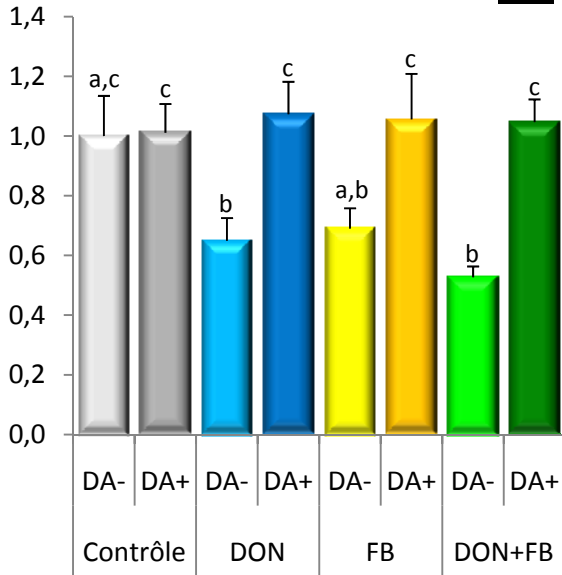
Les lymphocytes des porcelets exposés aux régimes mono- et co-contaminés montrent tous une diminution significative, par rapport au contrôle, de la capacité à proliférer après stimulation avec l'ovalbumine (**Figure 20**). La prolifération reste aussi faible que celle des lymphocytes contrôles non-stimulés. Pour les régimes supplémentés avec les DA, le contrôle est non significativement différent de son homologue non supplémenté, mais l'index prolifératif est néanmoins inférieur. Une nette amélioration de la prolifération a été obtenue lorsque les groupes contaminés DON et DON+FB contenaient les DA, en comparaison des régimes respectifs sans DA (DON+DA à J35, 1,9 fois supérieur ; DON+FB+DA à J28 et à J35, 2,8 et 2,2 fois supérieur, respectivement).

➤ **Sur la réponse spécifique HUMORALE**

La composante humorale de la réponse vaccinale a été évaluée par la mesure du contenu en IgA et IgG plasmatiques reconnaissant spécifiquement l'antigène vaccinal, l'ovalbumine. Comme pour la prolifération des lymphocytes, la concentration en Ig spécifiques de l'ovalbumine était insignifiante avant la vaccination des animaux (Jour 1, cf. chapitre 1). Leurs concentrations ont fortement augmenté suite à la deuxième administration d'ovalbumine, en particulier pour les IgG. Dans la **Figure 21**, nous présentons de la même manière que pour les résultats de la prolifération, les concentrations des IgA et IgG dirigées contre l'ovalbumine, 12 jours après la seconde injection de l'antigène.

Concernant les IgA, une élévation significative de leurs concentrations a été observée dans les plasmas des animaux ayant ingéré le DON seul (**Figure 21**, +73% et +61% à J28 et J35 respectivement). En revanche, le DON combiné aux FB n'a pas entraîné de différences significatives avec le contrôle. L'ajout de DA dans le régime mono-contaminé en DON a permis de réduire cette altération des IgA.

IL-8



MIP-1 β

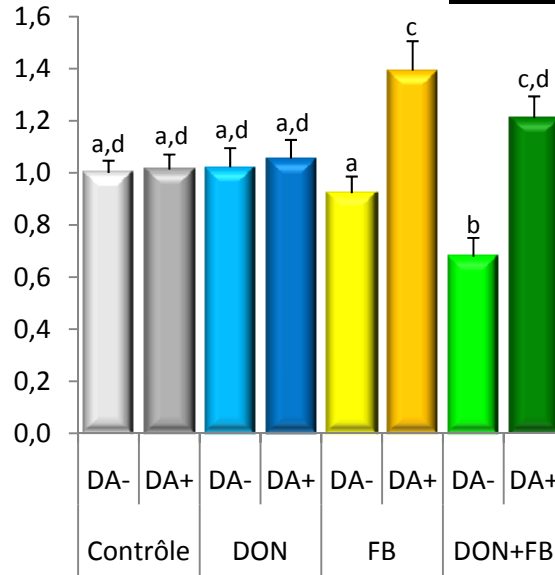
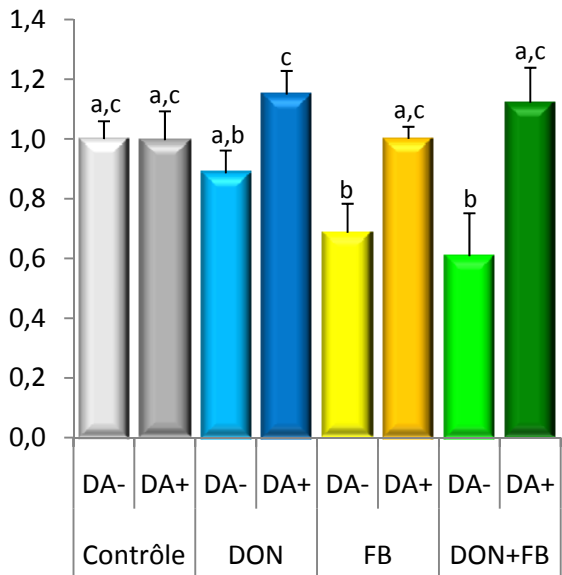


Figure 22 :

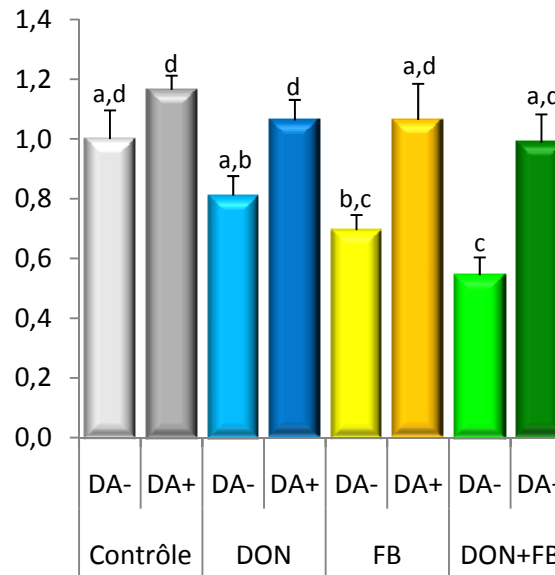
Effet de l'exposition aux mycotoxines avec un régime alimentaire supplémenté ou non en agents désactivateurs sur l'expression des ARNs spléniques codant pour des cytokines

Notes : DA, Agents Désactivateurs. Les valeurs sont présentées en moyenne \pm erreur type pour 5 animaux. *a,b,c,d* Les valeurs ne présentant pas le même exposant sont significativement différentes ($p < 0,05$).

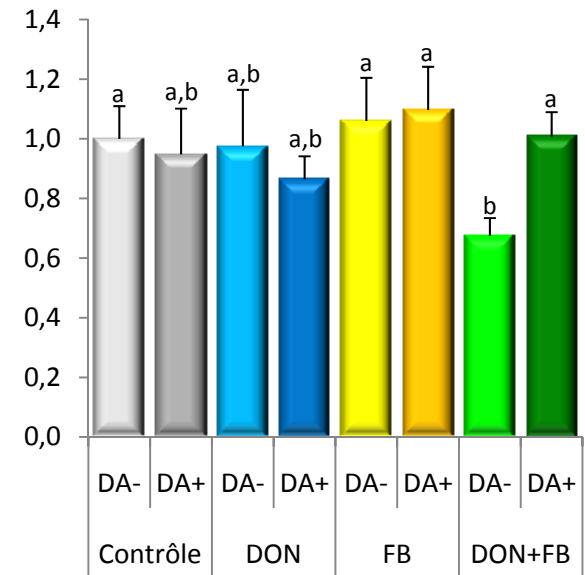
IL-1 β



IL-6



IL-12p40



Concernant les IgG, une diminution significative de leurs concentrations a été observée dans les plasmas des animaux ayant ingéré les FB seules, et surtout les FB associées au DON (**Figure 21**, -43% et -30% à J28 et J35 pour FB, respectivement, et -58% et -49% à J28 et J35 pour DON+FB, respectivement). L'ajout de DA n'a que très faiblement amélioré le changement induit par les FB seules, mais a partiellement restauré la dépression observée par les FB associées au DON (1,6 fois et 1,3 fois mieux à J28 et J35 respectivement).

➤ ***Sur l'expression des ARNm codant des CYTOKINES***

Afin de compléter le tableau sur le développement de la réponse vaccinale, nous avons évalué l'expression dans la rate, organe lymphoïde secondaire, des ARNm codant pour les cytokines IL-1 β , IL-6, IL-12p40, IL-8 et MIP-1 β . Ces cytokines sont connues pour être impliquées, en tant que messagers entre les cellules immunitaires, dans le développement de la réponse immunitaire spécifique.

Tous les traitements en mycotoxines ont affecté à J35 l'expression de ces messagers dans la rate, en particulier le traitement co-contaminé (**Figure 22**, -39%, -46%, -33%, -47% et -32% d'expression des transcrits codant respectivement pour IL-1 β , IL-6, IL-12p40, IL-8 et MIP-1 β dans la rate des animaux traités avec DON+FB en comparaison des animaux contrôles). L'ajout des DA dans les régimes contaminés en mycotoxines a en revanche nettement amélioré l'expression de ces ARNm, avec un profil de cytokines similaire à celui des deux groupes contrôles (**Figure 22**). Toutefois, une exception a été remarquée, avec le traitement FB+DA provoquant une augmentation significative de l'expression de MIP-1 β .

DISCUSSION

Les objectifs de nos travaux conduits avec différents groupes expérimentaux étaient (i) d'évaluer la toxicité induite par une combinaison de mycotoxines, le déoxynivalénol (DON) et la fumonisine (FB) deux fusariotoxines d'intérêt majeur, en comparaison de leur toxicité individuelle ; (ii) d'évaluer ces expositions chez le porc et à de faibles doses, le modèle animal choisi étant pertinent par rapport à son exposition naturelle via la composition de sa ration alimentaire, sa sensibilité et ses similitudes avec l'homme, et le choix des doses étant approprié par rapport aux situations rencontrées dans les élevages ; (iii) d'évaluer l'efficacité d'agents désactivateurs (DA) dans la lutte menée contre les effets nocifs de ces mycotoxines ; (iiii) et d'analyser la réponse des animaux sur des paramètres plus fins et plus ciblés que l'étude de paramètres zootechniques.

Toutefois, nous ne discuterons ici que de l'effet des agents désactivateurs, conçus par la société BIOMIN, considérant que les effets des mycotoxines, seules ou en combinaison, sur la réponse systémique et intestinale ont déjà été discutés dans le chapitre 1. Pour rappel, un des agents désactivateurs cible spécifiquement la mycotoxine DON, et l'autre spécifiquement les FB.

Les régimes contaminés en mycotoxines n'ont pas eu d'effets significatifs sur la croissance des porcelets tout au long de la période expérimentale. Pourtant l'ajout des DA dans les régimes a eu un effet non spécifique mais bénéfique sur la prise de poids des animaux, en particulier pour ceux des groupes contrôle et DON. Cet effet sur le gain de poids a déjà été reporté lors de l'utilisation de produits similaires développés par BIOMIN (Hanif *et al.*, 2008). Dans notre étude, nous pouvons attribuer le peu d'effets observés sur les paramètres hématologiques et biochimiques à l'utilisation de faibles doses de mycotoxines. Néanmoins, ces effets mineurs ont été totalement neutralisés par la présence des DA dans les aliments contaminés. La baisse du nombre de neutrophiles dans les régimes FB et FB+DON n'a pas été observée dans les régimes respectifs supplémentés. De même, les modifications biochimiques provoquées par les FB seules sur la concentration en créatinine, et par le DON seul sur la concentration en albumine, n'ont pas été observées dans les régimes respectifs supplémentés.

La perturbation du métabolisme des sphingolipides par les fumonisines a largement été décrite depuis de nombreuses années (Wang *et al.*, 1991), et reconnue comme responsable de la toxicité de ces toxines. Comme précédemment reporté dans le manuscrit, les fumonisines inhibent la céramide synthase et provoquent une accumulation des bases sphingoides, sphinganine (Sa) et sphingosine (So). Ainsi, la détermination du ratio Sa/So dans les fluides et tissus biologiques représente un bon marqueur d'exposition et de toxicité (Tran *et al.*, 2006). Comme attendu, les animaux exposés aux régimes contenant des FB présentaient un ratio largement supérieur aux animaux non exposés aux

FB. A noter que la présence de DON avec les FB n'a pas entraîné de potentialisation ou encore d'effet antagoniste ; le ratio étant égal à celui du régime mono-contaminé pendant les 35 jours d'exposition. A l'inverse, la supplémentation en DA dans ces deux régimes a semblé totalement réprimer l'action des FB sur le métabolisme des sphingolipides, comme en témoigne la mesure des ratios Sa/So. Ainsi, il est fortement probable que la carboxylestérase, isolée par BIOMIN et pulvérisée sur les aliments contaminés, ait hydrolysée totalement les fumonisines dans les conditions du tractus gastro-intestinal.

