Influence of deoxynivalenol and T-2 toxin on the intestinal barrier and liver function in broiler chickens

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www.mytox.be

This thesis is lovingly dedicated to my father

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ABBREVIATION KEY

α-ZAL	α-zearalanol
α-ZEL	α-zearalenol
ABC	ATP-binding cassette
ACN	acetonitrile
AcNIV	mono-acetylnivalenol
a-DON	mono-acetyldeoxynivalenol
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the plasma concentration-time curve
β-ZAL	β-zearalanol
β-ZEL	β-zearalenol
BEA	beauvericin
BEMEFA	Belgian Association of Feed Manufacturers
BLAST	basic local alignment search tool
BW	body weight
Caco-2	human colorectal adenocarcinoma cell
CAST	Council for Agricultural Science and Technology
cDNA	copy-deoxyribonucleic acid
Cl	clearance
CLDN	claudin
C_{max}	maximum plasma concentration
Ct	threshold cycle
CYP	cytochrome P450
d	day
Da	dalton
DAcDON	di-acetyldeoxynivalenol
DAcNIV	di-acetylnivalenol
DAS	diacetoxyscirpenol
DNA	deoxyribonucleic acid
DOM-1	deepoxy-deoxynivalenol
DON	deoxynivalenol
dsDNA	double stranded DNA
EC	Ethical Committee
EDTA	ethylenediaminetetra-acetic acid
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay

ERK	extracellular signal-regulated kinases
F	absolute oral bioavailability
FA	fusaric acid
FB	fumonisin B
FUC	fusarochromanone
FUP	fusaproliferin
FUS	fusarenone-X
g	g-force
GAP	Good Agricultural Practice
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GCY	glucose, yeast extract and peptone
GI(T)	gastro-intestinal (tract)
GOIs	genes of interest
h	hour
H6PD	hexose-6-phosphate dehydrogenase
HcK	hematopoietic cell kinase
HCT	human cell-line derived from colon-carcinoma microtissues
HIF-1α	hypoxia-inducible factor 1 subunit alpha
HKG	housekeeping gene
HMOX	heme-oxygenase
HPLC	high-performance liquid chromatography
HPRT	hypoxanthine-guanine phosphoribosyl transferase
HT-2	HT-2 toxin
IPEC	intestinal porcine epithelial cell
IS	internal standard
iv	intravenous(ly)
JAMs	junctional adhesion molecules
JNK	c-Jun N-terminal kinases
k_{el}	elimination rate constant
kg	kilogram
LC	liquid chromatography
LD_{50}	lethal dose for 50% of subjects
LOD	limit of detection
LOQ	limit of quantification
µg	microgram
µl	microliter
µm	micrometer
MAPK	mitogen-activated protein kinase
MAS	monoacetoxyscirpenol

MDR1	multiple drug resistance 1
MDZ	midazolam
mg	milligram
min	minute
ml	milliliter
MON	moniliformin
mRNA	messenger RNA
MRP2	multidrug resistance-associated protein 2
MS/MS	tandem mass spectrometry
MUCL	Mycothèque de l'Université Catholique de Louvain
n	number
NADPH	nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
NEO	neosolaniol
ng	nanogram
NIV	nivalenol
1-OH-midazolam	1-hydroxy-midazolam
4-OH-midazolam	4-hydroxy-midazolam
OTA	ochratoxin A
p.a.	post administration
PBS	phosphate buffered saline
PCD	programmed cell death
PCR	polymerase chain reaction
P-gp	P-glycoprotein
PKR	RNA-activated protein kinase
po	per os, oral(ly)
PXR	pregnane X receptor
(q)RT-PCR	(quantitative) Real-Time Polymerase Chain Reaction
Raf-1	RAF proto-oncogene serine/threonine-protein kinase
Rhoa	Ras homolog gene family, member A
RNA	ribonucleic acid
ROS	reactive oxygen species
RPL7	60s ribosomal protein L7
s	second
SD	standard deviation
SRM	selected reaction monitoring
T-2	T-2 toxin
$t_{1/2el}$	elimination half-life
TEER	trans-epithelial electrical resistance

TLR	toll-like receptor
T_{max}	time to maximum plasma concentration
UPLC	ultra performance liquid chromatography
Vd	volume of distribution
vol	volume
v/v	volume/volume
wk	week
XOR	xanthine oxidoreductase
ZAN	zearalanone
ZEN	zearalenone
ZO	zonula occludens
ZOH	α and β isomers of ZEN
ZONAB	zonula occludens-associated nucleic acid binding protein

GENERAL INTRODUCTION

1. The poultry industry with emphasis on broiler chickens

The poultry industry is one of the largest and fastest growing agro-based industries in the world. Table 1 summarizes the chicken meat production worldwide.

Table 1. World chicken meat production (million tones) (adapted from Evans et al., 1012).

Region	2000	2005	2006	2007	2008	2009	2010	2011	2012
Africa	2.8	3.4	3.4	3.7	4.0	4.2	4.4	4.6	4.7
Americas	27.2	32.7	33.7	35.3	37.4	36.7	38.4	39.2	39.4
Asia	18.7	22.5	23.5	24.9	26.4	27.2	28.6	29.9	31.0
Europe	9.4	10.7	10.8	11.7	12.1	13.4	13.8	14.2	14.5
Oceania	0.7	0.9	1.0	1.0	1.0	1.0	1.1	1.3	1.3
WORLD	**58.7**	**70.2**	**72.3**	**76.7**	**80.8**	**82.5**	**86.2**	**89.2**	**90.9**

The production of chicken meat is expected to grow due to export to 'emerging markets', namely China, India, Russia and the Middle-East.

Good management of a chicken husbandry is indispensable. The main goal is to convert feed into viable, disease-free poults as efficiently as possible. Over the last decades intensive selection programs were set up for broilers. Thanks to this selection, broiler chickens are actually the most effective meat-producing animals next to fish (Decuypere et al., 2010). Stable equipment has to be appropriate for *ad libitum* feed and drinking water availability. In addition, accurate light schedules have to be applied to allow maximum feeding but also taking the animal welfare into account. Owing to the high-density housing, ventilation and litter management are important matters to reduce stress and possible lesions (Elson, 2010). Besides management and biosecurity, vaccination is necessary to control infectious diseases in the modern poultry industry. Although vaccines never assure complete protection, they are essential to protect the birds against pathogens that can reduce their performances

(Cserep, 2009). Moreover, it is generally accepted that prevention is more cost effective than treatment. In practice, vaccination in poultry is applied to uniform populations and not to individuals. Besides viral infections, parasite and bacterial infections are important concerns in the poultry industry. Intervention strategies are chosen based on their economic and biological efficiency. Spray medication or drinking water medication are the most applied methods for mass treatment (Collett, 2009).

Progress in vaccination schedules and antimicrobial chemotherapy has made respiratory problems less important than gut health issues. According to own assessments and from veterinary diagnostic labs it can be concluded that actually gut health problems are the most important health problem in broilers.

A lot of pathogens responsible for gut lesions have already been described and methods of disease control have evolved with the intensification of the poultry industry. Initially, the focus was on clinical diseases of serious nature, but now the focus is on the non-defined subclinical diseases and birds' welfare. Hot topic is the influence of mycotoxins on the performance of broilers, with special attention to hepatic, intestinal and reproduction problems.

2. Mycotoxins as contaminants of animal feed

2.1 Classification and occurrence of mycotoxins

Fungi are able to contaminate a multitude of agricultural products such as cereal grains, maize, nuts and fruits. A large number of metabolites can be formed by these fungi including mycotoxins. Mycotoxins are called secondary metabolites because they are not necessary for the survival of the fungi (Keller et al., 2005). Toxins can, however, provide an ecological advantage for the fungus in certain environments. Due to mycotoxin production, plants can for example become more susceptible which makes the invasion of other fungi easier.

Mycotoxins are produced by filamentous fungi and are characterized by a low molecular weight. Thousands of mycotoxins exist, but only a few present significant food and feed

safety challenges. Contamination of feed with mycotoxins leads to heavy economic losses as a result of decreased animal production throughout the world (Zain, 2011).

Mycotoxins of importance for the poultry industry are mainly produced by fungi of the genera *Aspergillus*, *Fusarium* or *Penicillium*. From an economic point of view, *Fusarium* mycotoxins are the most significant (Devegowda and Murthy, 2005). The most commonly found *Fusarium* mycotoxins are the trichothecenes, zearalenone, fumonisin and moniliformin. The mycotoxins produced by *Fusarium* species from cereals are illustrated in Table 2. Many mycotoxin-producing *Fusarium* species are common causal organisms of *Fusarium* head blight, foot rot and root rot disease of cereals.

Table 2. Mycotoxigenic *Fusarium* species associated with cereals and their mycotoxins (adapted from Logrieco et al., 2002).

***Fusarium* species**	**Mycotoxins**
F. acuminatum	**T-2, MON**, HT-2, DAS, MAS, NEO, BEA
F. anthophilum	BEA
F. avenaceum	**MON, BEA**
F. cerealis	**NIV, FUS, ZEN**, ZOH
F. chlamydosporum	**MON**
F. culmorum	**DON, ZEN, NIV**, FUS, ZOH, a-DON
F. equiseti	**ZEN, ZOH**, MAS, DAS, NIV, DAcNIV, FUS, FUC, BEA
F. graminearum	**DON, ZEN, NIV, FUS, a-DON**, DAcDON, DAcNIV
F. heterosporum	**ZEN**, ZOH
F. nygamai	BEA, FB_1, FB_2
F. oxysporum	**MON**, BEA
F. poae	**DAS, NIV, FUS**, MAS, T-2, HT-2, NEO, BEA
F. proliferatum	**FB_1, BEA, MON, FUP**, FB_2
F. sambucinum	**DAS**, T-2, NEO, ZEN, MAS, BEA
F. semitectum	**ZEN**, BEA
F. sporotrichioides	**T-2, HT-2, NEO**, MAS, DAS
F. subglutinans	**BEA, MON**, FUP
F. tricinctum	**MON**, BEA
F. verticillioides	**FB_1**, FB_2, FB_3

Bold letters indicate the important mycotoxins. Abbreviations: a-DON (mono-acetyldeoxynivalenols: 3-aDON and 15-aDON); AcNIV (mono-acetylnivalenol); BEA (beauvericin); DAcDON (di-acetyldeoxynivalenol: 3,15-aDON); DAcNIV (di-acetylnivalenol: 4,15-AcNIV); DAS (diacetoxyscirpenol); DON (deoxynivalenol); FB_1 (fumonisin B_1); FB_2 (fumonisin B_2); FB_3 (fumonisin B_3); FUP (fusaproliferin); FUS (fusarenone-X); FUC (fusarochromanone); HT-2 (HT-2 toxin); MAS (monoacetoxyscirpenol); MON (moniliformin); NEO (neosolaniol); NIV (nivalenol); T-2 (T-2 toxin); ZEN (zearalenone); ZOH (α and β isomers of ZEN)

Different factors influence the occurrence of mycotoxins. The temperature, the relative humidity and the CO_2 concentration in the atmosphere are considered as the main pre-harvest factors (Ingram 1999; Miraglia et al., 2009). However, good agricultural practice (GAP) is essential to reduce contamination during harvest and storage (Magan and Aldred, 2007). Figure 1 illustrates the different factors affecting the mycotoxins' occurrence in the food and feed chain.

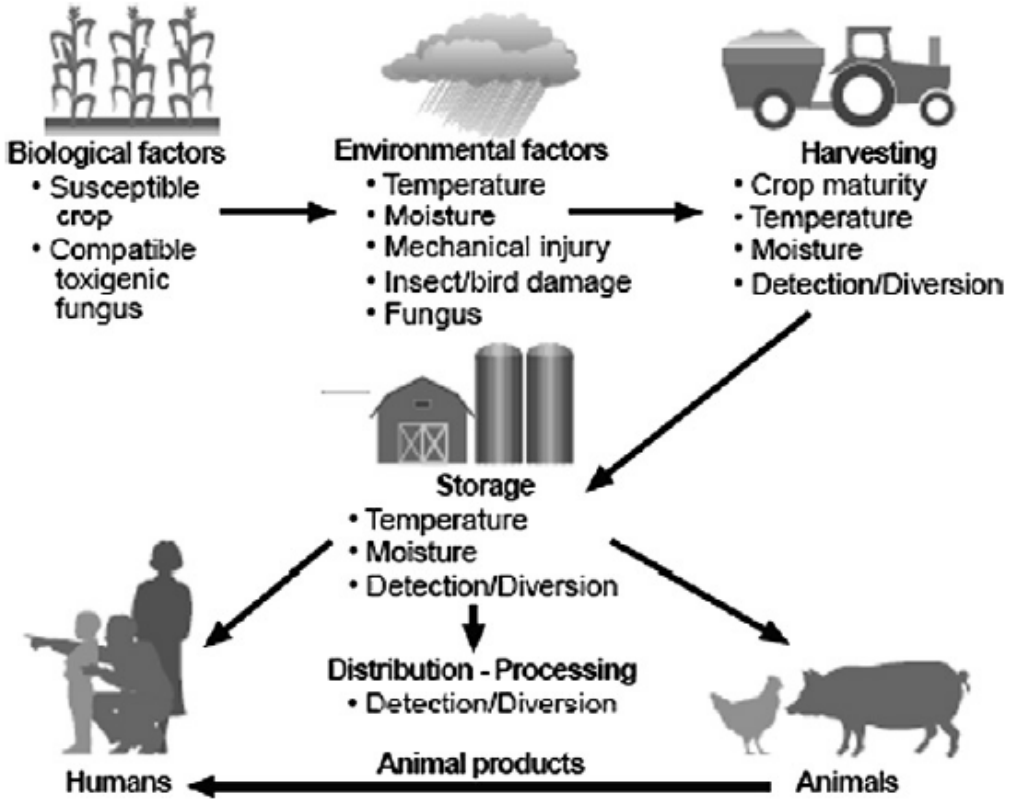


Figure 1. Factors affecting mycotoxin occurrence in the food and feed chain (adapted from CAST, 2003).

Binder (2007) reported the results of a two-year survey undertaken to evaluate the presence of mycotoxins in feed and feed raw materials. In total 2,753 assays were performed on 1,507 samples from European and Mediterranean markets. The following mycotoxins were analyzed: aflatoxin B1, ochratoxin A, deoxynivalenol, T-2 toxin, zearalenone and fumonisins. Results revealed that 52% of the samples were contaminated with deoxynivalenol, T-2 and zearalenone, as major contaminants. Therefore, in this thesis we mainly focused on these

three mycotoxins. Several other studies also reported the occurrence and also the co-occurrence of mycotoxins in feed in Europe (Streit et al., 2012).