Le foie et les poumons étant des organes cibles de ces mycotoxines, en particulier des FB, et l'intestin étant un organe naturellement exposé aux toxines suite à l'ingestion d'aliments contaminés, l'examen microscopique de ces tissus était ainsi nécessaire dans l'évaluation du risque mycotoxique. De plus, peu d'études ont reporté l'effet de faibles doses sur l'histopathologie des organes. Les résultats de nos analyses ont révélé que les mycotoxines seules ou en association induisaient des lésions hépatiques et pulmonaires, et altéraient l'intégrité intestinale (cf. chapitre 1). Les effets sur le foie et les poumons supposent une biodisponibilité et/ou une activation des mycotoxines suite à leur absorption intestinale. Ainsi, une efficacité optimale des agents détoxifiants suggère une biotransformation totale des mycotoxines dans les conditions du tube digestif, réduisant ainsi leur biodisponibilité. Le procédé ne doit pas non plus conduire à la production de métabolites toxiques. L'analyse des sections de foie des animaux ayant ingéré les régimes supplémentés en DA a révélé la présence de lésions hépatiques. De manière surprenante et inattendue, les animaux contrôles présentaient un score lésionnel élevé, significativement différent du score du groupe contrôle non supplémenté. Cette différence de scores entre les deux groupes contrôles était essentiellement attribuée à la présence de mégaloctoses, comme en témoigne l'examen des lames histologiques des contrôles DA+. Par conséquent, la présence de mégaloctoses chez les porcelets nourris avec les régimes contaminés et supplémentés, est vraisemblablement liée à un effet propre des agents détoxifiants qu'à un effet des mycotoxines. Dans ce sens, nous pouvons supposer que la biodisponibilité des FB a été fortement réduite, si l'on soustrait le score du groupe contrôle DA+ à celui du régime FB DA+. De la même manière, les scores obtenus pour les groupes DON et DON+FB supplémentés en DA pourraient être attribués d'une part à l'effet des DA, et d'autre part à un effet partiel du DON et/ou de son métabolite de biotransformation.

A l'inverse, la présence des DA dans les différents régimes n'a pas induit une augmentation de la prolifération des hépatocytes, et a grandement réduit l'index de prolifération observé dans le groupe co-contaminé en DON et en FB non supplémenté. L'addition des DA dans l'alimentation a également neutralisé l'effet des FB dans les poumons des animaux. Comme déjà mentionné dans ce mémoire et largement décrit dans la littérature, les FB ingérées à de fortes concentrations peuvent induire des

œdèmes pulmonaires chez le porc (Haschek *et al.*, 2001). A faibles doses, les effets sont beaucoup moins connus sur le poumon, mais dans notre étude que ce soit seules ou en combinaisons, les FB ont aussi provoqué des lésions pulmonaires. Ces lésions étaient absentes des groupes traités avec les produits détoxifiants. Ici également, nous pouvons supposer que la biodisponibilité des FB a été réduite, via son hydrolyse totale dans l'intestin par la carboxylestérase.

Bien que la principale voie d'exposition aux mycotoxines soit la voie entérale, l'impact de ces métabolites sur le tractus gastro-intestinal est une thématique encore peu étudiée. Nos résultats (cf chapitre 1) ont pourtant montré que même à faibles doses, les mycotoxines pouvaient affecter certaines fonctions et caractéristiques des cellules de l'épithélium. L'effet sur la diminution de la hauteur des villosités dans le jéjunum, après ingestion du régime mono-contaminé en DON, a été bloqué lors de l'ajout des DA dans ce régime. En revanche, une baisse de la hauteur des villosités a été notée pour le groupe contrôle supplémenté en DA, avec une valeur intermédiaire entre celles des deux groupes DON supplémenté ou non. Cette observation pourrait être liée à un plus faible nombre de cellules en mitoses dans la même région, puisque les entérocytes qui composent les villosités et les cryptes sont des cellules à division rapide. De plus, l'effet du DON avec ou sans DA sur les cellules en mitoses corrobore les données sur les villosités. Aucun effet sur ces paramètres n'a été constaté dans la partie iléale.

La supplémentation en DA dans le régime mono-contaminé en DON n'a pas permis de réduire l'effet sur la diminution de l'infiltration lymphocytaire dans le jéjunum. Elle l'a néanmoins réduite dans le groupe co-contaminé que ce soit dans le segment jéjunal ou iléal. A l'inverse de l'effet sur les lymphocytes, une plus forte infiltration de cellules plasmiques et d'éosinophiles dans le jéjunum a été détectée dans le régime mono-contaminé en FB. Aucune infiltration significative n'a été reportée pour les régimes supplémentés en DA. De la même manière, les effets sur ces populations cellulaires dans l'iléon ont été partiellement ou totalement résorbés en présence de DA.

Un des principaux objectifs de cette étude était d'étudier la réponse des animaux traités suite à un protocole d'immunisation. En effet, la présence de pathogènes et la vaccination sont des situations courantes dans les élevages, et considérant l'effet immunomodulateur des mycotoxines, le développement de la réponse spécifique suite à un challenge antigénique a été examiné. Comme expliqué dans le chapitre 1, nous avons montré que suite à l'immunisation des porcelets avec l'ovalbumine, les faibles doses de DON et de FB utilisées affectaient significativement la mise en place d'une réponse adaptée, et notamment lorsque ces toxines étaient en combinaison. Cette mise en place de la réponse vaccinale chez les animaux contrôles était optimale après la seconde injection d'ovalbumine. Les données après ce rappel vaccinal ont montré que la prolifération des lymphocytes en culture après stimulation avec l'antigène vaccinal était altérée par les mycotoxines. Un effet

bénéfique des DA n'a été observé qu'en fin d'expérience, et en particulier pour le régime co-contaminé.

En plus de l'analyse de la réponse cellulaire, nous avons analysé la composante humorale par le biais du titrage des immunoglobulines A et G reconnaissant spécifiquement l'ovalbumine. Comme largement décrit dans la littérature (Pestka *et al.*, 2003, 2004, 2005; Pinton *et al.*, 2008) et confirmé dans notre étude, l'ingestion continue en DON induit la synthèse des IgA. Cette altération de la concentration plasmatique des IgA anti-ovalbumine n'a pas été constatée après traitement du régime mono-contaminé en DON avec les DA. L'analyse des IgG spécifiques, qui est la classe d'anticorps principalement produite en réponse à un antigène, a montré une nette diminution de leurs synthèses après exposition aux régimes FB et DON+FB. Ces régimes supplémentés en DA ont présenté un meilleur profil d'IgG en fin d'expérience, bien que l'amélioration n'ait été que partielle. Une restauration partielle du titre d'anticorps anti-pseudo-rage a déjà été reportée chez des porcelets alimentés avec un régime co-contaminé en DON et zéaralénone (ZEA) et supplémenté avec l'agent désactivateur ciblant spécifiquement le DON, mais aussi avec un spécifique de la ZEA (Cheng *et al.*, 2006).

Considérant que les cytokines sont des messagers importants dans le développement des réponses immunitaires, nous avons évalué à la fin de l'expérience l'expression de transcrits codant pour des cytokines dans la rate des animaux. Au vu des résultats des régimes contaminés et non supplémentés sur les cytokines IL-8, MIP-1 β , IL-6, IL-1 β et IL-12p40, nous nous sommes restreints à l'analyse de leur expression pour les régimes supplémentés. Les effets observés après l'ingestion des mycotoxines ont été considérablement atténués en présence des DA pour ces cinq médiateurs de l'immunité.

En conclusion, dans notre étude le bénéfice apporté par ces méthodes de détoxification par biotransformation pouvait être de partiel à total en fonction des paramètres étudiés. Cette étude a permis de confirmer l'action efficace de la carboxylestérase sur la FB dans les conditions du tractus gastro-intestinal, et ainsi, de réduire la biodisponibilité de cette mycotoxine. Néanmoins, la supplémentation des deux DA dans l'aliment semblerait induire quelques effets non spécifiques et justifierait ainsi quelques études complémentaires.

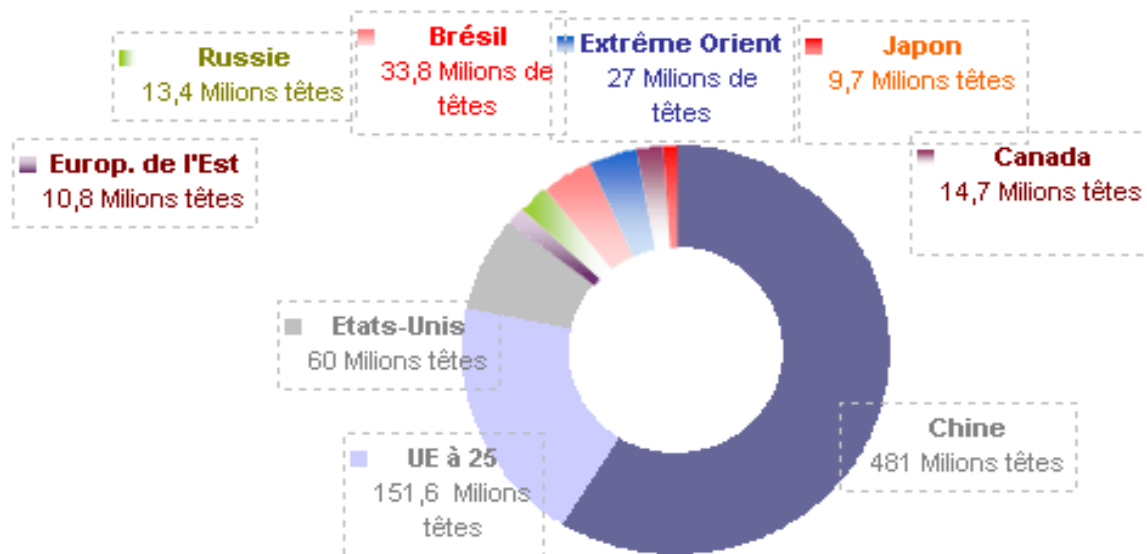
DISCUSSION GENERALE

Figure 23 :

(a) Cheptel porcin des principaux pays producteurs en 2005 (source : SNCP, Syndicat National du Commerce du Porc)

(b) Évolution des échanges mondiaux de viande de porc

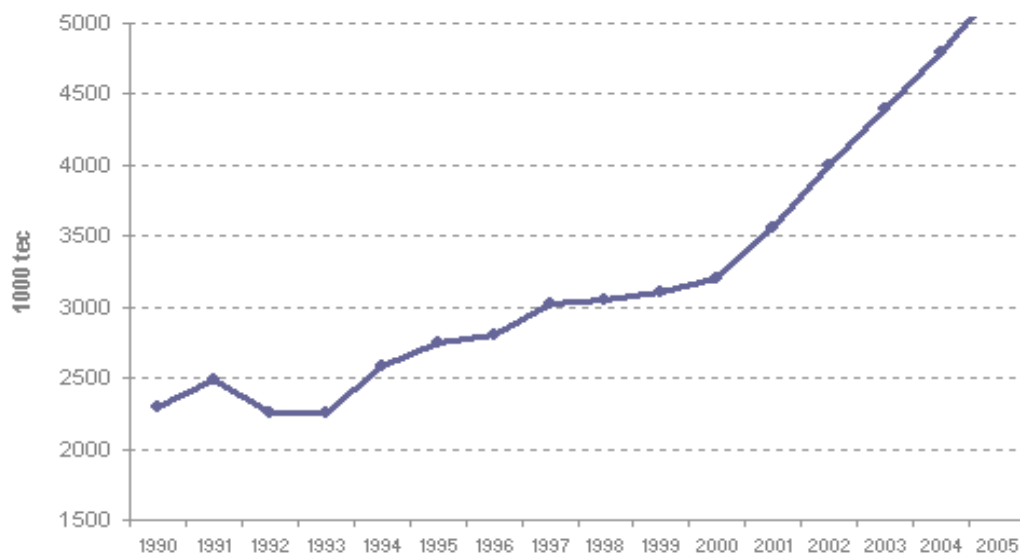
(a)



* Corée du Sud, Philippines, Taïwan

** Roumanie, Bulgarie, Croatie, Bosnie, RF de Yougoslavie

(b)



Dans ce travail de thèse, nous avons étudié l'effet des mycotoxines chez le porcelet, notre modèle animal lors des phases d'expérimentations, ainsi que des stratégies de lutte contre les mycotoxines. Nos résultats ont principalement été présentés sous la forme de publications scientifiques, et discutés par type d'étude. Ainsi dans cette discussion générale, nous nous attacherons à discuter du contexte et des critères expérimentaux, puis nous considérerons les résultats par systèmes de défense de l'organisme, d'abord le système immunitaire et ensuite le système digestif. Enfin, nous nous interrogerons sur de possibles apports des effets protecteurs des micro-organismes/enzymes de biotransformation.

1. Critères expérimentaux

LE MODELE ANIMAL

L'élevage de porcs et la production de viande porcine représentent un intérêt majeur dans l'économie mondiale, et ce secteur de l'agro-alimentaire ne cesse de croître depuis une décennie suite à l'intensification des échanges mondiaux (**Figure 23**). A l'échelle mondiale, la filière porcine est le chef de file des filières animales, avec 37% de la production mondiale de viande, devant les viandes de volailles et bovines (année 2008, *source FAO*). Ainsi, cette économie de marché suscite de nombreuses attentions quant à la qualité et au rendement de la production porcine, mais également en amont, à la qualité nutritionnelle de son alimentation. Du fait de sa composition riche en céréales (blé, orge, maïs, seigle), le porc est naturellement exposé à la contamination mycotoxique. Considérant qu'une grande partie de la production céréalière mondiale est destinée aux animaux d'élevage, dont le porc, la problématique des mycotoxines est au cœur du secteur de l'agro-alimentaire et entraîne des pertes économiques considérables (e.g. pertes totales associées aux mycotoxines estimées entre 630 millions et 2,5 milliards de dollars annuellement aux Etats-Unis (Wu, 2004)).

De plus, même si l'on peut affirmer que toutes les espèces peuvent être affectées par les mycotoxines, le porc est l'espèce la plus sensible à la plupart des mycotoxines. Cette observation est traduite par l'établissement de seuils en mycotoxines, fixés par la commission européenne, plus bas que pour les autres espèces cibles (**Tableau 23**). Cette sensibilité du porc aux effets des mycotoxines, est attribuée à l'absence de rumen qui joue un rôle important dans la détoxification des toxines. Les ruminants sont ainsi moins sensibles aux effets des mycotoxines, via l'action des micro-organismes du rumen. Cependant, bien que les ruminants soient tolérants, le stress lié à la haute productivité

Tableau 23 : Teneurs maximales recommandées en mg/kg (ppm) pour un aliment pour animaux ayant un taux d'humidité de 12% (basé sur la recommandation de la Commission Européenne du 17 août 2006 pour le déoxynivalénol, la zéaralénone, l'ochratoxine A et les fumonisines, **recommandation 2006/576/CE** ; basé sur la directive de la Commission Européenne du 31 octobre 2003 pour l'aflatoxine B1, **directive 2003/100/CE**)

ALIMENTS COMPLEMENTAIRES ET COMPLETS POUR :	Mycotoxines évaluées par l'UE				
	DON	ZEA	OTA	FB	AFB1
Porcs					
✓ Porcelets, Jeunes truies	0,9	0,1	0,05	5	
✓ Truies, Porcs d'engraissement	0,9	0,25	0,05	5	0,02
Volaille	5		0,1	20	0,02
Ruminants					
✓ Adultes (bovins, ovins, caprins)	5	0,5		50	0,02
✓ Jeunes (veaux, agneaux, chevreaux)	2	0,5		20	0,01
Bétail laitier	5	0,5			0,005
Equidés, Lapins	5			5	
Poissons	5			10	

Notes : DON, Déoxynivalénol ; ZEA, Zéaralénone ; OTA, Ochratoxine A ; FB, Fumonisines ; AFB1, Aflatoxine B1. Les champs vides représentent des mycotoxines non évaluées par l'UE chez certaines espèces, par manque de toxicité ou de données toxicologiques.

des vaches laitières et à la croissance rapide des bouillons ou des agneaux lourds diminue le potentiel de détoxification du rumen.

Cette sensibilité du porc aux mycotoxines varie également selon le stade de production et la présence de facteurs de stress dans le troupeau. Les porcelets sont généralement plus sensibles aux effets d'une contamination par les mycotoxines dans leur alimentation. Leur système immunitaire étant plus faible, ils sont moins aptes à combattre les effets néfastes causés par ces mycotoxines. S'ajoute également les animaux en stade de reproduction et ce, peu importe l'espèce. Il semblerait aussi que les mâles soient plus sensibles que les femelles à une intoxication par les mycotoxines (Cote *et al.*, 1985; Marin *et al.*, 2006).

Outre ces facteurs agronomiques qui justifient le choix de notre modèle animal, le porc présente des caractéristiques très proches de celles de l'homme pour différents systèmes biologiques. En effet, de nombreuses études attirent l'attention sur le choix du porc comme modèle expérimental de recherche, plutôt que le modèle rongeur par défaut. Il est acquis que l'utilisation des rongeurs en laboratoire repose plus sur une faisabilité économique et technique que sur un choix dicté par la science. Ainsi, du fait des similitudes en taille et en caractéristiques physiologiques des organes, l'espèce porcine *Sus scrofa* offre de nombreux avantages en tant que modèle animal dans l'étude des xénotransplantations, des maladies pulmonaires et cardiovasculaires, du système reproductif, des fonctions métaboliques, des désordres digestifs, ou encore des pathologies immunologiques (Miller and Ullrey, 1987; Rothkotter *et al.*, 2002; Guilloteau *et al.*, 2010; Verma *et al.*, 2011). Par ailleurs, cette espèce partage de fortes homologies de séquences avec celles de l'homme, permettant ainsi de développer et d'utiliser des outils de transcriptomique et de protéomique dérivés des banques de données humaines, dont les séquences sont très largement accessibles contrairement à celles du porc. Et enfin, notre équipe Immuno-Mycotoxicologie au sein du pôle ToxAlim de l'INRA a développé des approches *in vitro* (lignées de cellules épithéliales intestinales porcines, IPEC-1) et *ex vivo* (explants de jéjunum porcin (Kolf-Clauw *et al.*, 2009)) à partir de tissus porcins, et permettent ainsi de comparer et valider ces modèles avec le modèle *in vivo*.

L'EXPOSITION AUX MYCOTOXINES ET A LEURS DERIVES

1) Modes d'exposition choisies

Dans les phases d'expérimentations animales, le mode d'administration des mycotoxines a été différent d'une étude à l'autre. D'une part, nous avons formulé différents régimes alimentaires, artificiellement contaminés en mycotoxines, et les animaux étaient nourris avec ces régimes sans restriction dans la prise alimentaire. D'autre part, nous avons administré oralement, par gavage des animaux, des solutions de fumonisine B1 et de son dérivé hydrolysé, et ce en fonction du poids vif des porcelets. Les volumes à administrer étaient ainsi ajustés tous les deux jours pendant la période expérimentale.

Cette dichotomie dans le mode d'administration des mycotoxines a comme principale conséquence une différence dans l'exposition réelle aux toxines. En effet, dans le cas d'ingestion d'aliments contaminés *ad libitum*, nous pouvons aisément supposer que chaque animal dans un même régime n'a pas ingéré la même quantité de mycotoxines tout au long de la phase d'intoxication. Ce type d'exposition, dépendant de la quantité de nourriture consommée, pourrait être un des facteurs expliquant la variabilité de nos résultats. Toutefois, ces conditions reflètent exactement celles des élevages, et le peu d'effets négatifs observés dans certains cas sur les animaux peut être expliqué par un refus alimentaire, et donc une diminution de la consommation. Lors d'études d'association de mycotoxines, cette observation a déjà été reportée, et mise en cause dans le manque d'effets du régime combiné (effets moins qu'additif ou antagoniste). A l'opposé, le mode d'administration orale a permis d'exposer chaque animal d'un même groupe à la même concentration de substances tout au long de l'expérience. Ce protocole expérimental permet de réaliser des études courtes et de comparer précisément la toxicité de deux substances à doses égales.

2) Doses d'exposition choisies

De la même manière, dans nos approches expérimentales nous avons utilisé des concentrations de mycotoxines différentes. A noter que nous avons utilisé des aliments artificiellement contaminés (dans études chapitre 1 et 3) plutôt que des aliments formulés à base de lots de céréales naturellement contaminés. Considérant que le développement naturel de souches fongiques sur une céréale modifie ses qualités nutritionnelles, et qu'il est difficile d'éviter les multi-contaminations, ce choix nous a permis d'assurer une homogénéité dans la qualité des lots et d'attribuer les changements observés à la seule exposition alimentaire au DON et à la FB.

Tableau 24 : Analyse de denrées agricoles de janvier à décembre 2009 et détermination des niveaux de contaminations mondiales en mycotoxines (**enquête réalisée par la société BIOMIN**). Au total, 2727 échantillons ont été traités et évalués pour la présence de mycotoxines majeures. Les produits analysés étaient de nature diverses, comprenant des céréales (blé, maïs, orge, riz) et leurs co-produits (tourteaux de soja, gluten de maïs, DDGS), mais aussi des fourrages (paille, ensilage) et des produits finis.

ASIE	AFB1	ZEA	DON	FB	OTA
Nombre d'échantillons testés	988	936	829	1006	856
Positifs (%)	34	48	47	49	26
Moyenne des positifs (µg/kg)	109	255	826	1694	13
Maximum (µg/kg)	6105	7422	11836	32510	1582

EUROPE	AFB1	ZEA	DON	FB	OTA
Nombre d'échantillons testés	125	758	933	44	102
Positifs (%)	10	17	56	45	41
Moyenne des positifs (µg/kg)	3	94	725	2930	4
Maximum (µg/kg)	6	973	6000	11050	21

AMERIQUE DU NORD	AFB1	ZEA	DON	FB	OTA
Nombre d'échantillons testés	102	148	163	85	37
Positifs (%)	20	34	79	65	35
Moyenne des positifs (µg/kg)	48	538	1286	1744	4
Maximum (µg/kg)	831	8952	29300	11381	13

AMERIQUE DU SUD	AFB1	ZEA	DON	FB	OTA
Nombre d'échantillons testés	217	218	213	217	45
Positifs (%)	47	51	9	87	9
Moyenne des positifs (µg/kg)	9	185	372	4965	247
Maximum (µg/kg)	342	1740	1027	23100	355

MOYEN-ORIENT	AFB1	ZEA	DON	FB	OTA
Nombre d'échantillons testés	122	121	119	126	6
Positifs (%)	16	14	37	39	67
Moyenne des positifs (µg/kg)	66	94	402	716	22
Maximum (µg/kg)	388	392	1620	2948	31

AFRIQUE	AFB1	ZEA	DON	FB	OTA
Nombre d'échantillons testés	95	88	88	88	7
Positifs (%)	85	44	52	80	86
Moyenne des positifs (µg/kg)	91	60	569	1482	10
Maximum (µg/kg)	556	310	3859	10485	12

AFRIQUE DU SUD	AFB1	ZEA	DON	FB	OTA
Nombre d'échantillons testés	81	71	82	82	2
Positifs (%)	4	24	88	62	0
Moyenne des positifs (µg/kg)	3	93	1403	831	0
Maximum (µg/kg)	7	293	11022	4398	0

Dans l'étude des effets du DON et de la FB, seuls ou en combinaison, les doses ingérées par les animaux représentent des niveaux de contamination naturellement retrouvés dans les matières brutes utilisées dans les exploitations agricoles et d'élevages (**Tableau 24**), et sont proches des recommandations individuelles fixées par la commission européenne pour un aliment complet chez le porc (**Tableau 23**). Par ailleurs, notre objectif n'était pas d'induire des manifestations cliniques, comme très souvent reporté pour de fortes concentrations en mycotoxines, mais plutôt de déterminer l'effet d'une faible multi-contamination sur des paramètres plus ciblés ; paramètres dont les professionnels de la santé animale et de l'agro-alimentaire sont encore peu sensibilisés.

Parallèlement, si l'on se place dans le cadre d'expositions alimentaires chez l'homme telles qu'elles peuvent être rencontrées dans les pays en voie de développement ou dans des régions assurant leur autonomie via une production alimentaire locale, les doses que nous avons choisies présentent aussi un intérêt en termes de santé publique (Creppy, 2002). Il n'est pas rare dans ces populations que des épisodes d'intoxications aiguës en mycotoxines surviennent ou que des expositions chroniques à des doses faibles ou modérées engendrent des troubles digestifs (Bhat *et al.*, 1997) ou encore des effets cancérigènes (Li *et al.*, 2001).

Comme précédemment mentionné, l'étude sur la comparaison de la toxicité entre la FB1 et son dérivé hydrolysé nécessitait une analyse à concentrations égales, et aussi à concentrations provoquant une toxicité aiguë après ingestion de FB1. Pour cela nous avons décidé d'utiliser une dose de 2 mg FB1/kg de poids corporel, équivalente à une alimentation contaminée à approximativement 40 mg/kg d'aliment (en tenant compte de la consommation alimentaire d'un porcelet âgé de 4 à 6 semaines), et qui a été déjà reportée comme toxique chez le porc sur une courte période (Riley *et al.*, 1993).

3) Périodes d'exposition choisies

L'étude des effets du DON et de la FB, seuls ou en combinaison et supplémentés avec ou sans DA (Agents Désactivateurs), s'est réalisée sur une période couvrant 35 jours d'exposition aux différents régimes. Cette durée nous a permis d'examiner les effets des régimes suite à une exposition chronique à des doses subcliniques, et surtout de réaliser un protocole d'immunisation avec un antigène de laboratoire, l'ovalbumine. Les animaux au cours de ces 5 semaines d'expérimentation ont pu développer leur propre réponse vaccinale, telle que la réponse mise en place suite à un challenge antigénique. Dans notre équipe, plusieurs études ont en effet montré que les mycotoxines à des doses faibles ou modérées induisaient peu d'effets sur le système immunitaire non-activé, mais qu'une fois stimulé les mycotoxines ciblaient spécifiquement les processus intervenant dans la mise en place d'une réponse immunitaire spécifique (Taranu *et al.*, 2005; Pinton *et al.*, 2008; Meissonnier

et al., 2008b). Ce constat a été confirmé lors de ce travail de thèse, avec un effet amplifié lors de l'association des deux mycotoxines. Nous avons ainsi pu suivre l'évolution et l'altération de cette réponse vaccinale tout au long des 5 semaines, par le biais de prélèvements sanguins hebdomadaires. Cette durée d'exposition permet également de rendre compte de l'équilibre homéostatique sur une période assez longue, en déterminant si les animaux deviennent tolérants à l'ingestion chronique de ces contaminants alimentaires ou au contraire s'ils ne parviennent pas à réguler ou contrôler certaines variables et fonctions biologiques. En effet, certaines études, en particulier chez les rongeurs et pour le DON, ont montré des altérations importantes dans les premières heures d'une exposition aiguë (Amuzie *et al.*, 2008; Kinser *et al.*, 2004; Zhou *et al.*, 1997, 2003), mais nos résultats confirment également que certains critères restent affectés lors d'une exposition chronique (valeurs hématologiques et biochimiques, histopathologie, immunité, intégrité intestinale).

L'étude de FB1 et HFB1 a quant à elle été réalisée sur une période d'exposition plus courte, 14 jours. Ici, nous nous sommes placés dans des conditions d'exposition où la dose de FB1 utilisée et son administration quotidienne permettaient d'induire rapidement une toxicité aiguë et de maintenir cette toxicité tout au long des deux semaines d'expérience. Contrairement à l'étude précédente, nous n'avons pas immunisé les animaux, la durée de l'expérience ne s'y prêtait pas et s'orientait plus vers une étude de toxicologie générale.

LES SUPPLEMENTS ALIMENTAIRES : MICRO-ORGANISME/ENZYME A PROPRIETES DETOXIFIANTES

Deux types d'agents désactivateurs des mycotoxines ont été utilisés dans nos études. Le premier correspondait à une enzyme, plus particulièrement une carboxylestérase, qui a initialement été isolée à partir d'une bactérie de compost, du genre *Sphingopyxis*. Après séquençage du génome entier de cette bactérie et identification dans le cluster du gène codant pour cette enzyme et responsable de la dégradation de la FB1, le gène a été cloné puis exprimé en système hétérologue. Afin d'améliorer la solubilité de la protéine recombinante et d'éviter la formation de corps d'inclusion chez *E. coli*, l'enzyme a été exprimée chez *Pichia pastoris*, et récupérée dans les surnageants de culture (Heinl et *et al.*, 2010). La solution de fumonisine B1 hydrolysée (HFB1) a été obtenue après hydrolyse totale de la FB1 avec cette enzyme, et a été utilisée dans notre étude comparative de toxicité entre FB1 et HFB1. Dans l'étude avec la supplémentation en agents désactivateurs des régimes DON et FB, seuls ou en combinaison, l'agent ciblant les FB correspondait aux surnageants de culture lyophilisés, repris dans l'eau puis pulvérisés directement sur les lots d'aliments.