However, it is difficult to infer trends in the mycotoxin contamination of feedstuffs. One of the reasons is the fact that the occurrence patterns are expected to change as a consequence of rising average temperatures (Miraglia et al., 2009). For example, the prevalence of *Fusarium graminearum* is likely to increase in Northern Europe due to the expected changes in weather conditions for the upcoming years (Parikka et al., 2012). In addition, analysis methods used are different and sampling methods are rarely described. Nevertheless, sampling is considered as an important source of error in mycotoxin analysis due to the frequent inhomogeneous distribution of moulds and/or toxins (Whitaker, 2003).

An overview of the field contamination with DON, T-2 or ZEN for 2012 is given in Table 3. For this study the majority of the samples were taken in Belgium (144 samples). Other samples were collected in Germany (15 samples), France (20 samples), the Netherlands (6 samples) and Luxembourg (2 samples).

Table 3. Mycotoxin monitoring in Belgium, Germany, France, the Netherlands and Luxembourg for 2012 (adapted from Belgian Association of Feed Manufacturers (BEMEFA), 2012).

DON

DON level (µg/kg)	< 400	400-700	700-1000	1000-1250	1250-1750	1750-2500	>2500
number of samples	154	30	19	6	9	3	2

T-2

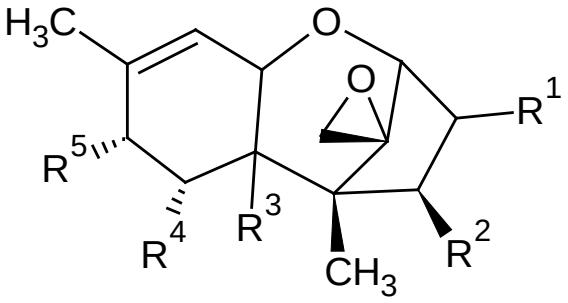
T-2 level (µg/kg)	<100	100-200	>200
number of samples	121	1	0

ZEN

ZEN level (µg/kg)	<75	75-100	100-200	>200
number of samples	155	4	2	5

2.2 Toxicity and metabolism of *Fusarium* mycotoxins

Trichothecenes contain sesquiterpene rings characterized by a 12,13-epoxy-trichothec-9-ene nucleus, responsible for their toxicity (Hussein and Brasel, 2001). This group of toxins is characterized by a molecular weight of approximately 200-500 Da (Pestka, 2007). The *Fusarium* trichothecenes can be divided in four groups: A, B, C and D. Type A does not contain a carbonyl group on C-8 (represented by R^5 in Figure 2). Group B is characterized by a carbonyl group on C-8. Members of group C have another epoxy-group between the C-7 and C-8 or C-8 and C-9 positions, respectively. A macrocyclic ring between C-4 and C-15 is typical for group D (Wu et al., 2010). Group A and B represent the most important members.



	R^1	R^2	R^3	R^4	R^5
DON	OH	H	OH	OH	O
3-aDON	OAc	H	OH	OH	O
15-aDON	OH	H	OAc	OH	O
T-2	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
HT-2	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$

Figure 2. Chemical structures of DON, T-2 and their major metabolites.

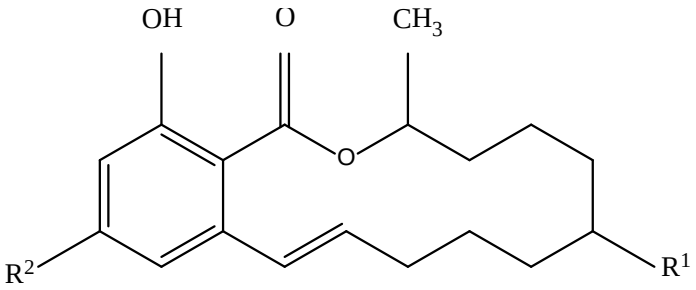
Most toxic type A trichothecene is T-2 toxin (T-2). The mycotoxin T-2 is rapidly metabolized to a variety of metabolites and eliminated in the excreta within 48h (Dohnal et al., 2008; Yoshizawa et al., 1980a). Different studies and animal models have shown that the major metabolic pathway is hydrolysis which occurs at the C-4 position, resulting in HT-2.

Structures of both T-2 and HT-2 are illustrated in Figure 2. HT-2 can be hydroxylated to 3'-OH-HT-2 in the liver or can be hydrolyzed to T-2 tetraol via T-2 triol. It is also possible that T-2 is hydroxylated immediately to 3'-OH-T-2. De-epoxidation and glucuronidation of the different compounds can also be a metabolic pathway (Weidner et al., 2012; Wu et al., 2010; Yoshizawa et al., 1980b). Especially in poultry, several uncharacterized metabolites of T-2 were found and they are still not identified (Young et al., 2007). Some metabolites are equally or even more toxic than T-2 (Dohnal et al., 2008). Increased toxicity of T-2 has even been observed owing to enterohepatic circulation (Coddington et al., 1989; Sokolovic et al., 2008). Retention of a large part of T-2 in the bile has been observed in broiler chickens, indicating the important role of the biliary excretion system in the elimination of this mycotoxin (Chi et al., 1978).

The B group includes deoxynivalenol (DON) and its acetylated derivates: 3-aDON and 15-aDON (Logrieco et al., 2002; Placinta et al., 1999). Figure 2 illustrates the structures of these B trichothecenes. DON is one of the least acutely toxic trichothecenes, but of particular interest owing to its high prevalence (Rotter et al., 1996). The majority of the ingested DON in broilers is quickly, nearly completely, absorbed in the stomach and proximal part of the intestines. De-epoxidation of DON to deepoxy-deoxynivalenol (DOM-1) occurs in the proximal part of the small intestines (Awad et al., 2011; He et al., 1992; King et al., 1984). 3-aDON can be metabolized to DON and DOM-1. For 15-aDON, following metabolites can be found: deepoxy-15-aDON, DON and DOM-1 (Young et al., 2007). Both 3-aDON and 15-aDON were reported equivalently or less toxic than DON for a longtime (Pestka, 2007). However, a recent study reported that 15-aDON is more toxic compared to DON and 3-aDON both *in vitro* and *in vivo* (Pinton et al., 2012).

Besides the group of trichothecenes, the mycotoxin zearalenone (ZEN) also is an important mycotoxin, more precisely as activator of the oestrogen receptor. ZEN is also known as 6-(10-hydroxy-6-oxo-*trans*-1-undecenyl)-β-resocylic acid μ-lactone (Bennett and Klich, 2003) (Figure 3). An *in vitro* study demonstrated that ZEN can be metabolized to α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL), with β-ZEL being the most prominent (Malekinejad et al., 2006). The affinity of these metabolites for the oestrogen receptor compared to ZEN can be ranged as follows: α-ZEL > ZEN > β-ZEL. A recent *in vivo* study showed that besides the above

mentioned metabolites, ZEN can also be transformed to α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) in broiler chickens (Yunus et al., 2012a). Only low rates of glucuronidation could be demonstrated in chickens compared to other animals (Malekinejad et al., 2006), suggesting that glucuronidation of ZEN is not the main metabolic pathway in chickens.



	R^1	R^2
ZEN	O	OH
α-ZEL	OH (α)	OH
β-ZEL	OH (β)	OH

Figure 3. Chemical structures of zearalenone (ZEN), α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL).

The toxicokinetic profiles of DON, T-2 and ZEN have already been investigated *in vivo* in pigs and ruminants. For poultry, only old data of toxicokinetic studies performed with radiolabeled toxins are available.

2.3 Implication for the health of broilers

Intake of rather high doses of mycotoxins may lead to mycotoxicosis, which can be characterized by several clinical signs (Binder, 2007). The type and concentration of the mycotoxin, the duration of exposure and the species, age, gender, immunological status of the animal are determinants for the symptoms. Synergistic effects between several mycotoxins can cause different diseases (Conkova et al., 2003).

The main target of *Fusarium* mycotoxins are rapidly proliferating and differentiating cells and tissues, with a high protein turnover, including the small intestines, liver and immune cells. The immunotoxicity of type A trichothecenes, especially T-2, is lower than the type B trichothecenes (Sharma, 1993). DON can both suppress and stimulate immunity, even at

equal doses (Rotter et al., 1996). T-2 and DON induce immunosuppression at high doses, but immunostimulation at low doses (Sokolovic et al., 2008). Adverse effects due to the presence of trichothecenes include decreased T- and B-lymphocyte counts, reduced antibody production and suppression of the lymphocyte proliferation (Girish and Smith, 2008). The exact mode of action of these mycotoxins on the immune system is beyond the aim of the research presented in this thesis.

Our focus was on the parameters leading to economic losses in the broiler industry, namely the zootechnical parameters feed intake and body weight. We also investigated the effects of DON and T-2 on the hepatic and intestinal function in broiler chickens. The possible effects of ZEN on these organs were not studied as ZEN is known to cause reproduction problems, which is of principal interest for laying hens and parent flocks.

2.3.1 DON mycotoxicosis

All animal species have been shown to be susceptible to DON, also called vomitoxin. However, the degree of susceptibility varies among the different species, ranged from the most sensitive to the most resistant: pigs>mice>rats> poultry ≈ ruminants (Prelusky et al., 1994). Differences in absorption, distribution, metabolism and excretion of DON among animal species might account for this differential sensitivity.

Acute mycotoxicosis due to consumption of DON is rarely seen in poultry. For DON in broiler chickens the LD_{50} is set at 140 mg DON/kg BW (Huff et al., 1981). However, chronic exposure to DON at low or moderate levels can cause anorexia, diarrhea, decreased live weight gain and altered nutritional efficiency (Pestka and Smolinski, 2005). For broiler chickens, different studies already reported the negative effects of DON on zootechnical parameters, such as feed intake and body weight (gain). Results of these studies are summarized in Table 4.

Table 4. Summary of the effects of DON on feed intake and body weight in broiler chickens.

Dose in feed	Exposure time	Decreased feed intake	Decreased body weight (BW)	Reference
16 mg/kg DON	21 days	reduced feed efficiency	no	(Kubena et al., 1989b)
3.4 mg/kg DON	35 days	no	no	(Bergsjo and Kaldhusdal, 1994)
15 mg/kg DON	21 days	no	no	(Kubena et al., 1997)
8.2 mg/kg DON, 20.3 mg/kg fusaric acid, 0.2 mg/kg ZEN	56 days	yes (finisher phase)	yes (finisher phase)	(Swamy et al., 2002)
21.2 mg/kg DON, 1.4 mg/kg NIV, 500 µg/kg 15-aDON, 300 µg/kg 3-aDON, <50 µg/kg HT-2, <20 µg/kg T-2, 406 µg/kg ZEN, < 0.2 µg/kg OTA, 219 µg/kg ergot alkaloids	35 days	yes	yes	(Dänicke et al., 2003)
5.9 mg/kg DON, 19.1 mg/kg fusaric acid, 0.4 mg/kg ZEN, 0.3 mg/kg 15-aDON --- 9.5 mg/kg DON, 21.4 mg/kg FA, 0.7 mg/kg ZEN, 0.5 mg/kg 15-aDON	56 days	no (starter period) yes, linearly (grower phase) recovery in the finisher phase	BW gain followed the same trend as the feed intake	(Swamy et al., 2004b)
10 mg/kg DON	42 days	no	no	(Awad et al., 2004)
12.6 mg/kg DON, traces of ZEN, traces of 15-aDON	84 days (broiler breeders)	no	no	(Yegani et al., 2006)
5 mg/kg DON	21 days	no	no	(Awad et al., 2006b)
10 mg/kg DON	42 days	no	no	(Awad et al., 2006a)
1.36-1.52 mg/kg DON, traces of 3-aDON, traces of ZEN and other *Fusarium* mycotoxins	35 days	yes, during the first three weeks	yes	(Dänicke et al., 2007)
18 mg/kg DON	21 days	no	yes, cubically	(Xu et al., 2011)
1 or 5 mg/kg DON	35 days	no	no	(Awad et al., 2011)
1.68 mg/kg DON, 0.145 mg/kg --- 12.209 mg/kg DON, 1.049 mg/kg ZEN	35 days	Yes	yes, during the first three weeks	(Yunus et al., 2012b)

Dotted line (----) separates two different experimental groups within one study.

Results seem to be variable, making it impossible to establish a simple dose-response relationship between growth depression and dietary concentrations of DON. In addition, artificially versus naturally contaminated feed seems to give different effects. In general, naturally contaminated feed led to more pronounced effects (Rotter et al., 1996). The presence of other toxins or other compounds in the feed can also be a possible explanation (Smith et al., 1997).

The way DON causes feed refusal has been investigated and was committed to a neuropharmacological effect. DON can act directly on the central nervous system, more specific on the serotoninergic system. Via this pathway DON was demonstrated to cause anorexia and emesis in pigs (Prelusky and Trenholm, 1993; Swamy et al., 2004a). In addition, an effect of DON on the peripheral serotonin receptors has been described in rodents (Fioramonti et al., 1993). Especially in broilers, higher cerebral levels of norepinephrine were found after exposure to DON, antagonizing the effect of serotonin on appetite suppression (Swamy et al., 2004a). This mechanism may explain the less severe feed refusal seen in broiler chickens. Conditioned taste aversion has also been reported in the presence of DON, mediated by the area postrema, which can be responsible for an emetic action too (Prelusky and Trenholm, 1993). The mechanism by which DON induces diarrhea was investigated in a human intestinal cell line and was associated with the inhibition of the sodium-glucose transport protein 1 transporter, resulting in a decrease of D-glucose associated water absorption and thus increased water content in the intestinal lumen (Maresca et al., 2002). A decreased glucose uptake was also observed in chickens after exposure to DON (Awad et al., 2008b; Awad et al., 2007b).

In addition, it has to be mentioned that broilers seem to be able to adapt to DON-contaminated diets (Awad et al., 2011). But also in pigs and rats the development of a kind of tolerance to low concentrations has been observed (Morrissey and Vesonder, 1985; Prelusky et al., 1994). Development of tolerance occurs with most anorexic compounds, more specifically with the compounds relying on a central serotoninergic mechanism (Silverstone, 1992).