Le second agent désactivateur utilisé dans cette étude correspondait à une bactérie, du genre *Eubacterium*, isolée initialement du rumen de bovins (Fuchs *et al.*, 2002; Schatzmayr *et al.*, 2006). Cette bactérie synthétise une époxydase capable de biotransformer le groupe époxyde des trichothécènes en un diène. Ainsi, le DON peut être enzymatiquement réduit par l'époxydase d'*Eubacterium* en un métabolite non toxique, le de-époxy-déoxynivalénol (DOM-1). Incorporée dans un produit commercial développé par BIOMIN, cette souche bactérienne a été la première à être appliquée comme un additif alimentaire contre les effets négatifs des trichothécènes. Tout comme l'enzyme expérimentale décrite ci-dessus, les cultures bactériennes obtenues après fermentation ont été stabilisées par lyophilisation, et incluses telles quelles dans nos régimes alimentaires.

Le développement de ces produits biotechnologiques nécessite néanmoins un long travail en amont dans la recherche et l'identification de souches capables de dégrader des mycotoxines, et doit ensuite satisfaire plusieurs critères en aval. Les principales exigences pour une application commerciale sont :

- une réaction de dégradation rapide (généralement évaluée au préalable dans des essais *in vitro* et/ou *ex vivo*), spécifique de la mycotoxine et irréversible
- une dégradation des mycotoxines en métabolites moins ou non toxiques
- une absence de pathogénicité des micro-organismes utilisés

- une réaction de dégradation optimale dans les conditions du tractus gastro-intestinal, quelque soit le potentiel d'oxydo-réduction, la teneur en oxygène et la valeur du pH
- un métabolisme des micro-organismes qui s'active rapidement dans le système digestif pour synthétiser l'(s) enzyme(s) détoxifiante(s)
- ne pas altérer les qualités organoleptiques et la valeur nutritionnelle des aliments

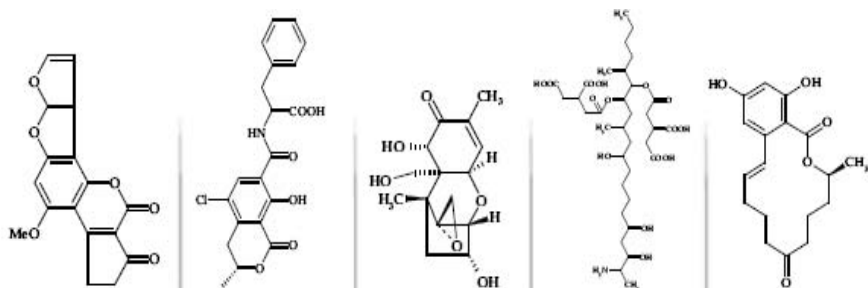
En plus de ces critères d'efficacité, le micro-organisme sélectionné doit être stabilisé avant application commerciale par différents processus tels que le séchage (e.g. à lit fluidisé), la lyophilisation, et également être incorporé dans des substances protectives (essentiellement des polymères organiques). Cela permet de garantir une stabilité suffisante du micro-organisme contre les conditions environnementales lors du stockage des aliments, et d'assurer son passage à travers l'acidité du tractus gastrique pour rejoindre indemne son site d'action, l'intestin. Le micro-organisme ne doit pas non plus être inhibé par la microflore intestinale, et dans le cas d'emploi de bactéries, la souche doit être résistante à certains antibiotiques qui pourraient être utilisés dans les élevages à des fins thérapeutiques. C'est pourquoi l'utilisation d'enzymes peut présenter un certain nombre d'avantages en termes d'exigences, par rapport à un micro-organisme entier.

Toutefois, ces approches de biotransformation affichent des atouts majeurs par rapport aux autres méthodes d'élimination des mycotoxines présentées dans l'étude bibliographique. Elles peuvent être facilement implémentées dans les procédés de fabrication d'aliments à un coût relativement bon marché, et sont de plus en plus reconnues et acceptées par les instances européennes (e.g. rapport de l'European Food Safety Authority (EFSA) sur la sécurité du produit BIOMIN *Eubacterium* BBSH797 en tant qu'additif alimentaire – N°EFSA-Q-2003-052 ; création récente d'un nouveau groupe d'additif sous la dénomination officielle de produits désactivateurs de mycotoxines). Les méthodes chimiques sont interdites dans l'UE, principalement du fait de la toxicité potentielle des métabolites produits et du manque de données sur l'innocuité des denrées traitées. Les adsorbants inorganiques ont soulevé certaines interrogations quant à leur potentiel d'adsorption non-spécifique, notamment vis-à-vis des nutriments, et également les possibles contaminations par des substances toxiques lors des extractions et purifications de ces adsorbants (e.g. cas de dioxine). La place occupée dans la ration alimentaire oblige également de réduire l'apport de certains nutriments aux dépens de ces additifs. Les adsorbants organiques, et plus particulièrement les parois de levures, présentent une solution alternative intéressante, mais comme les adsorbants inorganiques, l'efficacité d'adsorption a principalement été montrée vis-à-vis des aflatoxines, et la question de l'impact sur l'environnement lors de l'excrétion fécale du complexe mycotoxines/adsorbants se pose.

Figure 24 :

Effets toxicologiques provoqués chez le porc par l'ingestion des mycotoxines majeures (source *World Nutrition Forum, Salzburg 2010*)

AFB1, Aflatoxine B1
OTA, Ochratoxine A
DON, Déoxynivalénol
FB1, Fumonisine B1
ZEA, Zéaralénone



Symptômes

Atteinte des performances
(croissance, anorexie)

++

+

+++

+

Atteintes hépatiques

+++

+

++

Atteintes rénales

+++

+

Trouble de la reproduction
(avortement, infertilité)

+

+++

Oedème pulmonaire

+++

Atteintes du système
immunitaire

+++

+

++

+++

+

2. Les systèmes de défense de l'organisme

Les mycotoxines présentent un large spectre d'effets toxiques (**Figure 24**, exemple chez le porc). Les syndromes toxicologiques cliniques associés à l'ingestion de quantité moyenne à élevée sont bien caractérisés, ils s'étendent de mortalité aigüe, aux retards de croissance et aux troubles de reproduction. En revanche, l'ingestion chronique de faibles doses est beaucoup moins documentée, car n'induisant peu ou pas de manifestations cliniques, l'évaluation des effets nécessite l'examen de marqueurs plus spécifiques, notamment au niveau cellulaire. Pour la plupart des mycotoxines, le mécanisme de toxicité repose sur une inhibition de la synthèse protéique, que ce soit au niveau de la transcription ou de la traduction des séquences nucléiques. Ainsi, les cibles cellulaires de ces toxines peuvent être nombreuses et sont principalement des tissus et des cellules en prolifération et différenciation, présentant un taux de renouvellement protéique élevé, tels que l'on peut retrouver dans l'intestin grêle, le foie et le système immunitaire. En conséquence, certains processus cellulaires, tels que la réponse au stress oxydatif, le renouvellement et la perméabilité de l'épithélium digestif ou encore la réponse immunitaire face à des pathogènes peuvent être considérablement affectés lors d'une exposition répétée à des aliments faiblement contaminés.

L'IMMUNITÉ SYSTÉMIQUE ACQUISE ET SPÉCIFIQUE

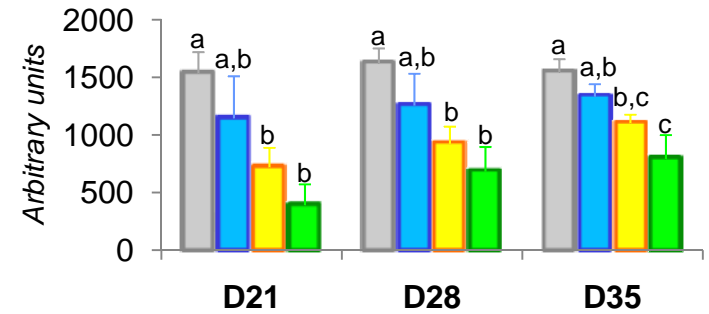
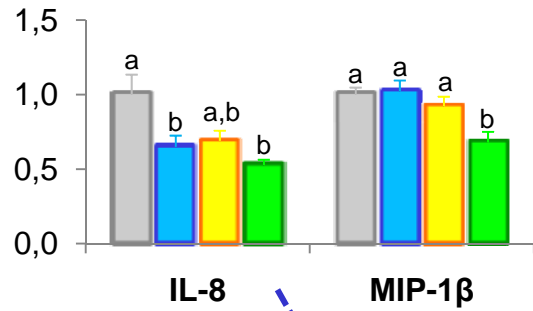
Au niveau du système immunitaire, les cibles cellulaires des mycotoxines sont les monocytes, les macrophages et les lymphocytes. En conséquence, l'immunosuppression induite par les mycotoxines peut avoir diverses conséquences en termes de santé animale :

- sensibilité aux maladies infectieuses : apparition spontanée d'infections à *Salmonella* ou *Campilobacter* chez des porcelets traités avec l'ochratoxine A (Stoev *et al.*, 2000) ; augmentation de la sévérité des lésions pulmonaires lors d'une infection à *Pasteurella multocida* chez des porcelets traités avec les fumonisines (Halloy *et al.*, 2005)
- réactivation des infections chroniques : réactivation de l'infection toxoplasmique dans le cerveau (augmentation du nombre de kystes) de rongeurs traités avec la toxine T-2 ou l'aflatoxine B1 (Venturini *et al.*, 1996)
- réduction de l'efficacité thérapeutique : baisse de l'efficacité d'un anti-coccidien chez des poulets traités avec la toxine T-2 et challengés avec *Eimeria tenella* (Varga and Vanyi, 1992)
- réduction de l'efficacité vaccinale : baisse du titre en anticorps spécifiques – de la maladie de Newcastle chez des poussins traités avec les aflatoxines (Gabal and Azzam, 1998) – de

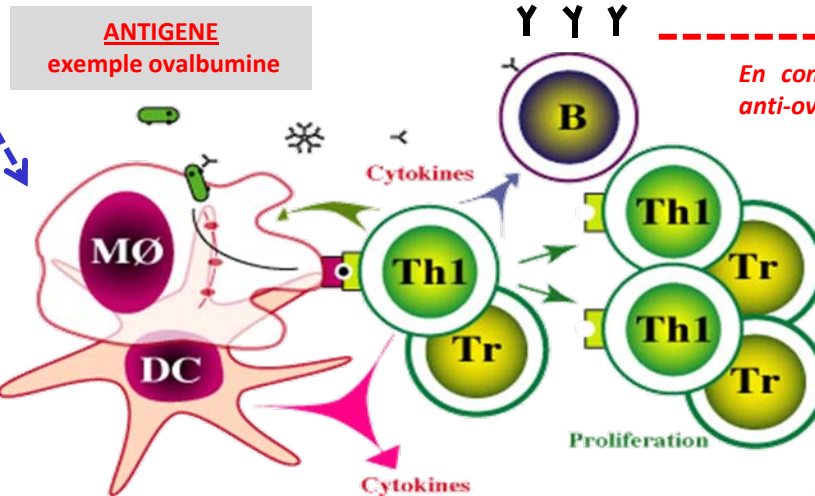
Figure 25 :

Hypothèses proposées dans l'altération de la mise en place de la réponse spécifique, d'après les résultats obtenus dans le chapitre 1 sur l'effet du DON et des FB, seuls ou en combinaison

■ control ■ DON ■ FB ■ DON+FB

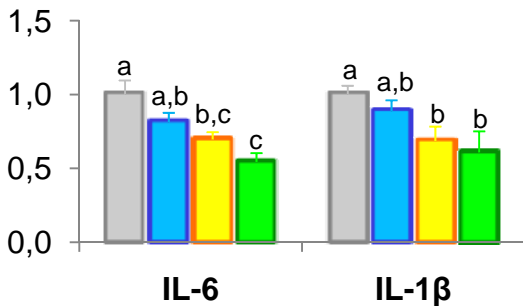


Cytokines impliquées dans le recrutement et la migration des cellules présentatrices d'antigène

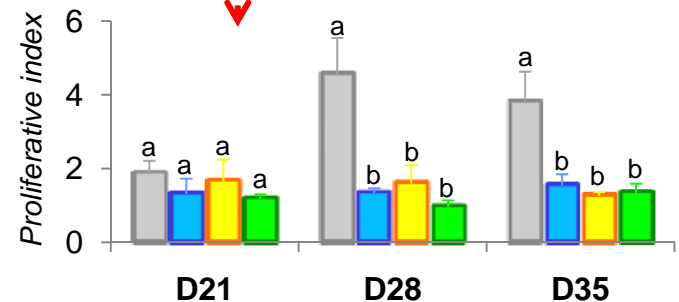


En conséquence, la production d'anticorps spécifiques anti-ovalbumine a été grandement diminuée

Incapacité des lymphocytes à proliférer après une stimulation avec l'antigène ovalbumine



Cytokines impliquées dans l'activation et la différenciation des lymphocytes B et T



Mycoplasma agalactiae chez des porcelets traités avec les fumonisines (Taranu *et al.*, 2005)
– de l'ovalbumine chez des porcelets traités avec la toxine T-2 (Meissonnier *et al.*, 2008a)

Cependant, certains aspects de l'immunosuppression provoquée par les mycotoxines restent très largement inexplorés. Par exemple, les mycotoxines sont très souvent produites simultanément, mais seulement très peu d'études reportent les effets de l'interaction de ces toxines sur le système immunitaire.

Une partie du travail de thèse a donc été de déterminer l'efficacité de la réponse vaccinale après exposition à une combinaison de deux toxines majeures, le DON et la FB. Nos résultats présentés dans le chapitre 1 suggèrent que les mycotoxines et spécialement la combinaison ont considérablement affecté le développement de la réponse vaccinale (**Figure 25**). L'absence d'effets sur la réponse totale non-spécifique, c'est-à-dire sur la population totale des lymphocytes du sang et sur les anticorps totaux issus de la population totale des plasmocytes, pourrait être attribuée aux faibles doses utilisées, et avant tout au fait qu'à ces doses, les mycotoxines agissent initialement sur les cellules en division, comme par exemple celles qui sont impliquées lors de l'activation du système immunitaire suite à un challenge antigénique.

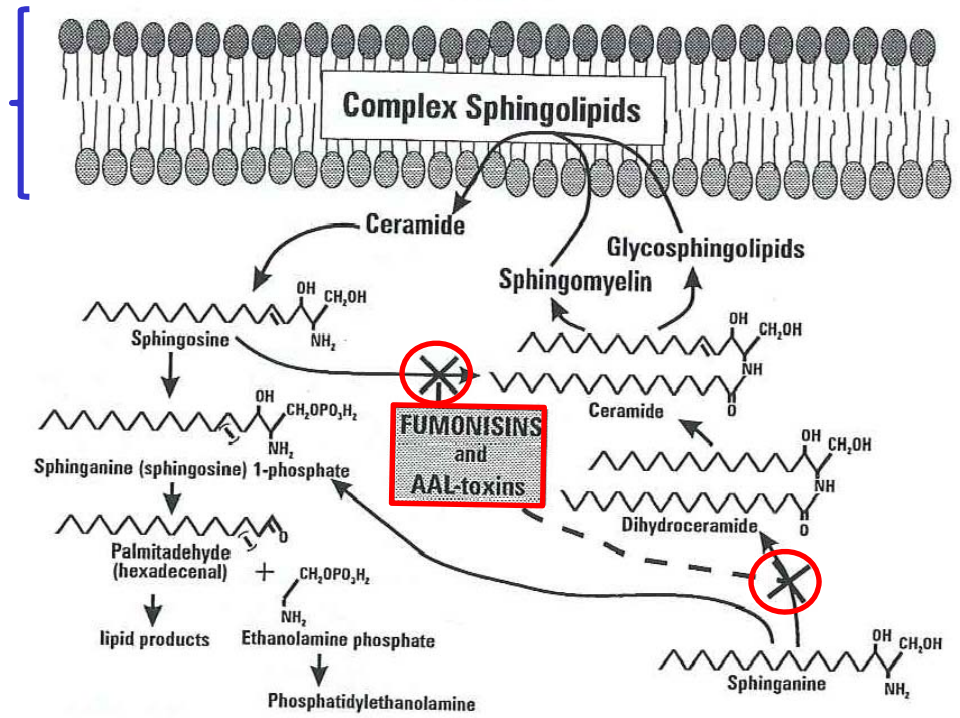
L'initiation de la réponse acquise fait intervenir les cellules dendritiques, mais aussi les macrophages ou les lymphocytes B, qui exercent des fonctions de sentinelles permanentes dans les tissus de l'organisme. Elles vont capturer l'antigène dans les tissus grâce aux récepteurs TLR (Toll-Like Receptor), puis devenir matures, on parlera alors de cellules présentatrices d'antigène (CPA). Cette maturation s'accompagne de modifications morphologiques et phénotypiques (expression de différents récepteurs et de molécules de co-stimulation). Les CPA vont ensuite migrer vers les organes lymphoïdes secondaires, tels que la rate, où elles vont rencontrer les lymphocytes T. Suite à la présentation de l'antigène aux lymphocytes T via les molécules du complexe majeur d'histocompatibilité (CMH) de classe I et II, et à l'activation par des molécules de co-stimulation et des médiateurs tels que les cytokines, les lymphocytes T spécifiques de l'antigène vont proliférer.

Dans notre étude, les différents résultats que nous avons obtenus suggèrent que la présentation de l'antigène ovalbumine et l'interaction avec les cellules T a été considérablement affecté par l'ingestion chronique des mycotoxines, en particulier quand le DON et la FB étaient associés, et à en conséquence entraîner une réponse vaccinale défectueuse. Nos données sur l'expression des cytokines spléniques montrent une diminution des transcrits codant pour IL-8 et MIP-1 β . Ces chémokines sont synthétisées lors de l'activation des macrophages avec l'antigène, et assurent le recrutement d'autres cellules immunitaires sur le site de reconnaissance de l'antigène grâce à des propriétés chimiotactiques. Il est ainsi possible de supposer que la mobilisation des CPA n'était pas

Figure 26 :

Mécanisme d'inhibition de la céramide synthèse par les fumonisines et implication dans le métabolisme des sphingolipides

**MEMBRANE CELLULAIRE
EUCARYOTIQUE**



Voie enzymatique (céramide synthèse) bloquée par les fumonisines

optimale. Les autres données sur l'expression des cytokines dans la rate ont également montré une diminution des transcrits codant pour IL-1 β , IL-6 et IL-12p40. Ces trois interleukines sont également produites par des cellules présentatrices d'antigène et permettent dans les organes lymphoïdes d'induire l'immunité à médiation cellulaire grâce à leurs propriétés d'activation et de stimulation de la différenciation des lymphocytes T naïfs (Trinchieri, 2003). De même ici, il est possible d'admettre que l'absence de prolifération des lymphocytes, que nous avons mis en culture et stimulés *in vitro* avec l'ovabumine, était liée à une reconnaissance de l'antigène et une activation des lymphocytes T défectueuses. Ces observations sont supportées par les données individuelles du DON et des FB dans la littérature. En effet, le DON a été montré *in vitro* comme capable d'inhiber l'expression des molécules du CMH de classe II et des molécules de co-stimulation, sur des macrophages (Wache *et al.*, 2009) et sur des cellules dendritiques (Hymery *et al.*, 2006) dérivés de monocytes humains. De la même manière, il a été démontré que le DON interférait avec la maturation phénotypique et la capture des antigènes de cellules dendritiques issues de monocytes porcins (Bimczok *et al.*, 2007), et affectait le potentiel de phagocytose de macrophages de souris et de dinde (Ayril *et al.*, 1992; Kidd *et al.*, 1995). En ce qui concerne les FB, l'exposition à la FB1 altérait la maturation de CPA intestinales *in vivo* chez le porc, ainsi que la capacité des cellules T à être stimulées (Devriendt *et al.*, 2009) ; et également diminuait l'activité de phagocytose de macrophages de poulet (Qureshi and Hagler, 1992) et la viabilité de macrophages murins (Dresden-Osborne and Noblet, 2002) *in vitro*. L'immunosuppression liée à l'exposition de la FB1 peut être expliquée par son mode d'action sur le métabolisme des sphingolipides, via l'inhibition de la céramide synthase. Comme plusieurs fois mentionné dans ce manuscrit, cette inhibition provoque l'accumulation des bases sphingoides, sphinganine (Sa) et sphingosine (So), mais aussi de leurs métabolites Sa-1-phosphate et So-1-phosphate (Riley *et al.*, 2001; Merrill *et al.*, 1997, 2001), et ces composés ont été déjà décrits comme immunomodulateurs. Par exemple, une augmentation de la sphinganine pourrait inhiber la protéine kinase C, impliquée dans l'activation des cellules T (Martinova and Merrill, 1995). Plusieurs études se sont focalisées sur le rôle de la sphingosine-1-phosphate (S1P) dans la réponse immunitaire, et ont montré que ce médiateur lipidique pouvait interférer dans la différenciation des monocytes en CPA matures et compétentes (Martino *et al.*, 2007), inhiber la sécrétion de cytokines dans des cellules dendritiques matures, et ainsi bloquer la différenciation des lymphocytes Th1 (Idzko *et al.*, 2002), ou encore inhiber la prolifération des cellules T (Jin *et al.*, 2003). En plus de ces considérations, les sphingolipides sont des constituants majeurs des membranes cellulaires, les fumonisines par leur action sur la céramide synthase bloquent la voie de synthèse des sphingolipides complexes tels que les glycosphingolipides ou les sphingomyélines (Soriano *et al.*, 2005) (**Figure 26**), et par conséquent

pourraient moduler l'expression des marqueurs et/ou des récepteurs à la surface des membranes des cellules immunitaires.

Nos résultats expérimentaux sur les immunoglobulines spécifiques nous laissent penser que la forte diminution observée dans la concentration plasmatique en IgG anti-ovalbumine serait ainsi une conséquence de ces effets adverses des mycotoxines sur l'initiation et la mise en place de la réponse immunitaire spécifique. En effet, le développement de la réponse humorale se produit lorsque les lymphocytes T activés par les CPA interagissent ensuite avec les lymphocytes B dans les organes lymphoïdes. L'activation des cellules B par les T permet ainsi la formation de plasmocytes qui sont capable de produire des anticorps dirigés contre l'antigène. Par ailleurs, l'IL-6 stimule les cellules B activées à se différencier en plasmocytes, et dans notre étude la diminution de l'expression de l'IL-6 dans la rate pourrait être corrélée à cette déficience en anticorps spécifiques, en particulier quand les deux mycotoxines étaient présentes simultanément.

Nous avons également analysé la concentration plasmatique en IgA anti-ovalbumine. Cette classe d'anticorps est principalement impliquée dans le système de défense des muqueuses, tel que dans le tractus intestinal, respiratoire ou uro-génital. L'élévation des IgA totales ou spécifiques ont été largement reportée dans la littérature après une exposition au DON (Drochner *et al.*, 2004; Pestka and Smolinski, 2005; Goyarts *et al.*, 2005; Pinton *et al.*, 2008). Cette immunoactivation pourrait laisser croire que le DON possède des propriétés bénéfiques en termes de stimulation de l'immunité et de protection contre certains pathogènes. Or, lors d'expositions répétées en DON chez un modèle murin, cette induction des IgA semble provoquer une néphropathie avec des atteintes similaires à celles observées dans le cas de néphropathies à IgA, une des glomérulonéphrites les plus fréquentes chez l'homme (Pestka, 2003). De plus, une sensibilité accrue envers des infections pulmonaires et entériques a pu être associée à cette stimulation en IgA (Li *et al.*, 2005, 2007).

Lors de l'association du DON avec la FB, aucun changement n'a été en revanche observé dans la teneur plasmatique des IgA. Nous avons donc supposé que la FB interférait dans le mécanisme induit par le DON et menant à cette accumulation d'IgA. Il a été montré que ce mécanisme était associé à une augmentation de l'expression de l'IL-6 (Yan *et al.*, 1997; Pestka and Zhou, 2000). Comme mentionné précédemment, l'IL-6 est une cytokine qui favorise la réponse humorale et ainsi la production d'anticorps. Etant donné que la production d'IgA est principalement observée dans les compartiments muqueux, nous avons admis que l'augmentation des transcrits de l'IL-6 dans l'intestin grêle des animaux exposés au régime mono-contaminé en DON (cf chapitre 1) pouvait être responsable des taux élevés d'IgA spécifiques. De plus, aucune modification de l'expression intestinale de l'IL-6 a été notée dans les régimes FB et DON+FB, corroborant ainsi les résultats d'IgA pour ces régimes. Récemment, il a été montré qu'une supplémentation alimentaire en

sphingomyéline augmentait la quantité d'IgA dans le gros intestin, suggérant que la sphingomyéline contrôlait la sécrétion d'IgA (Furuya *et al.*, 2008). Comme affirmé précédemment, les FB peuvent inhiber la synthèse des sphingolipides complexes dérivés des céramides, tels que la sphingomyéline, grâce à leur mécanisme d'inhibition au niveau de la céramide synthase. Cette inhibition a aussi été reportée sur l'intestin (Enongene *et al.*, 2000; Loiseau *et al.*, 2007) et donc nous pouvons émettre l'hypothèse que la déplétion en sphingomyéline pourrait être responsable de l'absence d'effets sur les IgA quand le DON est associé aux FB.