2.3.2 T-2 mycotoxicosis

Symptoms of T-2 mycotoxicosis are almost the same as for DON. The differences are only in the extent and the severity of the changes. The mycotoxin T-2 is considered the most acutely toxic member of the family of the trichothecenes and exposure can occur through different routes (Sokolovic et al., 2008). The LD_{50} of T-2 is 6.3 mg/kg BW in broiler chickens (Chi et al., 1977b). A wide range of toxic effects can be caused by chronic exposure to T-2 in animals: weight loss, emesis, diarrhea, lesions in liver and digestive system,… (Li et al., 2011). Especially in chickens reduced egg production, impaired egg hatch and feather alterations are other symptoms of chronic exposure to T-2 (Diaz et al., 1994; Wyatt et al., 1975). Most relevant chronic toxicity studies in poultry were summarized in a scientific opinion published by the European Food Safety Authority (EFSA) (Anonymous, 2011). Effects of T-2 on zootechnical parameters as reported by the EFSA are illustrated in Table 5.

T-2 is a neurotoxin, able to damage the blood-brain barrier and cause changes in the activity of serotonine which explains the reduced feed intake (Wang et al., 1998). An increase in brain indoleamines, e.g. serotonin, induced by T-2 can contribute to feed refusal (MacDonald et al., 1988). In addition, T-2 causes lesions in the oral cavity which can also be a factor responsible for a decreased feed intake (Wyatt et al., 1973). Other authors reported that one single dose of 5 mg/kg T-2 or feeding at concentrations of 1 to 5 mg/kg T-2 for at least one week, are necessary to cause lesions in the mouth (Sokolovic et al., 2008).

Table 5. Summary of the effects of T-2 on feed intake and body weight in broiler chickens (adapted from Anonymous, 2011).

Dose in the feed	Exposure time	Decreased feed intake	Decreased body weight (BW)	Reference
1, 2, 4, 8 or 16 mg/kg T-2	21 days	yes (from a concentration of 4 mg/kg T-2)	yes (from a concentration of 4 mg/kg T-2)	(Wyatt et al., 1973)
0.2, 0.4, 2 or 4 mg/kg T-2	63 days	yes (from a concentration of 4 mg/kg T-2)	yes (from a concentration of 4 mg/kg T-2)	(Chi et al., 1977a)
4 mg/kg T-2	21 days		yes	(Kubena et al., 1989a; Kubena et al., 1989b)
8 mg/kg T-2 and 3.5 mg/kg aflatoxin	21 days		yes	(Kubena et al., 1990)
0.11, 0.53 or 1.05 mg/kg T-2	35 days	no	no	(Sklan et al., 2001)
2 mg/kg T-2	28 days	no	yes	(Diaz et al., 2005)
0.5, 1.5, 4.5 or 13.5 mg/kg T-2	17 days	yes (from a concentration of 4.5 mg/kg T-2)	yes (from a concentration of 4.5 mg/kg T-2)	(Rezar et al., 2007)
Starter (D0-D21): 1.04 mg/kg T-2, 0.49 mg/kg HT-2 Finisher (D22-D39): 0.12 mg/kg T-2, 0.02 mg/kg HT-2	39 days		yes at 21 days	(Weber et al., 2010)
0.31 mg/kg T-2 0.26 mg/kg HT-2	21 days	no	no	(Pal et al., 2009)

From the collected data about dietary exposure to trichothecenes we can conclude that the variable effects of trichothecenes on performance of poultry indicate that zootechnical traits might not be a sensitive indicator of their toxicity. Only clinical signs and production parameters but no suitable biomarkers were evaluated in these studies. Therefore, research has to be focused on the negative impact of trichothecenes on the intestines and the liver at the molecular level and a possible link to reduced performance.

2.4 Implications for men's health

Besides direct intake of mycotoxin contaminated cereals and related food products, another route of exposure for men that has to be considered for *Fusarium* toxins is the transfer from animal feed to poultry tissues, possibly leading to residues in animal products. There is limited information available on the occurrence of trichothecenes in eggs or meat. For laying hens, very low transmission levels of DON or T-2 to the eggs have been reported, namely 0.19% and 0.17% of the administered dose, respectively (Chi et al., 1978; Prelusky et al., 1987). El-Banna et al. (1983) fed hens with rations contaminated with 5 mg/kg DON for 192 days and no traces of DON were found in the tissues or eggs using a GC-MS method (gas chromatography-mass spectrometry) with a detection limit of 10 µg DON/kg. DON and DOM-1 were analyzed in the liver of broilers after feeding concentrations of DON up to 5 mg/kg feed for five weeks. Both DON and DOM-1 were not detected in the liver (Awad et al., 2011). From these studies we may conclude that if the guidance levels as stated by the EFSA are respected for DON, risks are limited. For T-2, no similar studies have been published to our knowledge.

2.5 Principles of mycotoxin management

Basically, the best way to minimize the risk for a mycotoxin to come into the food chain would be to prevent its formation in crop production and/or during storage of the feedstuffs. However, under field conditions the presence of mycotoxins can never be fully excluded (Bhat et al., 2010). Whereas there are many factors involved in mycotoxin contamination, the climate is the main driving force of fungal colonization and mycotoxins production (Miraglia et al., 2009; Paterson and Lima, 2010). It is generally accepted that rainfall just before and during flowering of the crop favours the infection of crops with fungi belonging to the *Fusarium* species. It has also been demonstrated that the weather conditions during winter may have an influence on the survival of primary inoculums (Landschoot et al., 2011, 2012). Management of mycotoxins in livestock comprises all stages from 'farm to fork' (Miraglia et al., 2009). Pre-harvest prevention strategies are the use of

mold resistant crop varieties, GAP and the application of fungicides (Siegel and Babuscio, 2011). However, the use of fungicides not automatically results in a reduction of the mycotoxin contamination (Edwards, 2004). Exposure of *Fusarium* to sublethal concentrations of some fungicides might even stimulate mycotoxin production (Audenaert et al., 2011). Post-harvest methods comprise optimal storage conditions (effective drying, temperature control,…) (Magan and Aldred, 2007).

Before further processing of the feedstuffs, mycotoxin concentration and pattern must be determined (Döll and Dänicke, 2011) as mycotoxins are resistant to different production steps (Scudamore et al., 2008). Trichothecenes are known to be heat stable and are not degraded during normal food processing or autoclaving (Bullerman and Bianchini, 2007; Pestka and Smolinski, 2005). In Europe, maximum levels for products intended for animal feed production have been set by the European Commission. Table 6 illustrates the guidance level determined in the Commission Recommendation of 17 August 2006 for DON and ZEN (Anonymous, 2006). For T-2, the Belgian Federal Agency for the Safety of the Food Chain currently imposes a limit of 0.4 mg/kg feed for T-2 and HT-2 in poultry feed. On the other hand, Table 7 shows the very recent European Commission Recommendation of 27 March 2013 for T-2 and HT-2 (Anonymous, 2013).

If the mycotoxin concentration is lower than the guidance level, feedstuff can be used for feeding. However, if the mycotoxin concentration is higher than the recommended levels, action is necessary. There are different possibilities: technical decontamination prior to feeding or *in vivo* decontamination in the animal (Döll and Dänicke, 2011). Physical, chemical or biological treatments of contaminated feed is not efficient and expensive (Jouany, 2007). *In vivo* decontamination is frequently applied in livestock using feed additives, called mycotoxin detoxifying agents.

A scientific report was recently submitted to the EFSA with the title: 'Review of mycotoxin detoxifying agents used as feed additives: mode of action, efficacy and feed/food safety' (Anonymous, 2009). In this report, mycotoxin detoxifying agents are divided in two different categories: adsorbing agents and biotransforming agents. This distinction is made on the fact that the first group is able to bind the mycotoxins in contaminated feed in the

gastrointestinal tract of the animal. Ideally, this complex does not dissociate in the gastrointestinal tract of the animal, resulting in an efficient elimination via faeces and hereby preventing or minimizing exposure of animals to mycotoxins. Adsorbing agents can consist of aluminosilicates (e.g. bentonite, montmorillonite, zeolite, phyllosilicates), activated carbon, complex indigestible carbohydrates (cellulose, polysaccharides from the cell walls of yeasts and bacteria such as glucomannans and peptidoglycans) and synthetic polymers (cholestyramine and polyvinylpyrrolidone) (Anonymous, 2009). The biotransforming agents, such as bacteria, yeasts, fungi and enzymes, are responsible for the degradation of mycotoxins into less or non-toxic metabolites.

The EFSA stated that these detoxifiers have to be registered as feed additives belonging to the class of 'substances for reduction of the contamination of feed by mycotoxins'. Prior to registration, both efficacy and safety of the detoxifying agents have to be proven (Anonymous, 2010).

Table 6. The guidance values on the presence of deoxynivalenol and zearalenone in products intended for animal feeding (relative to a feedingstuff with a moisture content of 12%), as determined in the Commission Recommendation 2006/576/EC.

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg
Deoxynivalenol	Feed materials (*) -Cereals and cereal products (**) with the exception of maize by-products -Maize by-products Complementary and complete feedstuffs with the exception of: -Complementary and complete feedingstuffs for pigs -Complementary and complete feedingstuffs for calves (<4 months), lambs and kids	 8 12 5 0.9 2
Zearalenone	Feed materials (*) -cereals and cereal products (**) with the exception of maize by-products -maize by-products Complementary and complete feedingstuffs -complementary and complete feedingstuffs for piglets and gilts -complementary and complete feedingstuffs for sows and fattening pigs -complementary and complete feedingstuffs for calves, dairy cattle, sheep (including lamb) and goats (including kids)	 2 3 0.1 0.25 0.5

(*) Particular attention has to be paid to cereals and cereal products fed directly to the animals that their use in a daily ration should not lead to the animal being exposed to a higher level of these mycotoxins than the corresponding levels of exposure where only the complete feedingstuffs are used in a daily ration.

(**) The term 'cereals and cereal products' includes not only the feed materials listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in part B of the Annex to Council Directive 96/25/EC of 29 April 1996 on the circulation and use of feed materials (OJ L 125, 23.5.1996, p.35) but also other feed materials derived from cereals in particular cereal forages and roughages.

Table 7. Indicative levels for the sum of T-2 and HT-2 toxin in cereals and cereals products, as determined in the Commission Recommendation 2013/165/EU.

	Indicative levels for the sum of T-2 and HT-2 (µg/kg) from which onwards/ above which investigations should be performed, certainly in case of repetitive findings (*)
1. Unprocessed cereals (**)	
1.1. barley (including malting barley) and maize	200
1.2. oats (with husk)	1000
1.3. wheat, rye and other cereals	100
2. Cereal products for feed and compound feed (***)	
2.1. oat milling products (husks)	2000
2.2. other cereal products	500
2.3. compound feed, with the exception of feed for cats	250

(*) The levels referred to in this Annex are indicative levels above which, certainly in the case of repetitive findings, investigations should be performed on the factors leading to the presence of T-2 and HT-2 toxin or on the effects of feed and food processing. The indicative levels are based on the occurrence data available in the EFSA database as presented in the EFSA opinion. The indicative levels are not feed and food safety levels.
(**) Unprocessed cereals are cereals which have not undergone any physical or thermal treatment other than drying, cleaning and sorting.
(***) The indicative levels for cereals and cereal products intended for feed and compound feed are relative to a feed with a moisture content of 12 %.

3 The gastro-intestinal tract and the liver as a target for trichothecene mycotoxins

The digestive tract of poultry consists of a crop, which is a dilatation of the esophagus. The main function of the crop is storage of feed. Absorption of drugs in esophagus or crop is minimal since both parts of the digestive tract are characterized by keratinized stratified squamous epithelia (Lumeij, 1994). The avian stomach consists of two different parts: a proventriculus or glandular stomach and a ventriculus or gizzard, where grit is stored that aids in the physical grinding of feed. After the stomach, following parts of the digestive system can be distinguished: small intestines, two ceca, a rectum and a cloaca (Martinez et al., 2002) (Figure 4). The small intestine can be divided in three different parts in broiler chickens, but in the literature some discrepancy exists how this should be done. Therefore, our applied criteria are described below. The first part is just behind the gizzard and is also called the duodenal loop or duodenum. In the chicken the duodenum is the most important site for nutrient digestion and absorption (Vermeulen et al., 2002). The second part is situated at the level of the Meckel's diverticulum and is called jejunum. The last part, the ileum, is situated before the ileo-cecorectal transition. In chicken, ingesta can pass through the whole digestive tract very quickly. Fifty percent of ingesta pass in 12h and after 24h all passage is complete. Besides the movement of ingesta from proximal to distal, also three reverse peristaltic cycles are observed in chickens: from the gizzard to the proventriculus and the crop, from the duodenum to the gizzard and from the rectum to the caeca (Hoerr, 1998).

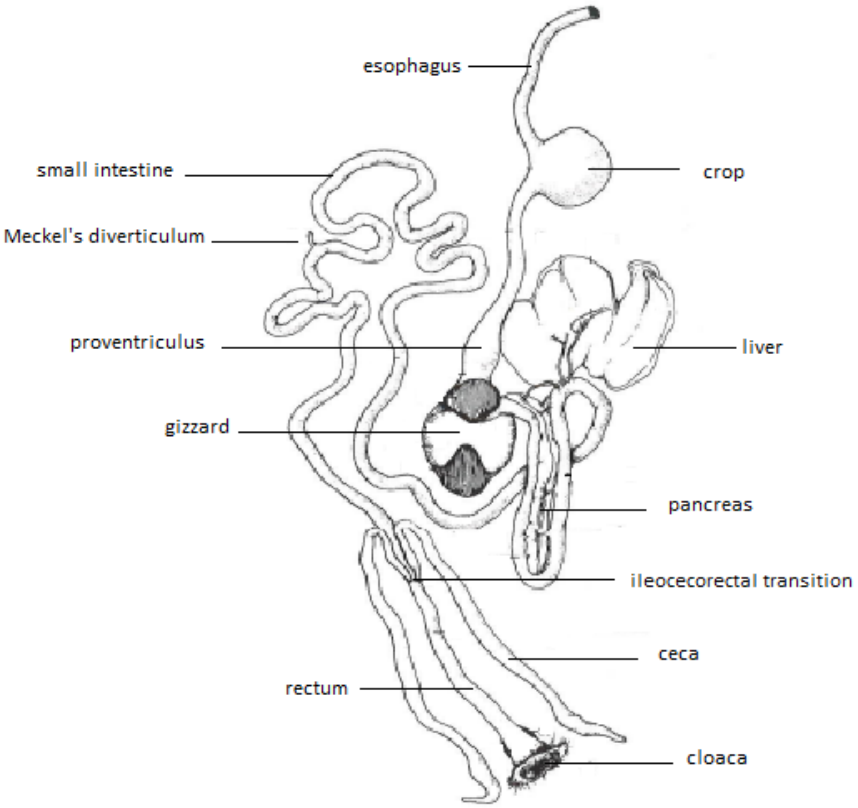


Figure 4. Anatomy of chicken's digestive tract (adapted from Duke, 1984).