MÉCANISME CELLULAIRE DE MODULATION DE L'IMMUNITÉ

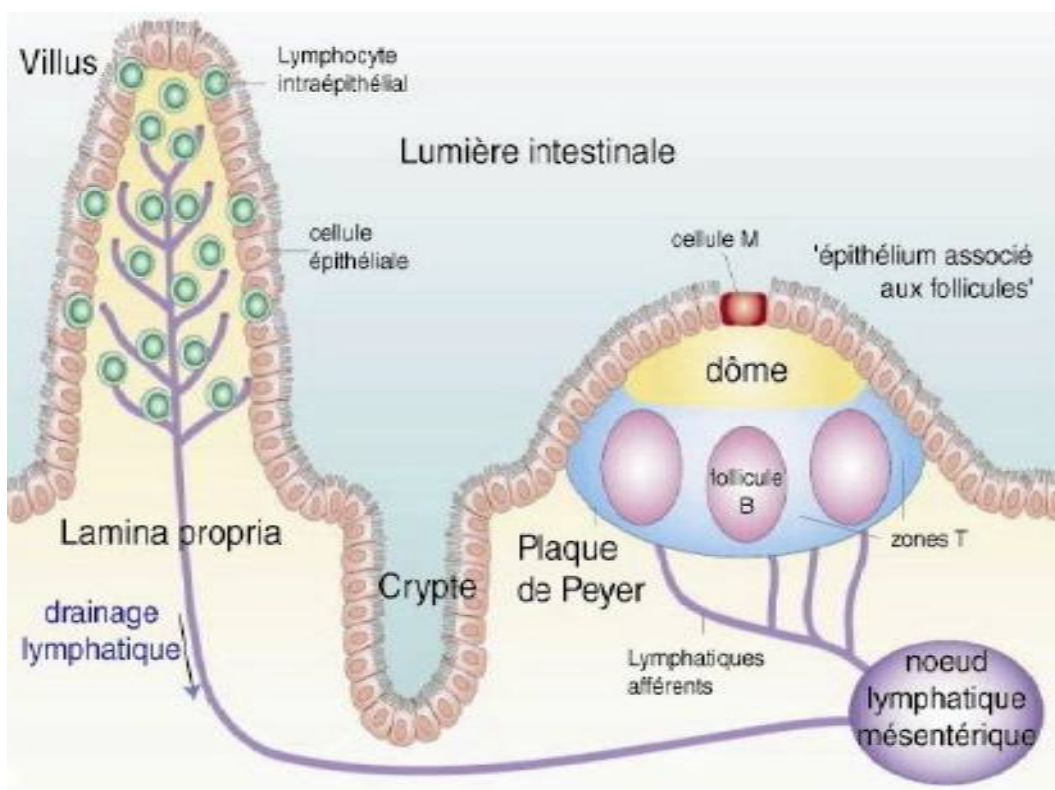
Les nombreux effets physiologiques observés après l'exposition aux mycotoxines ne sont pas dûs à une action directe de ces métabolites sur les cellules ou sur les tissus. En effet, ces altérations dans les fonctions cellulaires sont les conséquences du mode d'action des mycotoxines, qui bien que différent selon les toxines, ont en commun la capacité de rapidement perturber les voies de signalisation cellulaire, et ainsi d'affecter en aval l'expression de certains gènes. La voie des MAP kinases (MAPK, Mitogen-Activated Protein Kinase) constitue l'une des voies de signalisation les plus importantes dans la transduction des signaux au niveau cellulaire, et couvre un large éventail de fonctions (Cobb, 1999; Dong *et al.*, 2002). Ainsi, plusieurs études ont reporté que les mycotoxines activaient rapidement la voie des MAPKs, expliquant ainsi certains effets dans divers processus physiologiques, tels que la perméabilité intestinale, l'immunité, la carcinogénicité, ou encore l'apoptose (Pinton *et al.*, 2010; Jia *et al.*, 2004; Wattenberg *et al.*, 1996; Shifrin and Anderson, 1999; Chang *et al.*, 2009; Wu *et al.*, 2005). En ce qui concerne les effets additifs observés pour le DON et la FB sur la composante immunitaire, l'activation des MAPKs reportée individuellement pour les deux toxines pourrait être la cause de ces effets lorsque les mycotoxines sont associées. Que ce soit pour le DON ou la FB, suite à l'activation des MAPKs une augmentation de la production des prostaglandines et des leucotriènes, liée à la plus forte activité du métabolisme de leur précurseur l'acide arachidonique, a été démontrée (Moon and Pestka, 2002; Pinelli *et al.*, 1999). Ces médiateurs lipidiques sont connus pour être impliqués dans l'inflammation, l'immunomodulation, la régulation des facteurs de transcriptions ou l'activation des protéines kinases (Smith *et al.*, 1996). Dans le cas des FB, il a été montré que la FB1 activait les MAPKs via un mécanisme indépendant de l'accumulation des bases sphingoïdes Sa et So (Wattenberg *et al.*, 1996). Dans ce sens, il a été reporté que la phosphorylation des MAPKs se produisait 5 min après traitement avec la FB1, alors que 2 heures étaient nécessaires pour observer une augmentation de la concentration en Sa et So (Pinelli *et al.*, 1999).

Toutefois, les effets physiologiques observés après ingestion de mycotoxines ne peuvent pas se réduire à cette seule explication. L'activation des MAPKs est un phénomène complexe, rapide et transitoire, et qui implique de nombreuses étapes et médiateurs dans les multiples voies de signalisation (ERK, p38 et JNK). Par exemple, l'élévation des IgA par le DON a été attribuée à la surexpression de la cyclo-oxygénase-2 (suivie de la modulation de l'IL-6) après activation des MAPKs (Moon and Pestka, 2003). Or, la FB1 qui induit également l'expression de la cyclo-oxygénase-2 (Meli *et al.*, 2000) n'a pas provoquée d'augmentation des IgA dans notre étude que ce soit seule ou en combinaison avec le DON. Par conséquent, l'étude des effets des mycotoxines dans les voies de

signalisation cellulaire nécessite d'être prudent dans l'interprétation, et de savoir distinguer entre les effets spécifiques et non spécifiques en réponse aux stimuli.

Figure 27 :

Vue globale de la muqueuse intestinale, et des compartiments et des acteurs clés du système de défense de l'intestin



L'IMMUNITÉ INTESTINALE

En plus de sa fonction principale d'absorption des nutriments, le système digestif constitue le système immunitaire le plus important de l'organisme. Pourtant, sa fonction de défense immunitaire est souvent sous-estimée. Trois lignes de défense peuvent être distinguées :

- ✓ La flore intestinale : constituée d'une considérable diversité bactérienne, la flore intestinale représente la première barrière de protection contre les intrus indésirables
- ✓ La muqueuse intestinale : constitue la principale interface entre l'environnement extérieur et l'intérieur de l'organisme ; contrôle de façon très sélective le passage des substances de l'intestin à la circulation sanguine et lymphatique
- ✓ Le système immunitaire intestinal : abrite le GALT (Gut-Associated Lymphoid Tissue) qui contient à lui seul plus de 70% des cellules immunitaires de l'organisme ; développe sa propre réponse immunitaire de type innée ou adaptative (**Figure 27**)

Comme déjà mentionné dans ce mémoire, bien que l'exposition naturelle des mycotoxines se produise via l'ingestion d'aliments contaminés, l'effet de ces contaminants sur le tractus digestif est peu documenté. Toutefois, cet aspect depuis quelques années a semblé susciter l'attention de la communauté scientifique, au vu du nombre de recherches croissante dans le domaine. Il est donc important de savoir dans quelle mesure la consommation de mycotoxines peut affecter l'immunité intestinale, et par delà les conséquences en termes de santé humaine et animale. Par exemple, il a été montré dans notre laboratoire que l'ingestion de faibles doses de fumonisines augmentait la colonisation intestinale chez des porcelets infectés oralement par une souche pathogène opportuniste d'*E. coli* (Oswald *et al.*, 2003). Chez ces animaux, une translocation bactérienne accrue de la souche pathogène vers les organes extra-intestinaux a également été notée. Cette étude indique donc que le système immunitaire des porcelets traités avec les mycotoxines était incapable de contrôler l'infection, et était ainsi plus sensible aux pathogènes intestinaux.

Nos échantillons prélevés lors des phases animales sur l'intestin (jéjunum et iléon) ont principalement fait l'objet d'analyses histologiques et transcriptomiques. Nous avons essayé de déterminer le profil d'expression de gènes codant pour des cytokines tout au long de l'intestin grêle. Comme précédemment indiqué, les cytokines sont des médiateurs de l'immunité et sont impliquées dans différents processus, tels que l'inflammation, la réponse immunitaire innée, la réponse adaptative cellulaire et humorale. Au niveau de l'intestin, à l'instar des cellules immunitaires les cellules épithéliales intestinales sont également une importante source de cytokines (Stadnyk, 2002). Il était donc intéressant d'examiner la réponse locale, en particulier au niveau du jéjunum, et celle

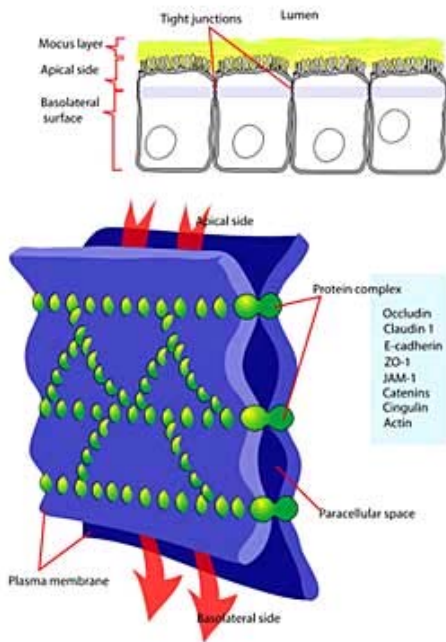
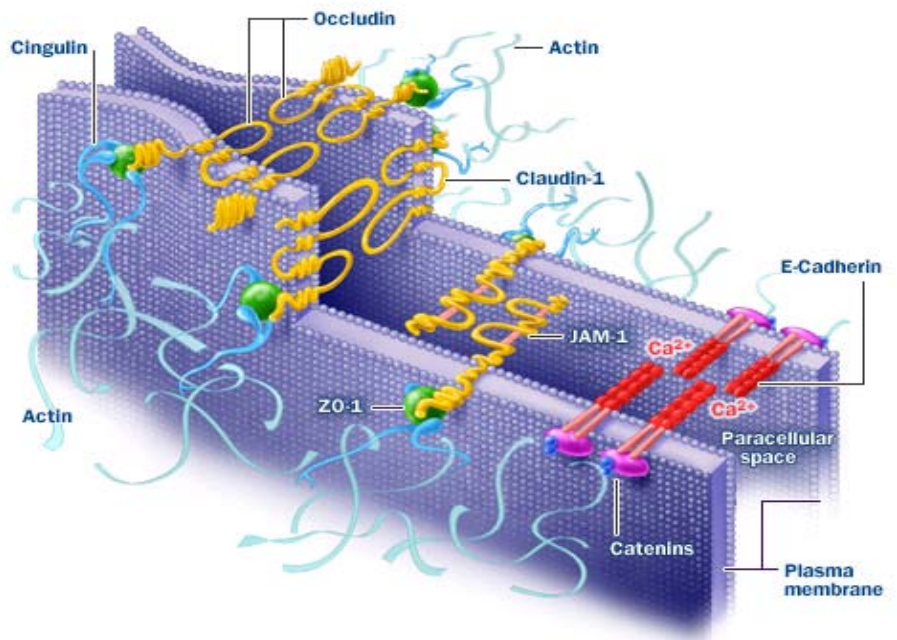


Figure 28 :

Réseau qui constitue le système de perméabilité paracellulaire de l'intestin, impliquant les protéines de jonction



plus spécifique impliquant le GALT (plaques de Peyer dans l'iléon et ganglions lymphatiques mésentériques), en réponse à l'ingestion d'aliments ou de solutions contenant des mycotoxines.

Dans le chapitre 1, le résultat de l'expression des gènes a révélé une augmentation (significative ou tendance) des transcrits de plusieurs cytokines (IL-1 β , TNF- α , IFN- γ , IL-6, IL-2, IL-10) chez les animaux traités avec les faibles doses de DON et FB, que ce soit dans le jéjunum ou l'iléon. Ces observations étaient plutôt intéressantes étant donné que pour la même étude nous avons observé une diminution de certains de ces transcrits dans la rate. Cette régulation positive des cytokines suggère chez ces porcelets une inflammation chronique de leurs intestins tout au long des cinq semaines d'exposition. L'augmentation des cytokines pro-inflammatoires a déjà été reportée dans le cas de maladies inflammatoires chroniques intestinales (IBD, Inflammatory Bowel Disease) chez l'humain, telles que la maladie de Crohn's ou la colite ulcéreuse (Papadakis and Targan, 2000). De plus, l'observation d'intestins de porc exposés à de faibles concentrations de toxines de *Fusarium* évoquait une inflammation de la muqueuse de la membrane intestinale, et ce dès 14 jours d'exposition (Obremski *et al.*, 2008). Ainsi, comme il a été récemment souligné, l'ingestion d'aliments contaminés en mycotoxines pourrait être considérée comme un risque potentiel dans la prédisposition aux inflammations chroniques de l'intestin (Maresca and Fantini, 2010).

A noter que l'induction de l'expression de l'IL-6 dans l'intestin grêle des animaux nourris avec le DON seul pourrait être corrélée avec l'élévation des IgA spécifiques que nous avons observés sur la partie systémique. Cette observation a été mentionnée dans la section précédente et corrobore également l'absence d'effets en présence des FB.

Outre le rôle des cytokines dans le maintien de l'inflammation chronique, ces médiateurs joueraient également un rôle dans le maintien de la perméabilité des barrières épithéliales et hémato-encéphaliques (Capaldo and Nusrat, 2009). En effet, il a été montré que l'inflammation des muqueuses lors des IBD était précédée d'une augmentation de la perméabilité paracellulaire intestinale. Ainsi, les manifestations répétées de diarrhées dans le cas des IBD pourraient être attribuées à cette perte de fonction de barrière. La fonction de barrière intestinale est maintenue par les jonctions serrées et adhérentes intercellulaires, formant un réseau complexe qui minimise l'espace entre les cellules adjacentes (**Figure 28**). Notre équipe a récemment reporté que le déoxynivalénol était capable d'altérer cette fonction de barrière, en agissant sur la protéine de jonction serrée, la claudine-4. Ces résultats ont été mis en évidence dans des modèles *in vitro*, sur les cellules IPEC-1 (Intestinal Porcine Epithelial Cell line) et Caco-2 (human colon carcinoma cell line), et *ex vivo* et *in vivo* sur des segments jéjunaux de porc (Pinton *et al.*, 2009, 2010). Dans ces études, l'altération de la perméabilité et l'identification de la claudine-4 ont été confirmées par mesure de la résistance électrique transépithéliale (TEER) et de la perméabilité paracellulaire (au passage de

dextran et d'*E. coli*), et par immunofluorescence et immunoblot avec des anticorps spécifiques de la claudine-4. De plus, l'équipe a dernièrement démontré que cette modification de la perméabilité par le DON était dépendante de l'activation des MAPKs, plus précisément la voie de signalisation ERK ½ (Pinton *et al.*, 2010).

L'expression et la localisation de ces protéines de jonctions, comprenant la famille des claudines, des cadherines et l'occludine, pourraient être régulées par certaines cytokines pro-inflammatoires, telles que l'IL-1β, le TNF-α et l'IFN-γ (Wisner *et al.*, 2008; Han *et al.*, 2003; Ye *et al.*, 2006; Al-Sadi and Ma, 2007). Lors de l'analyse des transcrits dans les tissus intestinaux prélevés sur nos animaux traités, une augmentation de l'expression de ces trois cytokines a été observée, principalement au niveau de la partie iléale. Par ailleurs, l'étude immunohistochimique sur les coupes d'intestin a révélé une détection plus faible de l'E-cadherine, en particulier au niveau de l'iléon. Ces différentes observations nous ont donc amené à réaliser des immunoblots sur l'iléon des animaux traités. Comme reporté dans le chapitre 1, l'expression des protéines E-cadherine mais aussi occludine était significativement réduite, notamment chez les animaux ayant consommé le régime co-contaminé en DON et FB. En revanche, nous avons également cherché à déterminer l'expression de la claudine-4 et malheureusement, nous n'avons pu confirmer les résultats antérieurs de l'équipe. Nous pouvons supposer que la nature des échantillons, jéjunum *versus* iléon, pourrait intervenir dans la dichotomie de ces résultats. De même, on ne peut exclure une internalisation de la structure de la claudine-4, attribuée à la contractilité du complexe actine/myosine induit par les cytokines (Capaldo and Nusrat, 2009), délocalisant ainsi la protéine de jonction de la surface apicale des entérocytes sans pour autant modifier son expression.

Cette probable perturbation de la perméabilité cellulaire pourrait avoir des conséquences importantes, en particulier après exposition à un aliment multi-contaminé, en autorisant le passage d'antigènes ou de substances indésirables de la lumière intestinale vers les compartiments systémiques ; ce phénomène ayant déjà été observé avec *E. coli* chez des porcelets traités avec les fumonisines (Oswald *et al.*, 2003).

Un aspect intéressant dans ces travaux de thèse est la réponse intestinale chez les porcelets exposés à une faible ou à une forte dose de fumonisine B1. Dans l'étude sur les co-contaminations, le régime individuel en fumonisines contenait 4,1 mg FB1/kg d'aliment. Dans l'étude comparative de la toxicité de FB1 et de HFB1, la dose administrée était de 2 mg FB1/kg poids vif/jour, soit à l'âge des porcelets une dose équivalente comprise entre 37 et 44 mg FB1/kg d'aliment. Cet écart d'un facteur 10 entre les deux doses utilisées a de manière surprenante mais non inattendue entraîné une différence dans le profil d'expression des cytokines dans l'intestin. Néanmoins, il est nécessaire de

souligner que les conditions d'intoxication dans les deux phases d'expérimentation animales étaient relativement différentes. En effet, dans l'étude de la co-contamination les porcelets étaient de sexe masculin, contrairement à l'étude FB1 *versus* HFB1 où ils étaient de sexe féminin. Le facteur sexe a déjà été reporté dans plusieurs espèces, avec une sensibilité aux mycotoxines plus forte chez les mâles que chez les femelles (Cote *et al.*, 1985; Marin *et al.*, 2006; Orsi *et al.*, 2007; Tamimi *et al.*, 1997; Domijan *et al.*, 2007). Cette différence de sexe entre les deux études n'était pourtant pas un choix délibéré, mais simplement une contrainte imposée par notre éleveur local lors de la mise en place de l'étude FB1/HFB1. Au niveau des autres différences d'expérimentation, la durée et le mode d'exposition était dans l'une de cinq semaines et par l'alimentation, et dans l'autre de deux semaines et par gavage.

A l'inverse de l'augmentation de l'expression des cytokines décrite dans la section précédente, une diminution de l'expression des mêmes cytokines a été notée chez les animaux traités avec la forte dose de FB1. Cette immunosuppression a été observée dans tous les segments du système digestif examinés. A noter que nous n'avons pas déterminé dans l'étude de la co-contamination la quantité de transcrits dans les ganglions mésentériques. Cette différence d'immunomodulation intestinale est intéressante et soulève ainsi des interrogations quant à la toxicité induite par des concentrations différentes en mycotoxines. Toutefois, dans les deux cas il semblerait que quelque soit les doses administrées, l'effet physiologique provoqué affectent de manière considérable les animaux. Même si la probabilité de rencontrer des aliments naturellement contaminés avec 40 mg FB1/kg est faible, ces résultats montrent qu'à cette dose les animaux sont probablement plus susceptibles aux infections du fait d'un système immunitaire défectueux. L'effet de cette dose sur l'expression des protéines de jonctions n'a pas été évalué mais ce point justifierait quelques recherches complémentaires si l'on considère que la diminution de l'expression de ces protéines est liée à l'augmentation de l'expression des cytokines. Néanmoins, il ne serait pas non plus surprenant que la perméabilité cellulaire soit affectée à cette concentration, en partant du principe que la dose est suffisamment élevée pour abolir plusieurs fonctions physiologiques.

3. Stratégies de lutte pour réduire les effets toxiques du déoxynivalénol et des fumonisines – approches de biotransformation

L'EUBACTERIUM SPECIFIQUE DES TRICHOHECENES

Depuis sa découverte en 1997, l'*Eubacterium* spécifique des trichothécènes (Binder *et al.*, 2000, 2001; Fuchs *et al.*, 2002) et nommée BBSH797 en l'honneur de l'équipe de recherche qui l'a isolée (BBSH, Binder Binder Schatzmayr Heidler), a été le premier additif détoxifiant des mycotoxines formulé à partir d'un micro-organisme vivant.

De nombreuses alternatives existent pour réduire l'exposition aux mycotoxines mais peu sont commercialement envisageables (*voir section ci-après la carboxylestérase*). En ce qui concerne les trichothécènes (TCT) et en particulier le déoxynivalénol (DON), les approches d'adsorption sont relativement inefficaces, et ainsi comme récemment suggéré les méthodes de détoxification par des micro-organismes semblent être les plus adaptées aux TCT (Awad *et al.*, 2010). L'*Eubacterium* BBSH797 a la capacité de dégrader le DON en un métabolite beaucoup moins toxique le de-époxy-DON (DOM-1) (Schatzmayr *et al.*, 2006; Sundstøl Eriksen *et al.*, 2004; Zhou *et al.*, 2008). Le groupe époxyde est considéré comme essentiel dans la toxicité des TCT. L'efficacité de la bactérie dans l'environnement intestinal a déjà été reportée dans un modèle *ex vivo* porcin traité en DON (Schatzmayr *et al.*, 2006). Dans nos travaux, l'incorporation de cette bactérie a permis de réduire significativement certains effets provoqués par l'ingestion du DON. Aucun marqueur d'exposition ne nous a permis de confirmer une dégradation efficace du DON dans les intestins des animaux. Néanmoins, nous pouvons affirmer que l'additif bactérien a réduit en partie la biodisponibilité du DON, au vu de la neutralisation partielle des effets provoqués par le DON (hauteur des villosités, IgA spécifiques, prolifération des lymphocytes). Il est à souligner également que la dose de DON utilisée semblait induire moins de toxicité que la dose choisie pour la FB, ne nous permettant pas ainsi de conclure objectivement sur l'efficacité du procédé.

Il serait intéressant de répéter cette expérience avec une multi-contamination en trichothécènes de type A et B à des doses suffisamment élevées pour entraîner des altérations cellulaires et tissulaires.

LA CARBOXYLESTERASE SPECIFIQUE DES FUMONISINES

L'un des principaux intérêts de la collaboration de l'industriel BIOMIN portait sur l'évaluation de l'efficacité et de la non-toxicité du processus de détoxification des fumonisines par la carboxylestérase, isolée de *Sphingopyxis*. Les résultats que nous avons présentés tout au long du mémoire de thèse sont l'aboutissement d'un long travail en amont, réalisé par notre partenaire, dans le screening et l'isolation de micro-organismes capables de dégrader les FB ; dans l'élucidation et l'identification du gène responsable de cette dégradation ; et dans l'expression et la stabilisation de ce gène.

La contamination naturelle en fumonisines est un problème majeur dans certaines régions de la planète, telles qu'en Amérique Centrale et du Sud, en Europe du Sud ou encore en Asie. Cette mycotoxine est importante en termes d'ubiquité, de toxicité et de difficulté à être éliminée des denrées. De plus, l'intérêt grandissant ces dernières années pour le bioéthanol en tant que source d'énergie renouvelable, a entraîné une inclusion croissante des co-produits de l'industrie de l'éthanol dans les rations alimentaires animales. Or, les mycotoxines pourraient être concentrées jusqu'à trois fois dans ces co-produits (DDGS, Dried Distillers' Grains and Solubles) en comparaison du grain initial (Wu and Munkvold, 2008). Ainsi, considérant que les fumonisines contaminent naturellement et principalement les grains de maïs, et que le bioéthanol est presque entièrement produit à partir des cultures de maïs, l'apparition accentuée et à des teneurs plus élevés de cette toxine dans l'alimentation animale pourrait très rapidement devenir un problème en termes de santé animale, d'agronomie et d'économie.

De nombreuses stratégies ont été évaluées et/ou développées à des fins commerciales dans le but de contrer les effets néfastes des fumonisines (cf études bibliographiques). Les méthodes physiques via un traitement direct sur les grains contaminés ont prouvé leurs efficacités. Néanmoins, l'application commerciale de ces approches reste très limitée. Parmi ces limites, la durée (lors du tri ou tamisage des grains), le coût (séchage des grains après séparation par flottaison) et les pertes importantes de matériel (tamisage) lors de ces traitements sont des inconvénients majeurs lors d'une utilisation industrielle. La dilution des lots contaminés avec ceux non contaminés est désormais une pratique interdite dans l'UE. Les fumonisines, mais aussi la plupart des mycotoxines sont également stables à des températures proches de celles utilisées dans la transformation conventionnelle des aliments. Les méthodes chimiques, bien que relativement efficaces, sont toutefois proscrites des traitements des aliments ; excepté l'ammoniation des denrées qui est tolérée dans certains pays (principalement sur les arachides). Cette interdiction se justifie principalement par le potentiel de toxicité des métabolites obtenus après traitements chimiques

(réversibilité de la réaction dans les conditions du tractus gastro-intestinal), par les potentielles interactions mycotoxines/matrices (mycotoxines « cachées », sous-estimation du contenu en toxines) et par l'impact sur le contenu énergétique et nutritionnel des aliments. Très peu de composés adsorbants (argiles, parois de levure) se sont montrés efficaces dans l'adsorption des fumonisines, ou alors se sont révélés relativement onéreux pour une application commerciale (e.g. polymères organiques).

Cette infructueuse prospection vis-à-vis des fumonisines a motivé le développement de méthodes alternatives et/ou complémentaires telles que la biotransformation de cette toxine. Dans nos études, nous avons pu tester et vérifier deux aspects importants dans la mise en place de ces approches. Premièrement, nous avons évalué le potentiel de toxicité de la fumonisine B1 totalement hydrolysée (HFB1) en comparaison de la molécule mère. L'une des recommandations de la FAO est que le processus n'entraîne pas la formation de métabolites toxiques. Par ailleurs, les données de la littérature sur la toxicité de HFB1 avaient suscité quelques controverses, notamment dans les années 90, d'où la nécessité d'avoir réalisé cette étude toxicologique comparative. Nos résultats ont clairement montré le très faible potentiel toxique de ce métabolite en comparaison de la FB1, que ce soit avant ou après absorption intestinale, c'est-à-dire l'impact sur le compartiment intestinal ou systémique. Ces observations convergent dans le sens des dernières recherches sur la toxicité de HFB1, ou plutôt de HFB1 pure, et réfutent également les résultats sur le matériel nixtamalisé affirmant la HFB1 responsable de la toxicité. Mais comme expliqué dans le chapitre 2, cette toxicité avait finalement été attribuée à la présence de résidus ou de FB1 « cachée » (fixée à la matrice et non détectée en HPLC) dans les préparations de matériel nixtamalisé, plutôt qu'à la présence de HFB1 (Park *et al.*, 2004; Burns *et al.*, 2008). Dans ce sens, le groupe de Voss, ayant dans un premier temps prétendu la dangerosité de HFB1 via la nixtamalization (Voss *et al.*, 1996, 1998), est revenu récemment sur ses positions lors de l'utilisation de HFB1 pure (Voss *et al.*, 2009). De plus, lors du *World Mycotoxin Forum 2010* organisé aux Pays-Bas, j'ai eu l'occasion de discuter brièvement de ces résultats avec Ronald Riley, scientifique du groupe de Kenneth Voss, me confirmant ainsi la validité de nos travaux. D'autres collaborateurs ont également démontré sur les cellules Caco-2, que du fait de la plus faible polarité de HFB1 par rapport à FB1, son passage à travers la barrière intestinale était facilité, augmentant ainsi sa biodisponibilité et suggérant un potentiel danger (Caloni *et al.*, 2002; De Angelis *et al.*, 2005). Notre collaboration avec BIOMIN nous a permis dans un projet parallèle, d'évaluer les résidus en FB1 et HFB1 dans des échantillons de fèces prélevés lors de la phase animale, et également dans différents échantillons biologiques (plasma, foie, reins, poumons). Ces analyses sont pour l'instant en cours mais nous permettront de déterminer le degré d'absorption intestinale

de HFB1. Toutefois, après ingestion de la solution en HFB1, très peu d'effets ont été observés après distribution du métabolite dans le compartiment systémique hépatique.

Le second aspect dans l'évaluation de cette approche de détoxification biologique, est bien évidemment l'efficacité de la carboxylestérase lors d'ingestion d'aliments contaminés en fumonisines, avec pour objectif d'hydrolyser les fumonisines dans le tractus digestif, et ainsi de limiter la biodisponibilité de ces mycotoxines. Nos travaux, présentés dans le chapitre 3, ont montré sur certains paramètres que la supplémentation en carboxylestérase pouvait significativement neutraliser les effets provoqués par les régimes contaminés. Des améliorations partielles (lésions hépatiques, prolifération des lymphocytes spécifiques, IgG spécifiques) et totales (hématologie et biochimie, lésions pulmonaires, expression des cytokines de la rate, population et prolifération cellulaire de l'intestin) ont été observées dans les régimes contenant les FB et supplémentés avec la carboxylestérase. Toutefois, le bénéfice s'est principalement ressenti par rapport aux effets induits par le régime co-contaminé en DON et en FB. En effet, de faibles doses étaient utilisées dans cette étude et les effets dus à la co-contamination étaient spécialement plus prononcés et significatifs que les effets individuels. Cependant, il est à préciser que le bénéfice dans le régime co-contaminé est partagé par la présence des deux agents désactivateurs, spécifiques du DON et de la FB.

Cette diminution de la toxicité après ajout de la carboxylestérase est attribuée à sa capacité d'avoir hydrolysé totalement les FB dans l'intestin des animaux. En effet, au vu des résultats tout au long de l'expérience du contenu en bases sphingoïdes, l'activité de l'enzyme ne semble pas avoir été altérée dans les conditions du tractus gastro-intestinal et aurait permis une hydrolyse totale des FB.