In addition, the liver also plays an important role as first line defense mechanism by the process called first-pass effect. The liver has an exposed position within the body as the gateway of the portal blood draining the gastrointestinal tract. Almost all the drugs absorbed in the gastro-intestinal tract have to enter the portal vein and encounter the hepatocytes (Hu and Li, 2011).

Both liver and intestines can interact on the oral bioavailability of drugs or xenobiotics. Consequently when mycotoxins interact with the intestinal or hepatic function, bioavailability of these drugs and xenobiotics can be influenced.

3.1 Effects at the gastro-intestinal level

Mode of action

Trichothecenes have an affinity for the 60S subunit of ribosomes, which leads to inhibition of the protein synthesis at the initiation, elongation or termination step (Rocha et al., 2005) (Figure 5). T-2 is active at the initiation phase, while DON acts as inhibitor of the elongation and/or termination step (Awad et al., 2008a; Sokolovic et al., 2008). Besides the effects on the protein synthesis, trichothecenes exert other effects on eukaryotic cells such as inhibition of the RNA and DNA synthesis as well as adverse effects on the mitochondrial function (Minervini et al., 2004; Ueno, 1984).

Trichothecenes can also induce apoptosis, a programmed cell death (PCD) response both *in vitro* and *in vivo* (Minervini et al., 2004; Pestka et al., 2004; Yang et al., 2000). DON was classified as a strong PCD inducer, while T-2 is a weak inducer (Shifrin and Anderson, 1999). The induction of apoptosis may require both translational arrest and mitogen-activated protein kinase (MAPK) activity. MAPK's are components of a signaling cascade that regulate cell survival in response to stress (Iordanov et al., 1997). T-2 induces apoptosis by activation of c-Jun N-terminal kinases (JNK), p38 and MAPK's, but the precise mechanism has not yet been elucidated (Sokolovic et al., 2008). The process is also called 'ribotoxic stress response' (Iordanov et al., 1997). Sergent et al. (2006) demonstrated that DON inhibits intestinal cell proliferation at concentration corresponding to those found in nature. DON induces namely the phosphorylation of p38, extracellular signal-regulated kinases (ERK) and JNK and concomitantly disrupts the intestinal permeability (Sergent et al., 2006). The trichothecene-mediated signal transduction pathway in mammalian cells is shown in Figure 5.

The link between ribosomal RNA damage and the induction of MAPK signaling pathways remains an unrevealed paradox (Pestka, 2007). Two different kinases have been pointed out until now which potentially mediate signaling of the trichothecene-mediated MAPK activation, namely RNA-activated protein kinase (PKR) and hematopoietic cell kinase (Hck) (Zhou et al., 2003; Zhou et al., 2005). However, the presence of other signal transducing proteins can not be excluded and the exact mechanism of action has still to be elucidated.

Different studies also reported that trichothecenes are able to induce the production of free radicals, causing membrane and DNA damage (Atroshi et al., 1997; Leal et al., 1999; Minervini et al., 2005; Rizzo et al., 1994; Vila et al., 2002). Oxidative stress can thus be considered as a possible underlying mechanism involved in the toxicity of trichothecenes. However, some contradictory studies were published concerning the induction of oxidative stress by DON and T-2. A recent study reported that T-2 is a moderate oxidant mycotoxin, while DON is a non-oxidant mycotoxin (El Golli-Bennour and Bacha, 2011). T-2 can generate higher reactive oxygen species (ROS) levels which lead to DNA damage, activation of p53 and final apoptosis in human cervical cancer cells (Chaudhari et al., 2009). This DNA damage occurs as early as 10 min after exposure to the toxin, a time point much earlier than the protein synthesis inhibition. However, the mechanism responsible for the DNA damage has still to be elucidated. DNA fragmentation of leukocytes in broilers was observed after exposure to T-2 at a concentration of 13.5 mg/kg feed for 17 days (Rezar et al., 2007). The same effects were seen in broilers even at a lower concentration of 10 mg/kg T-2 after exposure for 17 days (Frankic et al., 2006). Awad et al. (2012) demonstrated that the genotoxic effects of DON in broilers are not correlated with the induction of oxidative stress in the liver. In contrast, diets contaminated with DON and ZEA induced oxidative stress in the liver of broilers, but no effect was seen in the duodenal mucosa of the animals (Borutova et al., 2008). Besides the p53 pathway, ROS can also activate MAPK's and thus also lead to apoptosis by this pathway (Martindale and Holbrook, 2002).

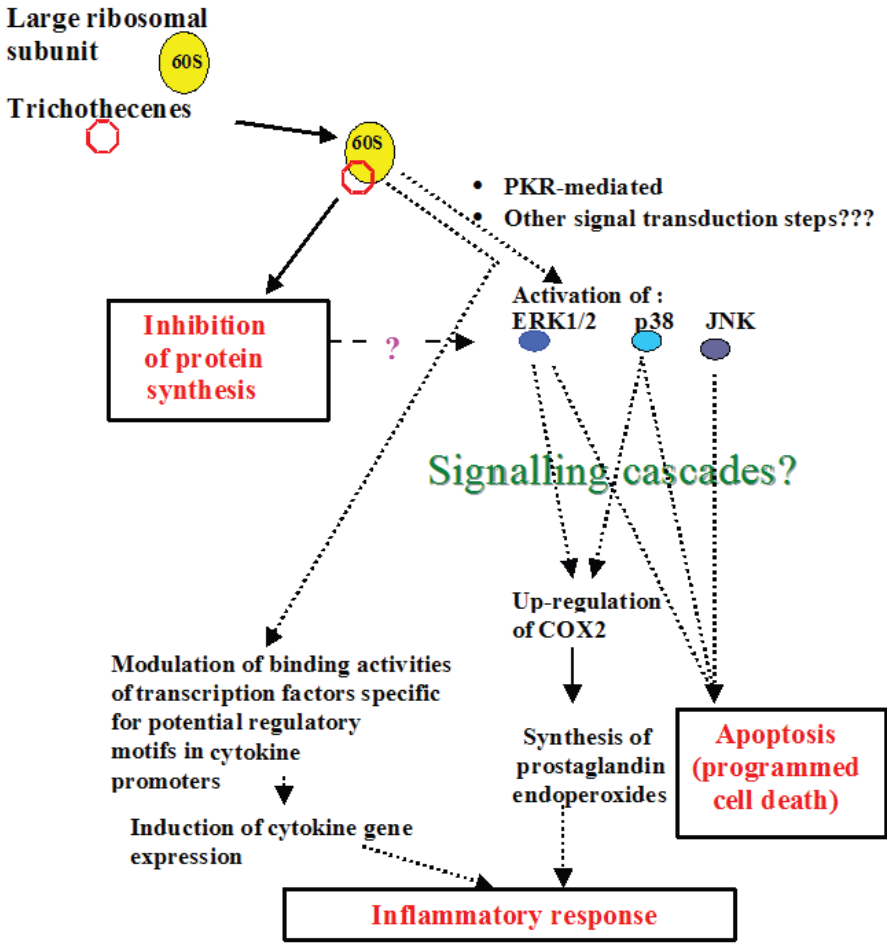


Figure 5. Trichothecene-mediated signal transduction and downstream processes in mammalian cells (adapted from Rocha et al., 2005).

Trichothecenes appear to have an impact on the cell cycle depending on the cell type. A cell cycle arrest in the G2/M-phase was observed in intestinal porcine epithelial cells from the mid-jejunum (IPEC-J2 cells) after incubation with 2000 ng/ml DON for 48h. In contrast, in intestinal porcine epithelial cells from the jejunum and ileum (IPEC-1 cells) a significant increase in PreG1 phase was observed under the same circumstances (Hegewald et al., 2010). Another study also demonstrated an arrest in G2/M-phase of the cell cycle after exposure to DON in a concentration range of 250 ng/ml and 1000 ng/ml in human HCT-116 and intestinal-407 epithelial cells (Yang et al., 2008). Prudence is however in order when extrapolating these *in vitro* results to *in vivo* situations.

3.1.1 Gut wall morphology

The intestinal mucosa functions as a kind of barrier which regulates the uptake of nutrients and water, but excludes potential pathogens and toxicants (Oswald, 2006). The epithelial surface of the intestine is characterized by a large contact area for absorption. The surface consists of a simple columnar epithelium, which is increased by the presence of depressions (crypts) and villi (DeSesso and Jacobson, 2001). This monolayer is also called the *mucosa* of the intestinal monolayer. Mature cells migrate along the crypt-villus axis towards the villus-top, underway these cells become differentiated cells (Booth and Potten, 2000; Simon and Gordon, 1995). Especially in chicks, cell proliferation not only occurs in the crypts but also along the villi (Uni et al., 2000). In poultry the flattened leaf-shaped villi become shorter and broader throughout the length of the small intestines. In mammals, normally a *submucosal* layer is found under the *mucosa*. However, in poultry the non-existence of a *tela submucosa* is a histological particularity. Furthermore, poultry also possess a thick *lamina muscularis mucosae* which is frequently in continuity with the underlying circular muscle (Hodges, 1974).

Histological alterations have been described after ingestion of different concentrations of trichothecenes. More precisely, cells on the tips of the villi are destroyed and crypt epithelium is injured (Hoerr, 1998). Shorter and thinner villi were observed in the duodenum and the jejunum of broiler chickens after exposure to 10 mg/kg DON in the feed for 6 weeks (Awad et al., 2006a). These authors related the histological changes to the irritant effect of DON. No effect on the jejunum villi, but only decreased height and width of villi in duodenum was found after 3 weeks exposure of broiler chickens to 5 mg/kg DON in the feed (Böhm et al., 2006). The same authors reported also that even concentrations of 1 mg/kg DON fed during 5 weeks resulted in a decreased villus height, decreased villus surface area and a reduced muscular thickness in the jejunum of broilers (Awad et al., 2011). Recently, a study could demonstrate that the increasing levels of DON linearly decreased the villus height in both the mid-duodenum and mid-jejunum of broiler chickens (Yunus et al., 2012b). T-2 exposure at a concentration of 0.982 mg/kg for 32 days resulted in shorter villi in the duodenum and shorter and thinner villi in the jejunum of turkey poults (Sklan et al., 2003).

The decrease in enterocyte height is highly indicative that these mycotoxins can alter the digestive and absorption function. Besides a direct irritant effect of the toxin, the inhibition of the protein synthesis by DON, which results in death of the epithelial cells, was also pointed out as explanation for the morphological changes.

The mitotic index in crypt epithelia was decreased after inoculation with T-2 in mice (Li et al., 1997). Reduced enterocyte migration was observed in the jejunum of turkey poults fed T-2 (Sklan et al., 2003). DON is also known to inhibit the intestinal cell proliferation (Sergent et al., 2006). Trichothecenes can cause apoptosis via different pathways and can influence the cell cycle, which can be an explanation for the reduced proliferation.

3.1.2 The intestinal functional barrier

Intestinal absorption can be described as the way molecules navigate across the enterocytes. The transport through the enterocyte barrier can be divided in active, passive and specialized transport; and into a paracellular and a transcellular route. Passive diffusion can be defined as the movement of molecules across the lipid bilayer without the need of energy. The driving force for this process is the concentration gradient (Park and Chang, 2011). Active transport is an energy-consuming system, mostly acting against the concentration gradient. An example of specialized transport is endocytosis, whereby macromolecules are taken up into the cells by vesicles. From the literature, trichothecenes are known to interact with the intestinal barrier function, interrupting both paracellular and transcellular routes.

3.1.2.1 Trichothecenes interact with the paracellular pathway

The epithelial barrier is formed by a lipid bilayer of enterocytes. The enterocytes are adhered to each other through complexes that form junctions between the cells, called tight junctions. These tight junctions regulate the traffic between the epithelial cells or the paracellular pathway. The intestinal barrier is a dynamic barrier, which is characterized by the fact that the tight junctions are able to open and close at any time in a response to a

variety of stimuli (Forster, 2008). Besides adhesive functions, these intercellular junctions are also involved in different signaling pathways that regulate the proliferation and the differentiation of the epithelial cells (Matter and Balda, 2003).

Tight junctions are constituted of different proteins as illustrated in Figure 6. Three different types of transmembrane proteins can be distinguished: occludins, claudins and junctional adhesion molecules (JAMs) (Matter and Balda, 2003). Occludin was the first identified integral membrane protein in chicken (Furuse et al., 1993). However, different studies demonstrated that occludin is not indispensable for the formation of tight junction strands (Furuse et al., 1996; Saitou et al., 1998), but more supports the role of claudins (Forster, 2008). The members of the claudin family on the other hand, really form the backbone of the tight junctions (Matter and Balda, 2003). So far, at least 24 members of the claudin family have been identified in different animal species and these proteins also seem to be expressed in a tissue-specific manner (Forster, 2008). Expression studies revealed that claudin 1 and 5 are specific for the mediation of transepithelial resistance in poultry and thus are important subtypes for the intestinal barrier function (Amasheh et al., 2005; Inai et al., 1999; Markov et al., 2010). The third group consists of the junction-associated adhesion molecules. Three different members have been characterized until know, but only the function of JAM-1 is well defined. JAM-1 is involved in the immune cell migration or cell adhesion (Bazzoni et al., 2000). Besides these transmembrane proteins, the tight junctions also possess a cytoplasmatic plaque responsible for the organization of different processes such as morphogenesis, cell polarity, cell proliferation and differentiation. This plaque consists of two different categories of proteins: the peripherally associated scaffolding proteins (ZO-1, ZO-2, ZO-3 and cingulin) and the signaling proteins (ZONAB, Rhoa and Raf-1) (Forster, 2008). Of special interest are the zona occludens proteins as they act as a kind of adaptors, which are connected to the transmembrane proteins and to other cytosolic components (Paris et al., 2008).