Cette approche semble donc prometteuse dans la stratégie de lutte contre les fumonisines. Par ailleurs, quelques micro-organismes ont déjà démontré des capacités à détoxifier les fumonisines, mais aucun à notre connaissance n'a pu être développé et proposé aux acteurs de la filière agricole. Cependant, le procédé mériterait quelques études complémentaires avant sa validation pour une utilisation commerciale. Par exemple, une étude plus approfondie sur l'exposition et la détection des résidus en FB et HFB dans différentes matrices biologiques après hydrolyse dans l'intestin pourrait apporter des informations complémentaires. De plus, la méthode de pulvérisation utilisée dans notre expérience n'est pas un procédé commercialement viable, et donc l'efficacité de l'enzyme devra être de nouveau prouvée avec une méthode alternative d'incorporation lors de la fabrication des aliments.

LA PRESENCE SIMULTANEE DE LA CARBOXYLESTERASE ET DE L'EUBACTERIUM

La combinaison des deux approches de détoxification a amélioré de manière significative la réponse globale des porcelets suite à la consommation concomitante des deux mycotoxines. Toutefois, dans notre expérimentation une variabilité non négligeable a été observée sur certains paramètres entre les deux groupes contrôles, supplémentés et non supplémentés. Les expérimentations *in vivo* et les gros animaux génèrent souvent une forte variabilité intra- et inter-individuelle mais il semblerait néanmoins que ces additifs ou les substances rentrant dans la composition de ces additifs aient certains effets non spécifiques. Le fait le plus marquant concerne la présence de mégalytoses dans le foie des animaux contrôles supplémentés. Pourtant, le produit lyophilisé contenant l'*Eubacterium* et incorporé dans nos aliments aurait la particularité de contenir des extraits d'algues et de plantes aux propriétés immuno- et hépato-protecteurs. La composition exacte de ce produit est confidentielle mais nos observations justifieraient quelques investigations complémentaires, l'utilisation de micro-organismes vivants pouvant provoquer des effets non souhaités.

CONCLUSIONS

Figure 29 :

Effets d'exposition à des mycotoxines chez l'homme et l'animal (adapté de Williams T., XIIth International IUPAC Symposium on Mycotoxins and Phycotoxins, 20-25 May 2007)

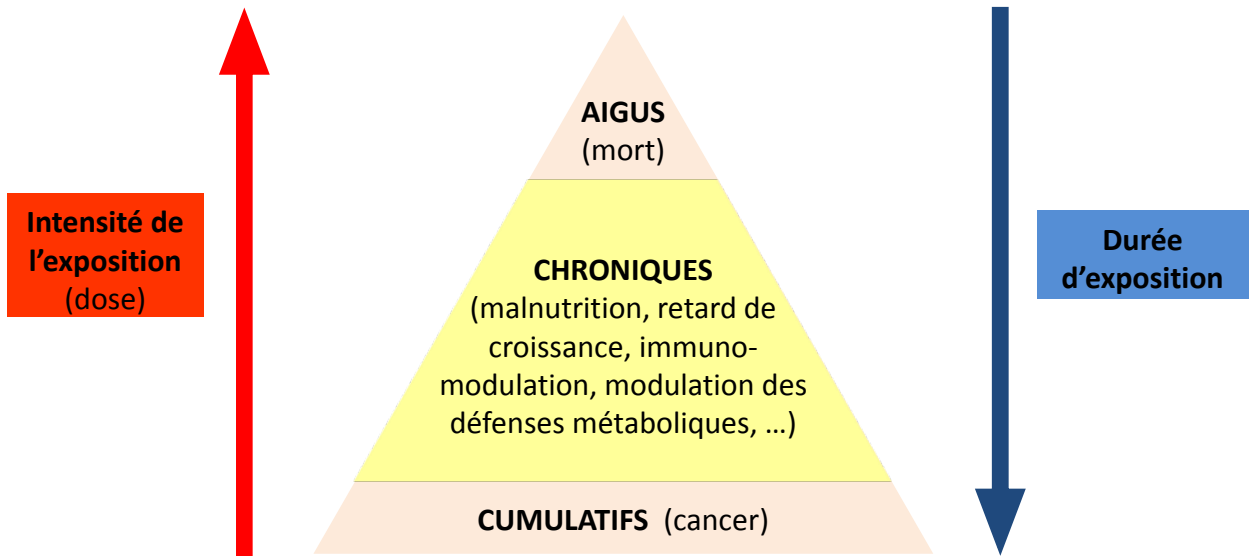
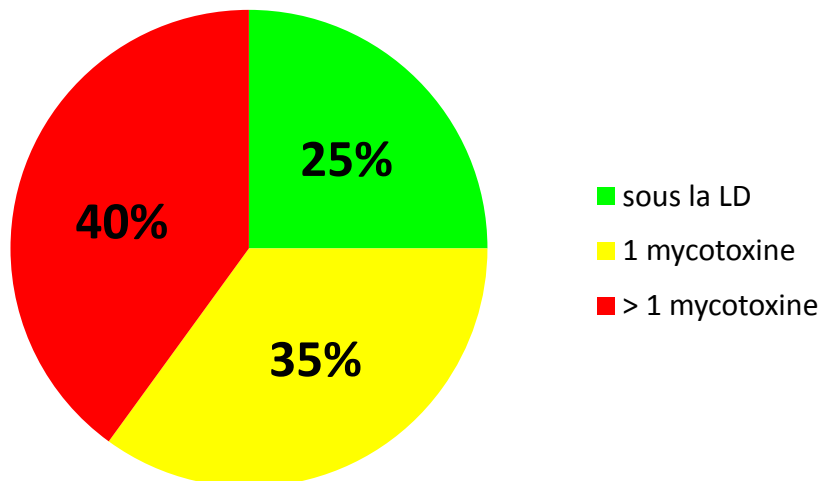


Figure 30 :

Graphique associé au **Tableau 24** sur l'analyse mondiale de denrées agricoles, comprenant 2727 produits de nature diverses. Représentation du nombre de mycotoxines détectées dans les échantillons, et par conséquent de la fréquence des multi-contaminations

Notes : LD, Limite de Détection



Les effets d'exposition à des mycotoxines peuvent être résumés en trois catégories (**Figure 29**) :

- De rares épisodes aigus, dus à des accidents de contaminations par de fortes doses de toxines pouvant entraîner la mort ;
- Des effets cumulatifs, lors d'expositions à long terme à des faibles doses et en association avec d'autres facteurs environnementaux ; des études épidémiologiques ont ainsi clairement identifié le rôle de l'AFB1 en association avec le virus de l'hépatite B dans le développement d'hépto-carcinomes ;
- Mais aussi une grande part d'effets chroniques, sans manifestations cliniques précises et qui demeurent aujourd'hui encore mal caractérisés quant à leurs conséquences en termes de santé humaine et animale.

Ce dernier point est de nos jours la situation la plus fréquente, où les mycotoxines contaminent naturellement diverses denrées agricoles à des seuils de contamination relativement bas. Les effets sont très mal connus du fait de l'absence de manifestations cliniques, et aussi du besoin d'utiliser des marqueurs plus fins et spécifiques pour caractériser ces effets. Ainsi, après stimulation du système immunitaire via un protocole de vaccination, nous avons montré que la réponse immunitaire mise en place, impliquant des populations et des médiateurs spécifiques, était significativement altérée avec de faibles concentrations en mycotoxines. Si nous n'avions pas activé le système immunitaire, nous n'aurions pas mis en évidence cette capacité des mycotoxines à altérer à faibles doses des composantes spécifiques de la réponse immunitaire. Cette sensibilité du système immunitaire aux mycotoxines dans ces conditions de stimulation antigénique, avait déjà été reportée dans des études antérieures faites par notre laboratoire. De la même manière, il a été démontré qu'à faibles doses les mycotoxines avaient la capacité de moduler l'activité d'autres cibles cellulaires associées à des fonctions vitales. Par exemple, des effets sur les fonctions intestinales en réponse à l'ingestion de faibles quantités de ces contaminants ont été reportés dans le travail de thèse et dans d'autres groupes de recherches. Bien que les données sur la réponse intestinale soient encore limitées, un impact réel des mycotoxines sur les fonctions d'absorption des nutriments, sur le profil de la flore commensale ou encore sur la prédisposition à des infections entériques a été démontré. Ce champ d'investigations suscite un intérêt grandissant dans nos sociétés, où il est de plus en plus considéré qu'un bon état de santé est lié à un bon fonctionnement de l'écosystème intestinal. Parmi les autres fonctions vitales que nous pouvons citer, les fonctions métaboliques du foie peuvent être affectées par les mycotoxines. Quelques études ont par exemple reporté des effets de faibles doses de mycotoxines sur le stress oxydatif et sur la modulation d'enzymes hépatiques impliquées dans la détoxification et le transport de xénobiotiques.

Ainsi, l'analyse classique des paramètres zootechniques ou biochimiques n'est pas suffisante dans l'analyse du risque mycotoxique. L'altération de ces paramètres est davantage la conséquence à une exposition aigüe, à des concentrations qui ne reflètent pas forcément les situations de terrain courantes, du moins dans nos pays en Europe.

La présence simultanée de plusieurs mycotoxines dans un aliment est également un autre point sensible dans l'évaluation du risque mycotoxique. Les multi-contaminations sont une réalité de terrain, et sont fréquentes comme le révèle très souvent les échantillonnages des denrées agricoles (**Figure 30**). Le risque d'être exposé à différentes mycotoxines en même temps est accentué par le fait qu'un produit fini est un mélange de plusieurs matières brutes. Ce potentiel de toxicité des produits multi-contaminés n'est pas pris en compte dans les réglementations/recommandations fixées par les instances nationales et européennes. Comme déjà mentionné, ces autorités ne tiennent compte que des études toxicologiques pour une toxine donnée, et fixent ainsi des seuils individuels. Le manque d'expériences *in vitro* et *in vivo* sur les effets des multi-contaminations peut expliquer ce constat en comparaison des expériences réalisées individuellement. Comme montré dans nos travaux et dans notre étude bibliographique, la caractérisation des interactions est en plus très souvent complexe. En effet, de nombreux facteurs (doses, espèces, sexe et âge, durée et mode d'administration) peuvent influencer les réponses, et même lors de l'étude d'une même association de mycotoxines les résultats peuvent différer. Toutefois, les effets totaux d'une co-contamination sont généralement nettement supérieurs aux effets totaux individuels, même lorsque l'interaction conduit à des effets moins qu'additifs ou antagonistes. Parmi ces études, il a également été démontré que la combinaison de mycotoxines à faibles concentrations pouvait affecter les animaux, alors que les concentrations individuelles étaient sous les concentrations reportées comme provoquant des effets négatifs.

Dans nos études, avec des doses reflétant des contaminations naturelles, les animaux ayant ingéré le DON et la FB simultanément étaient significativement plus affectés que ceux ayant consommé « seulement » les toxines individuelles. La réponse vaccinale chez ces animaux était approximativement 40-50% moins efficace que celle des animaux contrôles tout au long de l'expérience. Quelle est donc réellement l'impact des multi-contaminations dans les élevages, où la présence de pathogènes et la vaccination sont des situations fréquentes ?

Pour remédier aux effets toxicologiques induits par les mycotoxines, de nombreuses solutions ont été proposées et/ou développées tout au long de la chaîne de production alimentaire, depuis le champ avec des stratégies de prévention avant toute invasion fongique jusqu'aux élevages avec l'ajout d'additifs pour empêcher la biodisponibilité des toxines. Cette dernière catégorie d'approches

est utilisée lorsque la prévention de la contamination mycotoxique avant la récolte ou lors de la post-récolte et du stockage n'est pas possible, mais également en tant que stratégie préventive. Par notre collaboration avec l'industriel BIOMIN, nous avons donc évalué l'efficacité de produits classés dans cette catégorie d'additifs. L'activité de notre partenaire réside dans l'identification de microorganismes et/ou d'enzymes capables de détoxifier les mycotoxines. La diversité structurelle des mycotoxines et la problématique des multi-contaminations impliquent que les stratégies de lutte proposées soient efficaces et spécifiques contre chaque mycotoxine. Nos travaux sur l'évaluation des produits développés par BIOMIN ont montré une approche de détoxification prometteuse et qui permettrait de limiter considérablement la biodisponibilité, et ainsi les effets néfastes du DON et de la FB chez les animaux.

La nécessité de poursuivre dans cette mise en place de stratégies de prévention, de décontamination, de détoxification mais aussi de modèles de prédiction, est encouragée par le devenir de ces contaminations fongiques dans les prochaines décennies en conséquence du réchauffement climatique. L'impact du changement du climat (sécheresse, précipitations, catastrophes naturelles) suscite de nombreuses interrogations sur la future distribution et la prévalence des mycotoxines (Bryden, 2009, Paterson and Lima, 2009, Tirado et al., 2010).

Dans un contexte général, nos travaux s'inscrivent principalement dans le secteur de la production animale, où les dépenses liées à la prophylaxie, aux soins médicaux des animaux et également aux pertes attribuées à la baisse de productivité dans les élevages sont des points majeurs de la filière agricole. Mais la problématique des mycotoxines est aussi appréhendée de manière différente en fonction des zones géographiques. Dans les pays industrialisés, le problème est essentiellement d'ordre économique, affichant de lourdes pertes financières dans les différents secteurs de la filière agricole. A l'inverse, dû à une précarité et à un manque de moyens de prévention dans les pays en voie de développement, l'exposition aux mycotoxines reste encore un problème de santé publique dans ces pays. Toutefois, depuis l'intensification des échanges mondiaux, ces pays sont également confrontés au problème économique.

En conclusion, la contamination fongique, la production des mycotoxines et les conséquences liées à ces contaminants sont et resteront un problème d'actualité dans la filière agricole. Ces contaminations étant naturelles et largement tributaires du climat, elles sont difficilement contrôlables.

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