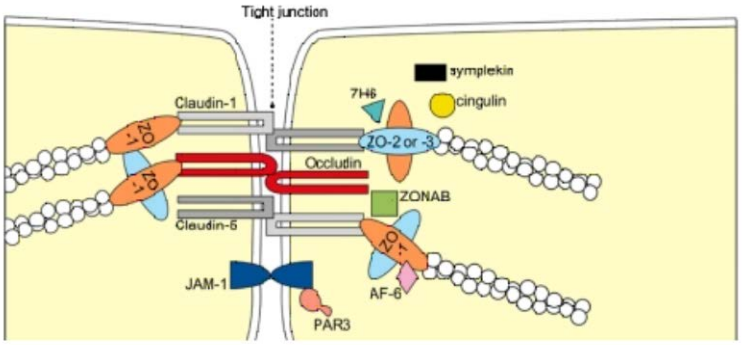


Figure 6. Molecular composition of tight junctions (adapted from Forster, 2008).

To measure the permeability of membranes, probe drugs are frequently used. However, the selection of a useful probe drug is not easy. Movement across the paracellular pathway depends on 5 different parameters, namely the concentration gradient, the surface area of the epithelium, the thickness of the epithelium, the time available for permeation and the intrinsic permeability properties of the barrier. To evaluate this last factor, a probe drug has to be selected with the guarantee that the four first factors are not changed. In addition, the probe drug may not be degraded or transformed by the gut flora (Arrieta et al., 2006). Another frequently used method in both *in vitro* and *ex vivo* studies is the measurement of the trans-epithelial electrical resistance (TEER) across the intestinal membrane. This technique is a useful approach in combination with other techniques, since the TEER measurements can be influenced by different factors that are difficult to take into account (Madara, 1998). For example, a decrease in TEER does not always equate with an altered paracellular pathway as migration of neutrophils across the intestinal epithelia may cause the same effects. Another example is edge damage of tissue which can occur due to manipulation of the epithelial explants when using Ussing chambers for the measurements of intestinal TEER. Edge damage consists of a loss of integrity of the tissue, resulting in a decreased TEER (Clarke, 2009).

The application of 2000 ng/ml DON for 48h on IPEC-1 and IPEC-J2 cells led to a desintegration of ZO-1 as observed by immunofluorescence, reinforced by a decreased protein expression of ZO-1 as shown by western blot analysis (Diesing et al., 2011; Hegewald

et al., 2010). In contrast, another study reported that ZO-1 and occludin staining and localization were not affected by DON treatment. However, a reduced intensity for the staining of claudin 3 was observed in Caco-2 cells and for both claudin 3 and 4 in IPEC-1 cells (Pinton et al., 2009). The overall morphology of the cells remained unchanged during this trial, suggesting a direct or specific effect on claudins rather than general cell damage. The authors suggested that the tight junction complex structure and function was regulated through the activation of the MAPK pathway by DON (see Figure 5). A concentration-dependent reduction of the expression of claudin 4 was demonstrated by western blot analysis in human Caco-2 cells which were exposed to 50, 500 or 5000 ng/ml DON for 24h (Van de Walle et al., 2010). The authors reported that this effect was due to the ability of DON to inhibit protein synthesis. There is a lack of literature on the effects of trichothecenes on the tight junctions after *in vivo* exposure. One study reported a reduced claudin 4 protein expression in pigs after 4 weeks treatment with DON at a concentration of 2.85 mg/kg feed for 5 weeks (Pinton et al., 2009). However, no data are available on the effects in broiler chickens.

Barrier disruption is an important etiologic factor of intestinal inflammation because it can lead to an increased permeability to luminal antigens with subsequent contact with toll-like receptors and potential activation of underlying immune cells (Forster, 2008; Maresca et al., 2008). TLRs play a key role in microbial recognition, control of adaptive immune responses, and induction of antimicrobial effectors pathways, leading to efficient elimination of host-threatening pathogens (Takeuchi et al., 1999).

3.1.2.2. Trichothecenes interact with efflux transporters involved in the transcellular pathway

Along the entire length of the gastro-intestinal tract, different drug transporters can be localized in the apical membrane of the enterocytes. These transporters are able to remove xenobiotics from the enterocyte linings and return them into the gastro-intestinal lumen. This secretion process is also called efflux transport (Chang et al., 2011). After their return

into the gut lumen, drugs continue to move along the GI tract and afterwards the molecules can re-enter the enterocytes or they can be eliminated with the faeces. It is generally accepted that members of ATP-binding cassette (ABC) are widely involved in the active process of efflux transport. The ABC family needs cellular energy for active transport of substrates against the concentration gradient (Higgins and Linton, 2004). Another requirement is that the transporter protein needs to recognize the specific drug as a substrate. Afterwards, the protein undergoes a conformational change which allows the substrate to be transported.

A study of the evolution of the ABC gene family showed that chickens only have 41 ABC genes which is lesser than any other higher vertebrates (Annilo et al., 2006). Several efflux transporters have already been identified in chickens such as P-glycoprotein (P-gp or MDR1) and multidrug resistance-associated protein2 (MRP2). In turkeys another efflux transporter has been described, namely breast cancer resistance protein (BCRP) (Haritova et al., 2008b). P-gp is highly conserved and is known to be expressed in tumor cells and thus to lead to multidrug resistance (Goldstein et al., 1989). However, this protein is also expressed in healthy intestines and in other organs such as e.g. the liver of animals (Haritova et al., 2010). Another protein which is also found at this level is MRP2. A difference in expression between these two efflux transporters has been observed in both mammals and chickens. P-gp expression pattern in chickens shows an increasing trend from proximal to distal in the intestinal tract. MRP2 on the other hand, is characterized by a decreasing trend along the entire length of the GI tract of poultry. Due to their apical localization in the different organs, efflux transporters are well positioned to function as a barrier for xenobiotics (Chang et al., 2011; Schrickx and Fink-Gremmels, 2008). Both induction and inhibition of these transporter proteins can occur, which respectively results in a decreased and increased bioavailability of a drug (Zhou, 2008). Different factors including disease, diet, endogenous or exogenous compounds can be responsible for the up- or down-regulation of the efflux pumps (Fardel et al., 2005). Numerous xenobiotics have been found to be good substrates for these transporters and can be responsible for the modulation of their expression (Green et al., 2005). Two fluoroquinolone antibiotics, namely danofloxacin mesylate and enrofloxacin have been demonstrated to restore partially the mRNA down-regulation of MDR1 due to *E.*

coli infection in broiler chickens. The antibiotics were, however, not able to restore MRP2 down-regulation in these infected animals (Haritova et al., 2008a). The same trend was observed for danofloxacin mesylate in turkeys (Haritova et al., 2008b).

Differences in drug bioavailability as described above can be a factor responsible for resistance to antibiotics. On the other hand, micro-organisms, such as bacteria, also possess ABC transporters to adapt to the environment and to develop resistance to the actions of toxic compounds (Chen et al., 2010; Sabri et al., 2006). However, the effects of mycotoxins on ABC transporters of micro-organisms have, to our knowledge, not been investigated in poultry until now.

It is suggested that P-gp is strongly involved in the protection of animals against different mycotoxins which are substrates of P-gp (De Angelis et al., 2005). The toxin nivalenol has been characterized as a substrate for both P-gp and MRP2 in Caco-2 cells (Tep et al., 2007). Also DON was demonstrated to be a substrate for both MDR1 and MRP2 in Caco-2 cells (Videmann et al., 2007). Immunohistochemical analysis showed a reduced expression of P-gp at the brush border of the small intestines in pigs receiving 1 mg DON/kg of feed. A higher dose of 3 mg/kg DON even resulted in a complete disappearance of P-gp (Van der Heyden et al., 2009). A study conducted with laying hens reported the use of the paracellular pathway by DON, however, the same authors did not exclude the transcellular pathway (Awad et al., 2007a).

3.1.2.3 Trichothecenes interact with drug metabolism in the GI tract

The bioavailability of drugs can be limited due to their limited absorption or extensive metabolism. Older literature stated that the liver is the main organ for metabolism of drugs. However, recent publications reported that the small intestines also play a major role (Paine et al., 1996; Thummel et al., 1996). It is important to mention that the intestines are the first site of exposure for xenobiotics and in addition, intestinal cells are exposed to higher concentrations of drugs compared to the liver cells as the intestine is the place where drugs are released from their dosage form (Hu and Singh, 2011). Some studies even reported that

the intestinal metabolism may be of greater importance than the hepatic metabolism (Fromm et al., 1996; Wu et al., 1995). The principal biotransformation enzymes in the gut wall include the phase I cytochrome P-450 (CYP450) subfamily, but also phase II reactions can occur. Phase I reactions are usually oxidation, hydrolysis and reduction, while phase II reactions are the conjugation of the parent compound and phase I metabolites.

The most important family of enzymes involved in phase I metabolism is the CYP450 superfamily of hemeproteins. The CYP superfamily is subdivided into families and subfamilies based on the amino acid sequence homology. Enzyme families 1 to 4 are primarily responsible for the biotransformation of drugs (Fink-Gremmels, 2008). In humans, the predominant CYP isoform in the small intestines is CYP3A4 (Zhang et al., 1999). An avian CYP450 of the CYP3A family has been cloned, namely CYP3A37. A homology of approximately 60% with human CYP3A4 and of 62% with pig CYP3A29 was found (Ourlin et al., 2000). However, relatively little is known about expression and activity of CYP3A enzyme in birds (Vermeulen et al., 2002).

An important remark is that P-gp and CYP3A may be functionally linked due to their co-localization in the intestinal tract and to their similar substrates, but also because they can be co-induced as a response to some xenobiotics (Watkins, 1997). Although some information is available about the influence of trichothecenes on intestinal P-gp, little data is, to our knowledge, available about the effects of these toxins on intestinal CYP enzymes in animals. One study reported that CYP1A1 was induced in Caco-2 cells after exposure to 0.03 µg/ml T-2 (Kruber et al., 2011). However, *in vitro* cell response to mycotoxins is not always a good predictor for the *in vivo* situation (Rocha et al., 2005).

3.2 Effects at the hepatic level

The liver is responsible for energy homeostasis, cholesterol metabolism, blood filtration, bile production and processing of nutrients and hemoglobin. Additionally, an important function of the liver is detoxification of toxic substances both from endogenous and exogenous origin (Wang and Tompkins, 2011). The liver receives blood directly from the gut, via the portal vein, and thus the liver can be directly exposed to intestinal absorbed compounds (Figure 7). To accomplish the role of detoxifier, the liver also disposes of metabolizing enzymes of phase I and II, as described earlier. The biotransformation of drugs before entering the systemic circulation is referred to as first-pass metabolism and the liver CYP enzymes are considered to be the major protagonists for this process.

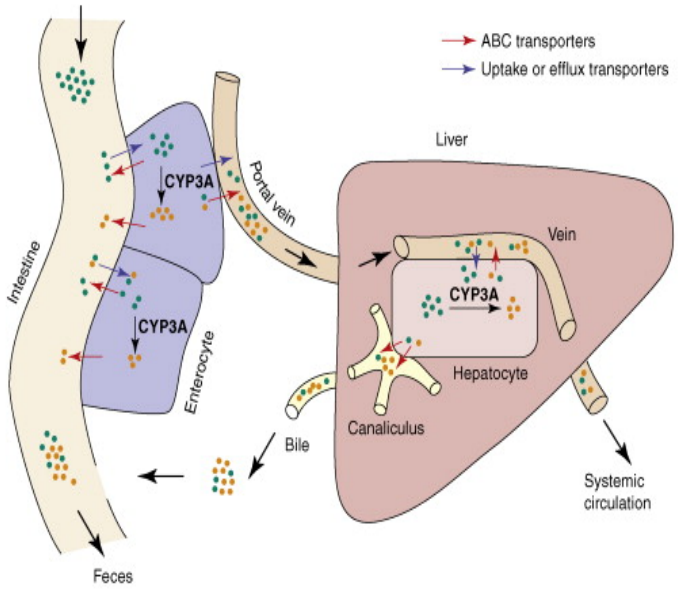


Figure 7. First-pass effect (adapted from van Herwaarden et al., 2009).

The effect of trichothecenes on hepatic CYP enzymes has been investigated *in vitro*. Hepatic CYP3A22 mRNA expression in primary hepatocyte cultures of piglets was induced after T-2 exposure (0.1 µg/ml) for 48h (Ge et al., 2010; Wang et al., 2011), while CYP1A expression was not affected under the same conditions (Wang et al., 2011).

Some *in vivo* studies have already been performed in other animal species than chickens to study the effects of chronic exposure to T-2. The concerned literature is summarized in Table 8.

Table 8. Summary of performed *in vivo* studies concerning the effects of dietary exposure to trichothecenes on hepatic CYP proteins.

Mycotoxin	**Exposure time**	**Species**	**Target CYP**	**Effect in the liver**	**Reference**
T-2 toxin and diacetoxyscirpenol: 1.0 mg/kg feed	1, 4 or 8 days	Rat	CYP450	Dose-dependent decrease in protein expression	Galtier et al., 1989
T-2 toxin: 0.5, 0.25, 0.1 mg/kg feed	5 days	Rabbit	CYP1A1 CYP1A2 CYP2A1 CYP2B4	Dose-dependent decrease in protein expression	Guerre et al.. 2000
T-2 toxin: 2.102 mg/kg feed	28 days	Pig	CYP1A CYP2B CYP2C CYP3A	Significant lower protein expression. Decreasing trend for the protein expression was observed, but no significant differences	Meissonnier et al., 2008
T-2 toxin: 0.903 mg/kg feed	14 days	Pig	CYP3A	Significant lower protein activity	Goossens et al., 2013

In the liver, the efflux pumps MDR1 and MRP2 as described earlier, are also present. The mRNA expression of MDR1 is lower in the chicken liver compared to the intestinal expression, while for MRP2 the opposite is seen (Haritova et al., 2010). These hepatic efflux pumps could possibly be altered due to mycotoxin exposure. However, at the start of this research no data was available about this subject.

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SCIENTIFIC AIMS

The European Union has established the most comprehensive regulations for mycotoxins in food and feed. However, these regulations do not comprise all the mycotoxins. In addition, mycotoxins rarely occur as a single contaminant which questions the applicability of the proposed individual safety levels. From economical point of view *Fusarium* mycotoxins are the most important in our temperate climate regions. Deoxynivalenol is considered the most prevalent *Fusarium* toxin, whereas T-2 toxin the most acute toxic one. Different studies already reported the effects of these mycotoxins on production parameters in poultry, but action at mRNA and protein level in target organs may be underestimated. Therefore, the general aim of this thesis was to investigate the effects of the *Fusarium* toxins deoxynivalenol and T-2 toxin on the intestinal barrier and hepatic function in broiler chickens.

In general, poultry are claimed to be relative resistant to mycotoxicosis. However, nor clear dose-response relationship can be found in this species, nor information on their disposition and toxicokinetic characteristics in the body. In practice, there is a need for suitable biomarkers to assess mycotoxin exposure *in vivo*.

It is generally accepted that the presence of mycotoxins can be reduced, but not completely avoided. Therefore, mycotoxin detoxifiers are frequently used as feed additives to prevent mycotoxicosis. Mycotoxin detoxifying agents can be divided in two different groups according to their mode of action, namely adsorbing and biotransforming agents. However, little is known about the safety and efficacy of these feed additives.

The scientific aims of part I of this thesis therefore are:

1. To perform toxicokinetic studies for deoxynivalenol, T-2 toxin and zearalenone in order to evaluate the oral bioavailability and toxicokinetic parameters of these different mycotoxins in broiler chickens (Chapter 1)

2. To evaluate different biomarkers to assess deoxynivalenol exposure after chronic feeding in broiler chickens, and to test the efficacy and safety of two types of mycotoxin detoxifiers, i.e. an adsorbing agent and a biotransforming agent (Chapter 2)

Being an interface between the outside world and the inside body, the gastro-intestinal tract is especially equipped for the absorption of essential nutrients, ions and vitamins. At the same time it must function as a first line of defense against exogenous toxins and harmful micro-organisms. In addition, the oral bioavailability of xenobiotics may be limited by biotransformation and/or efflux in the gut and liver. The principal enzymes responsible for biotransformation belong to the cytochrome P450 family, with the CYP3A subfamily being important. However, the expression and activity of CYP3A have not been studied in poultry.

CYP450 enzymes are also known to interact with efflux transporter proteins such as P-glycoprotein and other multidrug resistance-associated proteins. The transporter systems and biotransformation enzymes are present in both small intestines and liver. These organs consist of continuously proliferating and differentiating cells, and are thus important target organs of trichothecenes. Moreover, as mycotoxin adsorbing agents pass through the whole digestive tract, their influence on the intestinal barrier function may also be questioned. Effects of deoxynivalenol are mainly observed at the intestinal level in several animal species. On the other hand, significant effects of T-2 on biotransformation enzymes have already been reported in other animal species. Therefore, besides deoxynivalenol, also a study with T-2 was included in this thesis.

The scientific aims of part II of this thesis are thus:

1. To evaluate the influence of deoxynivalenol and an adsorbing agent on the intestinal barrier and liver function in broiler chickens (Chapter 3)
2. To evaluate the gene expression and activity of CYP3A in liver and small intestines of broiler chickens (Chapter 4)
3. To investigate the effects of T-2 toxin on the gene expression of CYP450 enzymes and drug efflux transporters and on the activity of CYP3A, both in liver and small intestines of broiler chickens (Chapter 5)

EXPERIMENTAL STUDIES

Chapter 1.

Toxicokinetic studies of three important *Fusarium* mycotoxins: deoxynivalenol, T-2 toxin and zearalenone

Adapted from: Osselaere, A., Devreese, M., Goossens, J., Vandenbroucke, V., De Baere, S., De Backer, P., Croubels, S. (2013). Toxicokinetic study and absolute oral bioavailability of deoxynivalenol, T-2 toxin and zearalenone in broiler chickens. Food and Chemical Toxicology 51, 350-355.

ABSTRACT

Mycotoxins lead to economic losses in animal production. A way to counteract mycotoxicosis is the use of detoxifiers. The European Food Safety Authority stated that the efficacy of detoxifiers should be investigated based on toxicokinetic studies. Little information is available on the absolute oral bioavailability and the toxicokinetic parameters of deoxynivalenol, T-2 and zearalenone in broilers. Toxins were administered intravenously and orally in a two-way cross-over design. For deoxynivalenol a bolus of 0.75 mg/kg BW was administered, for T-2 toxin 0.02 mg/kg BW and for zearalenone 0.3 mg/kg BW. Blood was collected at several time points. Plasma levels of the mycotoxins and their metabolite(s) were quantified using LC-MS/MS methods and toxicokinetic parameters were analyzed. Deoxynivalenol has a low absolute oral bioavailability (19.3%). For zearalenone and T-2 no plasma levels above the limit of quantification were observed after an oral bolus. Volumes of distribution were recorded, i.e. 4.99 L/kg, 0.14 L/kg and 22.26 L/kg for deoxynivalenol, T-2 toxin and zearalenone, respectively. Total body clearance was 0.12 L/min.kg, 0.03 L/min.kg and 0.48 L/min.kg for deoxynivalenol, T-2 toxin and zearalenone, respectively. After IV administration, T-2 toxin had the shortest elimination half-life (3.9 min), followed by deoxynivalenol (27.9 min) and zearalenone (31.8 min).

INTRODUCTION

Many fungi species are able to produce and secrete low-molecular-weight compounds, called mycotoxins. The occurrence of mycotoxin-producing moulds is a worldwide problem, but the nature and the quantity of produced mycotoxins depend on the interaction of several factors (Bhat et al., 2010). Moulds belonging to the *Fusarium* genus are commonly present in moderate climate zones (Edwards, 2011). These fungi are responsible for the production of trichothecenes, which are divided in four groups. Type A (e.g. T-2 toxin) and type B (e.g. deoxynivalenol) are the most important. Other mycotoxins like zearalenone are also produced by these *Fusarium* moulds (Devegowda and Murthy, 2005).

Due to the threat for the animal health, the occurrence of these mycotoxins in animal feed can lead to important economic losses. Especially the poultry industry is confronted with this problem, as cereal grains form the major part of the animals diet (Anonymous, 2011). Deoxynivalenol (DON), known colloquially as “vomitoxin”, causes decreased weight gain, anorexia and emesis in animals (Pestka, 2007). T-2 toxin (T-2) mostly induces cutaneous lesions of the oral cavity and intestinal membrane in poultry (Weber et al., 2010). A decrease in egg production has also been reported after chronic exposure to T-2 (Diaz et al., 1994). On the other hand, zearalenone (ZEN) induces reproductive disorders (Devegowda and Murthy, 2005).

Recommended maximum levels in poultry feed were stated by the European Union for DON in complete feed (5 mg/kg). For ZEN, maximum levels were set for cereals and cereal products at 2 mg/kg and for maize by-products at 3 mg/kg. For T-2 toxin on the other hand, no maximum levels for the feed were pointed out until now (Anonymous, 2006, 2011). Although these maximum levels are a good starting-point, other actions have to be undertaken to reduce the risk for mycotoxicosis. The use of mycotoxin detoxifying agents is a common way to prevent the negative impacts of mycotoxins (Diaz et al., 2005; Döll et al., 2004). These feed additives can be divided in two different classes, depending on their mode of action: adsorbing and biotransforming agents. The first group is able to bind the mycotoxin in the intestinal tract of the animal; the second group reduces the mycotoxin to (a) less toxic metabolite(s).

Evidently, the efficacy of these feed additives has to be proven. The European Food Safety Authority (EFSA) states that toxicokinetic studies have to be performed for the investigation of the bioavailability and the absorption/excretion of mycotoxins in combination with detoxifying agents. Trials have to be performed with respect to the recommended maximum levels for the toxins in animal feed. For each mycotoxin different end-points have been proposed to test the efficacy of mycotoxin detoxifying agents, except for the emerging toxin T-2. For DON and ZEN for example, the blood serum or plasma concentration levels of the main toxin and its metabolite(s) have to be determined (Anonymous, 2009). For DON, this is deepoxy-deoxynivalenol (DOM-1) as main metabolite, and for ZEN these are α-zearalenol (α-ZEL) and β-zearalenol (β-ZEL).

However, little or no information is available on the absolute oral bioavailability and the toxicokinetic parameters of mycotoxins in animals, especially for poultry. To the best of our knowledge there are only two reported studies: one on the excretion of ^{14}C-labeled DON in white leghorns chickens after a single oral bolus (Prelusky et al., 1986) and another study about the excretion and tissue distribution of tritium-labeled T-2 toxin in chicks (Chi et al., 1978). These studies only observed the presence of radio-labeled mycotoxins in function of the time, but no toxicokinetic parameters were calculated and thus no data are available concerning the absolute oral bioavailability of DON, T-2 and ZEN in broiler chickens. For this reason, toxicokinetic trials for DON, T-2 and ZEN were performed with broilers in a two-way cross-over design. The mycotoxins were administered by oral and intravenous bolus, in order to calculate the absolute oral bioavailability of the mycotoxins. The calculated toxicokinetic parameters can be used in further research to investigate the efficacy of mycotoxin detoxifying agents, e.g. to study their effect on the absorption and bioavailability of mycotoxins. Moreover, knowledge on the kinetic parameters of toxins is essential to understand their mode of action and to evaluate animal and human health risks. In addition, we also quantified the plasma levels of the metabolites DOM-1, α-ZEL and β-ZEL as proposed by EFSA, after administration of the recommended maximum levels for mycotoxins in animal feed.

MATERIALS AND METHODS

Animals

Twenty-four 3-week-old broiler chickens (Ross 308, local commercial poultry farm) were housed according to the requirements of the European Union (Anonymous, 2007). The animals were kept in three groups, each consisting of 8 animals (4 ♂ and 4 ♀), in floor pens with wood shavings. The applied light cycle was the same as on the commercial farm (20 hours light/ 4 hours dark). The experiments started after 1 week of acclimatization allowing the animals to adapt to the environment. During the whole experiment the animals were fed with blank feed *ad libitum*. This feed was commercially available broiler feed (Bromix Plus®) obtained from Versele-Laga (Deinze, Belgium). It was considered as blank feed after analysis for the presence of mycotoxins by a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Fytolab, Zwijnaarde, Belgium). Following mycotoxins were analyzed in the feed with their respective limit of detection: aflatoxin B1 (1 µg/kg), aflatoxin B2 (1 µg/kg), aflatoxin G1 (1 µg/kg), aflatoxin G2 (1 µg/kg), cytohalasin E (2 µg/kg), DON (100 µg/kg), 3-acetyl-DON (100 µg/kg), fumonisin B1 (50 µg/kg), fumonisin B2 (50 µg/kg), HT-2 (50 µg/kg), T-2 (20 µg/kg), nivalenol (100 µg/kg), ochratoxin A (0.5 µg/kg), ZEN (10 µg/kg), α-zearalenol (20 µg/kg), β-zearalenol (20 µg/kg). None of the mentioned toxins were detected above the limit of detection. The animal experiments were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2010/176). After the experiment, the animals were humanely euthanized with an intravenous injection of T61® containing embutramide, mebenzonium iodide and tetracain hydrochloride (Intervet, Brussels, Belgium).

Mycotoxins

The mycotoxins DON, T-2 and ZEN used for the toxicokinetic studies were purchased in powder form from Fermentek LTD (Jerusalem, Israel). Mycotoxins were dissolved in ethanol *pro analysis* and afterwards diluted in water of HPLC quality (1/8 for DON; 1/20 for T-2; 1/2 for ZEN, v/v) in order to obtain stock solutions (DON: 1 mg/ml, T-2: 50 µg/ml, ZEN: 0.5 mg/ml), which were used for dosing the broiler chickens.

The administered dose of DON was based on the recommended maximum concentrations in poultry feed (5 mg/kg feed) (Anonymous, 2006). A dose of 2 mg/kg was applied for ZEN, according to the maximum level for cereals and cereal products with the exception of maize by-products (Anonymous, 2006). For T-2, the results of the analysis of 67 contaminated feed samples by Monbaliu et al. (2010) were taken into account. During this trial the highest reported level of T-2 was 112 µg/kg (Monbaliu et al., 2010). Based on these results, the dose for T-2 toxin was set at 0.1 mg/kg feed. In addition, the daily feed intake of broilers was taken into account (150 g/kg BW). Thus, the administered doses for DON, T-2 and ZEN were respectively, 0.75 mg/kg BW, 0.02 mg/kg BW and 0.3 mg/kg BW (Table 1).

All standards used for the analytical experiments (DON, deepoxy-deoxynivalenol (DOM-1), T-2 toxin (T-2), HT-2 toxin (HT-2), ZEN, α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL) and zearalanone (ZAN)) were obtained from Sigma-Aldrich (Bornem, Belgium) and stored at ≤ -15°C. The internal standards (IS) $^{13}C_{15}$-DON, $^{13}C_{24}$-T-2 and $^{13}C_{18}$-ZEN were purchased as 25 µg/ml solutions in acetonitrile (ACN) from Biopure (Tulln, Austria) and stored at ≤ -15°C. Stock solutions of 1 mg/ml were prepared for each compound in methanol, except for T-2 (0.5 mg/ml in methanol) and for DON (1 mg/ml in acetonitrile). The standard of DOM-1 was purchased as a solution of 50 µg/ml in acetonitrile. Per group of compounds (i.e. DON and DOM-1, T-2 and HT-2, ZEN and its metabolites), combined working solutions were prepared by diluting appropriate volumes of the stock solutions with ACN/water (50/50, v/v). These working solutions were used to prepare matrix-matched calibrators and quality control samples in plasma. For the internal standards, working solutions of 1 µg/ml in ACN/water (50/50, v/v) were prepared.

Experimental protocol

The twenty-four animals were divided into three groups of eight animals, one group for each mycotoxin. The animal experiments were performed in a two-way cross-over design and mycotoxins were administered intravenously (IV) or orally (PO). In brief, for each mycotoxin, four animals received an oral bolus and four animals received the mycotoxin intravenously. After a wash-out period was applied (Table 1), the animals that previously received an oral

bolus, received at that time an intravenous bolus and vice versa. For the intravenous injection in the wing vein a 25G needle (0.5 x 16 mm, Becton Dickinson, Temse, Belgium) was used. The mycotoxin was administered orally using a crop tube. Due to the high concentration of lipids in chicken plasma after feeding (Ferlazzo et al., 2011), the obtained plasma is more viscous resulting in lower workability. Therefore, feed was withdrawn from 6 hours before, until 4 hours post-administration (p.a.) of the toxins. Following the toxin administration, blood samples were taken from the leg vein at different time points: at 0 (just before administration) and at several time points post-administration depending on the administered mycotoxin (Table 1). Blood was collected in heparinized tubes. Samples were centrifuged at 2851 g and 4°C for 10 minutes and plasma was stored at ≤ -15°C until further analysis.

Table 1. Design of the animal experiment.

Mycotoxin	Dose (mg/kg BW)	Wash-out period (days)	Time points of blood sampling
Deoxynivalenol	0.75	2	0, 10, 20, 30, 40 and 50 min, 1, 1.5, 2, 3, 4, 6 and 8h
T-2 toxin	0.02	2	0, 2, 5, 10, 15, 20, 30, 40 and 50 min, 1h
Zearalenone	0.30	7	0, 10, 20, 30, 40 and 50 min, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 30, 36, 48 and 72h

Plasma analysis

Quantification of the plasma levels of DON and its major metabolite DOM-1 was performed using an in-house developed and validated LC-MS/MS method (De Baere et al., 2011), while little modifications were applied for the sample preparation. Briefly, 250 µl of plasma were spiked with 12.5 µl of the IS working solution ($^{13}C_{15}$-DON, 1 µg/ml), followed by the addition of 750 µl of acetonitrile. Afterwards, the samples were vortexed (15 sec) and centrifuged (10 min, 7826 g, 4°C). The supernatant was evaporated using a gentle nitrogen stream (40 ± 5°C). The dry residue was reconstituted in 200 µl of a 95/5 (v/v) mixture of mobile phase A/B. The mobile phase A consisted of 0.1 % glacial acetic acid (VWR, Leuven, Belgium) in

water of UPLC quality. Mobile phase B consisted of methanol of UPLC quality. After vortex mixing and filtering through a Millex® GV-PVDF filter (0.22 µm), the sample was transferred to an autosampler vial, and an aliquot (10 µl) was injected onto the LC-MS/MS instrument.

The same sample preparation procedure was applied for the analysis of T-2 and its metabolite HT-2. $^{13}C_{24}$-T-2 was used as the IS. After the evaporation of the supernatant, the dry residue was reconstituted in 200 µl of a 70/30 (v/v) mixture of mobile phase A/B with mobile phase A consisting of 5 mM ammonium acetate (VWR, Leuven, Belgium) in water/methanol/acetic acid (94/5/1, v/v/v) and mobile phase B of 5 mM ammonium acetate in methanol/water/acetic acid (97/2/1, v/v/v). A 10-µl aliquot was injected onto the LC-MS/MS instrument (De Baere et al., 2011).

The plasma concentrations of ZEN and its metabolites α-ZEL, β-ZEL, α-ZAL, β-ZAL and ZAN were determined using an in-house validated LC-MS/MS method. To 250 µl of plasma were added 12.5 µl of the IS working solution ($^{13}C_{18}$-ZEN, 1 µg/ml) and 750 µl of acetonitrile, as for DON and T-2 analysis. The dry residue was then reconstituted in 200 µl of a mixture of water/methanol (85/15, v/v). After vortex mixing, the sample was passed through a Millex® GV-PVDF filter and transferred to an autosampler vial. An aliquot (10 µl) was injected onto the LC-MS/MS instrument.

The LC-MS/MS instrument consisted of a Surveyor® type MS pump Plus and Autosampler Plus HPLC in combination with a TSQ® Quantum Ultra™ mass spectrometer (Thermo Fisher Scientific, Breda, The Netherlands). Chromatographic separation of the analytes of interest was achieved on a Hypersil Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) using 0.01 % acetic acid in water (A) and acetonitrile (B) as mobile phase. A gradient elution was performed at a flow rate of 300 µl/min and a column temperature of 45°C. The mass spectrometer was operated in the selected reaction monitoring (SRM) mode and for each compound the two most intense precursor ion > product ion transitions were selected for quantification and qualification, respectively. The following transitions were used: DON: m/z 355.1 > 265.2 and 355.1 > 295.1, DOM-1: m/z 339.0 > 249.0 and 339.0 > 59.1 and $^{13}C_{15}$-DON: m/z 370.1 > 279.2 and 370.1 > 310.1; T-2: m/z 484.1 > 215.1 and 484.1 > 185.1, HT-2: m/z 442.0 > 263.1 and 442.0 > 215.1 and $^{13}C_{24}$-T-2: m/z 508.2 > 229.1 and 508.2 > 198.1; ZEN: m/z 317.3 > 131.0 and 317.3 > 175.0, α-ZEL: m/z 319.2 > 275.1 and 319.2 > 301.1, β-ZEL:

m/z 319.2 > 301.1 and 319.2 > 275.1, ZAN: m/z 319.2 > 205.0 and 319.2 > 275.1, α-ZAL: 321.2 > 277.1 and 321.2 > 303.1, β-ZAL: m/z 321.2 > 277.1 and 321.2 > 303.1 and for $^{13}C_{18}$-ZEN: 335.3 > 185.1 and 335.3 > 169.1.

The limit of quantification (LOQ) of DON, DOM-1, T-2 and HT-2 ranged from 1 to 2.5 ng/ml (De Baere et al., 2011), while for ZEN and its metabolites LOQ values between 1 to 5 ng/ml were obtained. All values below the LOQ were not included in the plasma concentration-time curves and the toxicokinetic analysis. Limits of detection (LOD) ranged from 0.01 to 0.63 ng/ml for DON, T-2 and their metabolites (De Baere et al., 2011) and for ZEN and its metabolites from 0.005 to 0.07 ng/ml.

Toxicokinetic analysis

The toxicokinetic parameters were analyzed using the software program WinNonlin, Version 6.2.0 (Phoenix, Pharsight corp., USA). A non-compartmental model was used to determine the area under the plasma concentration-time curve ($AUC_{0\rightarrow\infty}$), elimination rate constant (k_{el}), half-life of elimination ($t_{1/2el}$), volume of distribution (Vd), clearance (Cl), maximum plasma concentration (C_{max}) and time to maximum plasma concentration (T_{max}). The absolute oral bioavailability (F) was calculated from the following equation: $F(\%) = \frac{AUC0\rightarrow\infty OR}{AUC0\rightarrow\infty IV}$ x 100.

RESULTS

Toxicokinetic study of deoxynivalenol

The plasma concentration-time profiles of DON, after a single oral or intravenous bolus, are shown in Figure 1. No plasma concentrations of the main metabolite of DON, DOM-1, could be detected above the LOQ. Moreover, from 2 hours p.a. no levels above LOQ could be detected for DON. Main toxicokinetic parameters of DON are summarized in Table 2. The AUC after IV administration was much higher than after oral administration, which correlates with a low absolute oral bioavailability (F). F was determined to be 19.3 ± 7.42%.

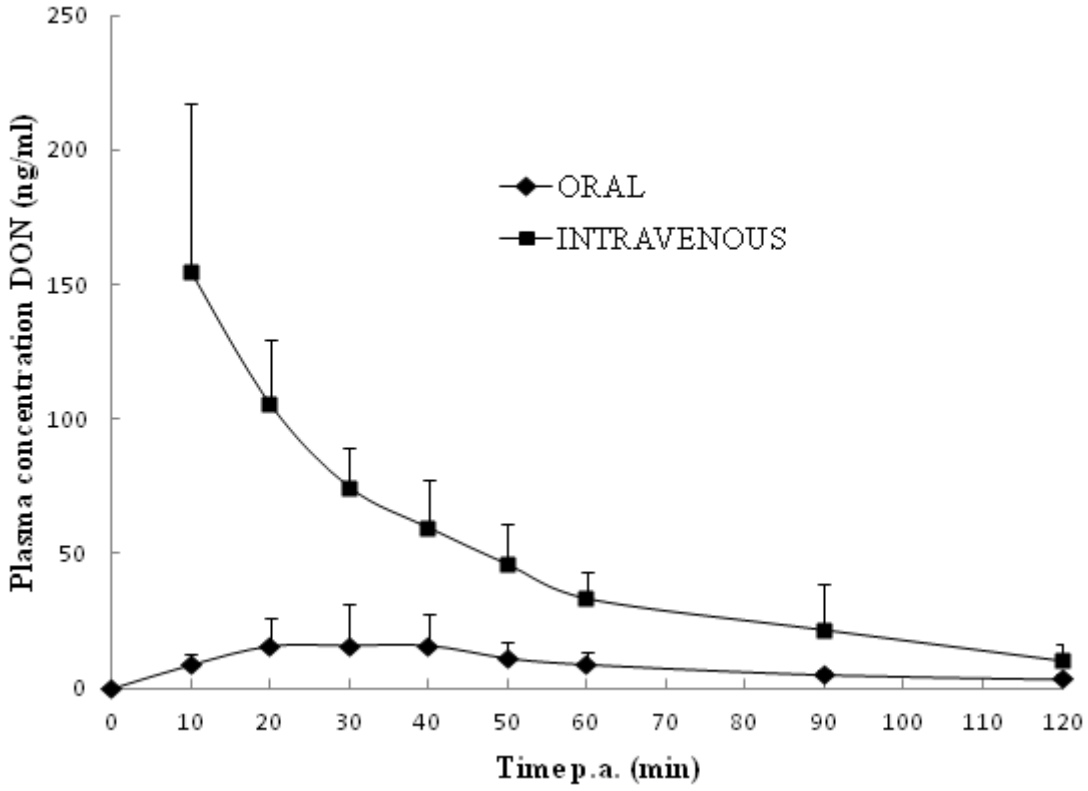

Figure 1. Plasma concentration-time profile of DON after a single oral or intravenous administration of DON (0.75 mg/kg BW) to eight broiler chickens. Results are presented as mean values + SD.

Toxicokinetic study of T-2 toxin

After a single oral bolus of T-2, no plasma levels above the LOQ could be observed for T-2 and its metabolite HT-2. The plots of the plasma concentration-time curve after an intravenous bolus of T-2 are depicted in Figure 2. Plasma concentrations of T-2 were only detected until 20 min and of the main metabolite HT-2 only 2 min post intravenous administration with a mean concentration of 3.9 ± 0.06 ng/ml. Five minutes post-administration only half of the animals showed plasma levels of HT-2 above the LOQ (LOQ = 2.5 ng/ml). Mean plasma concentrations of these four animals were 1.3 ± 0.28 ng/ml. Thereafter, no plasma concentrations of HT-2 above the limit of quantification could be observed (data not shown). Toxicokinetic parameters of T-2 are shown in Table 2.

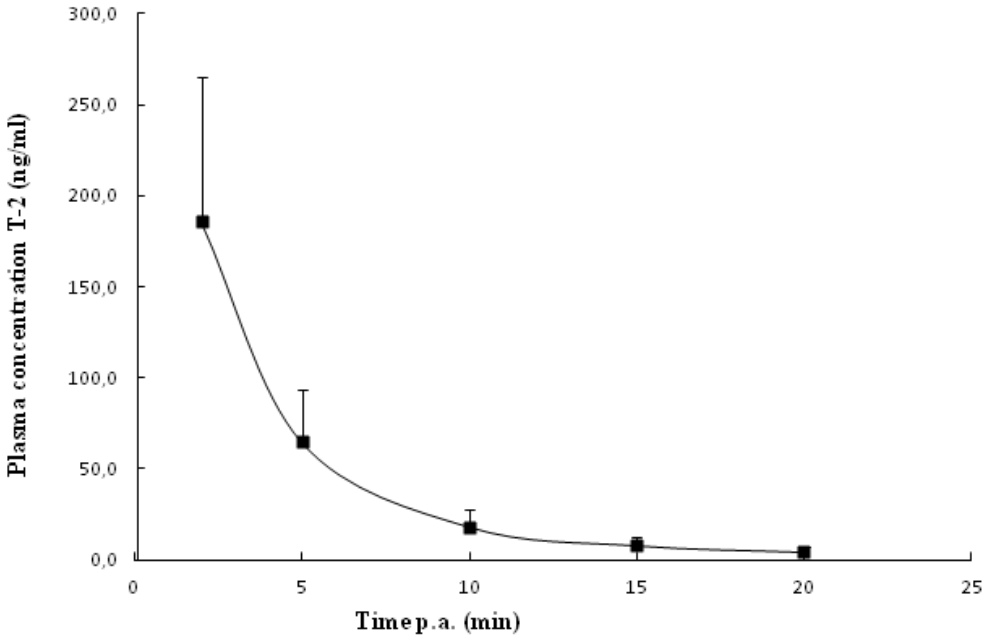

Figure 2. Plasma concentration-time profile of T-2 after a single intravenous administration of T-2 (0.02 mg/kg BW) to eight broiler chickens. Results are presented as mean values + SD.

Toxicokinetic study of zearalenone

After a single oral bolus of zearalenone (0.3 mg/kg BW) no plasma concentrations above the LOQ could be detected for ZEN and its metabolites. The plasma concentration-time profile after a single bolus of ZEN given intravenously is shown in Figure 3. No plasma levels of the metabolites could be detected (<LOD), except for α-ZEL. However, the detected concentrations of α-ZEL were below the limit of quantification of 5 ng/ml.

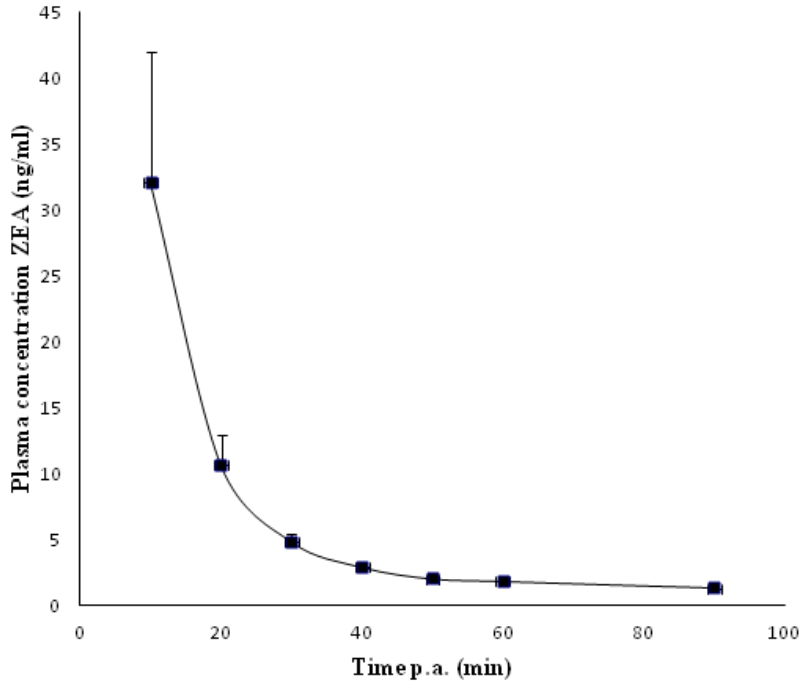

Figure 3. Plasma concentration-time profile of ZEN after a single intravenous administration of ZEN (0.3 mg/kg BW) to eight broiler chickens. Results are presented as mean values + SD.

Table 2. Main toxicokinetic parameters of DON, T-2 and ZEN after oral or intravenous administration of the toxin in broiler chickens (n=8). Results are presented as mean values ± SD.

Toxicokinetic parameters	DON (PO)	DON (IV)	T-2 (IV)	ZEN (IV)
AUC0-inf (ng.min/ml)	1281 ± 481.7	6198 ± 983.4	889 ± 363.2	626 ± 60.0
Cmax (ng/ml)	26.1 ± 14.64	167.9 ± 56.76	185.4 ± 79.79	32.1 ± 9.91
Tmax (min)	35.0 ± 8.37	-	-	-
Cl/F (PO) or Cl (IV) (L/min.kg)	0.65 ± 0.217	0.12 ± 0.024	0.03 ± 0.008	0.48 ± 0.050
Vd/F (PO) or Vd (IV) (L/kg)	35.72 ± 15.563	4.99 ± 1.168	0.14 ± 0.045	22.26 ± 15.148
kel (1/min)	0.02 ± 0.008	0.03 ± 0.006	0.19 ± 0.061	0.03 ± 0.021
T 1/2el (min)	38.2 ± 11.19	27.9 ± 6.89	3.9 ± 0.98	31.8 ± 20.60
F (%)	19.3 ± 7.42	-	-	-

(AUC, area under the curve; Cmax, maximum plasma concentration; Tmax, time to maximum plasma concentration; Cl, clearance; F, bioavailability; Vd, volume of distribution; kel, elimination rate constant; T1/2el, half-life of elimination)

DISCUSSION

Recently, new bolus models have been developed at our department for the *in vivo* efficacy testing of mycotoxin detoxifying agents (Devreese et al., 2012). The bolus models have been applied for the mycotoxin DON in broilers, but not for other mycotoxins such as T-2 toxin and ZEN. Little or no information is available to this day about the absolute oral bioavailability and toxicokinetic parameters of DON, T-2 and ZEN in broilers. This information could also be helpful for the evaluation of the health risk caused by the different toxins in chickens compared to other animal species.

To the best of our knowledge, only Prelusky et al. (1986) reported plasma levels of DON in chickens 2-2.5 hours after administration of a single oral bolus of ^{14}C-labeled DON to White Leghorns hens. Tissue distribution and excretion of tritium-labeled T-2 toxin in chicks was reported earlier (Chi et al., 1978). However, these studies did not analyze the parent toxins and their possible metabolites separately, but only the disappearance of the radioactivity in plasma.

Toxicokinetic studies were already performed for several mycotoxins in other animal species (Goyarts and Dänicke, 2006; Shin et al., 2009). Extrapolation of these results to chickens is however not possible, as poultry possess different physiology and biotransformation of xenobiotics, and chickens revealed to be less sensitive to the risk of mycotoxicosis compared to other animal species (Devegowda and Murthy, 2005). For these reasons three separate toxicokinetic studies were performed for the mycotoxins DON, T-2 and ZEN in broiler chickens. Plasma concentrations of the toxins and their main metabolites were analyzed using validated LC-MS/MS methods. Mass spectrometry has the important advantage to be sensitive and to present an unambiguous identification of specific compounds. All the mycotoxins were administered as a standard solution, thus interaction with other mycotoxins was avoided. Moreover, animals received blank feed during one week before the start of the study which excludes the presence of baseline levels of the toxins before the start of the experiments.

The toxicokinetic study of DON revealed a low absolute oral bioavailability of 19.3 ± 7.42%. Prelusky et al. (1986) already reported a rapid transport of DON-derived radioactivity

through the alimentary tract of chickens, contributing to the poor absorption of the toxin. In sheep even a lower F has been reported (7.5%) (Prelusky et al., 1985), but in pigs the bioavailability is higher after a single oral bolus (54%) and even much higher (89%) after chronic exposure to DON (Goyarts and Dänicke, 2006). These results are in accordance with the rank order of susceptibility to the harmful effects of DON; i.e. pigs > poultry and ruminants. In the present study, no clinical signs of intoxication were observed following administration of DON in broilers. A high plasma clearance (0.12 ± 0.024 L/min.kg) might also explain the relative tolerance of poultry to DON. In pigs, after a single intravenous administration of DON, the observed clearance was 0.00381 L/min.kg, which is indeed significantly lower compared to chickens (Goyarts and Dänicke, 2006). DOM-1 is known to be produced via intestinal microbial activity rather than by the liver or other organs. However, no plasma levels of DOM-1 above the LOQ could be detected in this study, maybe due to a low absorption. The conjugated forms of DON and its metabolites were not analyzed, as the main goal of this study was to evaluate the absolute oral bioavailability of the parent toxin. Moreover, the analysis of DON alone without DON-glucuronide was reported to be a suitable biomarker for DON exposure and toxicity (Wu et al., 2007).

The results of the toxicokinetic study of T-2 revealed that no plasma levels of T-2 and HT-2 could be detected after a single oral bolus of the mycotoxin. Retention of radioactivity in liver and bile after oral administration of tritium-labeled T-2 in chickens, indicate elimination of the absorbed toxin and its metabolites in the intestinal tract through the biliary excretion system (Chi et al., 1978). However, after one intravenous bolus of the toxin, plasma levels could be observed for T-2 and its main metabolite HT-2. Twenty minutes after the single bolus IV administration, no plasma levels could be observed for T-2 anymore, which can be explained by the limited volume of distribution (0.14 ± 0.045 L/kg) and the rather high clearance of this toxin (0.03 ± 0.008 L/min.kg). This results in a rapid elimination half-life (3.9 ± 0.98 min). On the other hand, T-2 toxin is known to be rapidly metabolized to different metabolites, which are more polar (Yoshizawa et al., 1980). This is in accordance with our results: HT-2 was detected already 2 minutes after administration of T-2. No other toxicokinetic studies with T-2 in broilers are available to compare our results with. Concentrations of the glucuronide-forms of T-2 and HT-2 were not investigated during our

study, as glucuronidation of these compounds was only reported in rats and pigs until now (Wu et al., 2009).

The toxicokinetic parameters of ZEN have already been studied in different animal species, especially in pigs due to the high sensitivity to ZEN of this animal species (Malekinejad et al., 2006). In the present study, no adverse effects were observed following the administration of ZEN or its solvent (ethanol) in broilers. During our study, no plasma levels could be observed for ZEN and its metabolites after one oral bolus of 0.3 mg/kg BW in broilers. ZEN is known to be rapidly absorbed after oral administration. However, the degree of absorption is difficult to determine in animals due to its extensive biliary excretion (Zinedine et al., 2007). After administration of a single bolus of 5.8 µg ZEN/kg BW in naturally contaminated wheat to broilers, ZEN and α-ZEL could be detected, but only in 6 of the 32 plasma samples and values were around the detection limits (10 ng/ml for ZEN and 5 ng/ml for α-ZEL) (Dänicke et al., 2001). A likely explanation could be the low oral bioavailability of ZEN, as reported in rats where F equals 2.7 % after oral administration of ZEN at a dose of 8 mg/kg BW (Shin et al., 2009). In our study, the maximum recommended level of 2 mg/kg for ZEN in cereals and cereal products was respected (Anonymous, 2006). Although this concentration was probably too low to obtain plasma concentrations after a single oral bolus,it has to be noticed that no levels above 1.8 mg/kg feed were reported for ZEN in Europe in cereal grains and animal feeds (Gromadzka et al., 2008). Moreover, in the study of Monbaliu et al. (2010), where European feed samples were analyzed with LC-MS/MS, the minimum and maximum level ranged between 0.058 and 0.387 mg/kg of ZEN.

However, after one intravenous bolus of ZEN, plasma levels were observed for ZEN and α-ZEL. In broilers and pigs, α-ZEL has been reported to be the main metabolite of ZEN (Dänicke et al., 2005; Dänicke et al., 2001). These findings are in contrast with the results of an *in vitro* study performed to investigate the hepatic transformation of ZEN. In both the hepatic microsomes and the post-mitochondrial fraction ZEN was mainly transformed to β-ZEL in chickens (Malekinejad et al., 2006). In turkeys α-ZEL was also reported to be the only detectable metabolite of ZEN after 2-weeks of feeding of 800 mg ZEN/kg diet (Olsen et al., 1986). However, in ruminants β-ZEL is known to be the major metabolite of ZEN (Dong et al., 2010), which can be a possible determinant of the species differences in susceptibility for

ZEN (Malekinejad et al., 2006). Compared to other animals, chickens revealed to have a lower glucuronidation capacity for ZEN (Malekinejad et al., 2006), for this reason glucuronide-forms of ZEN and its metabolites were not analyzed.

Compared to other animals, such as pigs (Cl= 0.048 L/min.kg; Vd= 10.48 L/kg) and goats (Cl= 0.003 L/min.kg; Vd= 7.32 L/kg), broilers seem to have the highest clearance (0.48 ± 0.050 L/min.kg) and also a high volume of distribution (22.26 ± 15.148 L/kg) for ZEN (Dong et al., 2010; Dänicke et al., 2005). These results confirm the statement that the susceptibility to the estrogenic effects of ZEN cannot be explained by circulating ZEN and its metabolites (Dong et al., 2010).

From our findings, we can conclude that oral bolus models respecting the maximum recommended levels in feed to test the efficacy of mycotoxin detoxifying agents *in vivo* cannot be applied for the mycotoxins T-2 and ZEN, due to their low absolute oral bioavailability in broilers. Administration of higher doses can offer a possibility, but can lead to higher risks for acute mycotoxicosis. On the other hand, it is also clear that plasma or blood concentrations of the tested parent toxins and their main reported metabolites as stated by the EFSA cannot be used as biomarkers to test the efficacy of mycotoxin detoxifiers in broilers, when maximum recommended levels in feed are respected.

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Chapter 2.

Evaluation of different biomarkers to assess deoxynivalenol exposure and efficacy and safety testing of mycotoxin detoxifiers

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ABSTRACT

Contamination of feeds with mycotoxins is a worldwide problem and mycotoxin detoxifying agents are used to decrease their negative impact. The European Food Safety Authority (EFSA) recently stated guidelines and end-points for the efficacy testing of detoxifiers. Our study revealed that plasma concentrations of deoxynivalenol and deepoxy-deoxynivalenol were too low to assess efficacy of two commercially available mycotoxin detoxifying agents against deoxynivalenol after 3 weeks continuous feeding this mycotoxin at concentrations of 2.44±0.70 mg/kg feed and 7.54±2.20 mg/kg feed in broilers. This correlates with the poor absorption of deoxynivalenol in poultry. A safety study with two commercially available detoxifying agents and veterinary drugs showed innovative results with regard to the pharmacokinetics of two antibiotics after oral dosing in the drinking water. The plasma and kidney tissue concentrations of oxytetracycline were significantly higher in broilers receiving a biotransforming agent in the feed compared to control birds. For amoxicillin the plasma concentrations were significantly higher for broilers receiving an adsorbing agent in comparison to birds receiving the biotransforming agent, but not to the control group. Mycotoxin detoxifying agents can thus interact with the oral bioavailability of antibiotics depending on the antibiotic and detoxifying agent, with possible implications for practical use of these agents.

INTRODUCTION

Food safety is an important issue receiving a lot of scientific attention worldwide. The presence of mycotoxins, produced as secondary metabolites of toxigenic moulds, in food and feed is one of the most important concerns. Also climate changes, characterized by an increase of temperature and relative humidity have a great impact on plants and their pathogens (Ingram, 1999; Miraglia et al., 2009). Both parameters, in combination with the rising level of CO_2 in the atmosphere have an influence on the mycotoxin production. The changed production of mycotoxins is correlated mainly to the predominance of *Fusarium graminearum*, a fungus responsible for the production of zearalenone and of mycotoxins belonging to the class of trichothecenes (Paterson and Lima, 2010).

Consequences of climate changes for the food system comprise all the stages from 'farm to fork' and thus not only the pre-harvest conditions, but also other parameters are important such as quality and temperature of the grain storage facilities. Poor management can lead to microbial activity and loss of quality, for this reason Good Agricultural Practice (GAP) is essential to minimize mycotoxin development (Magan and Aldred, 2007). Mycotoxins are very resistant to all kinds of production steps, even brewing, malting and extrusion-processes are not a threat for these contaminants (Scudamore et al., 2008).

Mycotoxins exert several direct adverse effects on human and animal health, while the excretion of some mycotoxins in animal products such as milk can also contribute to the consumers' exposure (Fink-Gremmels, 2008). Apart from the health problem, mycotoxins can cause important economic losses. Their toxic properties depend on the particular mycotoxin and its dose. Deoxynivalenol (DON) for example, which is one of the most prevalent mycotoxins (Monbaliu et al., 2010), causes vomiting and diarrhea when ingested in high doses, especially in pigs. In lower concentrations it induces weight loss and feed refusal (Miller et al., 2001). Poultry on the other hand are very resistant to DON, for example concentrations above 5 mg per kg diet are necessary to cause detrimental effects (Awad et al., 2006).

One of the most recent methods to counteract mycotoxicosis in animals is the use of mycotoxin detoxifying agents in feed. These detoxifiers are added to animal feed and act in

the digestive tract of the animal. The additives can consist of different components and depending on their mode of action they are 'adsorbing' or 'biotransforming' agents. The first class is able to bind mycotoxins and to reduce their gastro-intestinal absorption, the second one degrades mycotoxins to less or non-toxic metabolite(s) (Anonymous, 2010). Research has already been performed on the efficacy of these feed additives using both *in vitro* and *in vivo* assays (Avantaggiato et al., 2004; Avantaggiato et al., 2005; Galvano et al., 2001). Testing under laboratory circumstances is difficult since the intestinal conditions are almost impossible to reproduce. Adsorption isotherms and different models like intestinal cell lines and Ussing chambers using intestinal tissues, can be used to study these detoxifiers *in vitro* (Clarke, 2009; Lemke et al., 1998). Although *in vitro* trials are a rapid screening method to choose which agents can be further tested *in vivo*, animal trials remain essential.

The set up of *in vivo* trials is an important point of discussion. The European Food Safety Authority (EFSA) has recently published a scientific opinion about the efficacy and safety testing of mycotoxin detoxifying agents and focusing on the experimental design of *in vivo* studies (Anonymous, 2010). The EFSA has also pointed out several relevant end-points for the different mycotoxins. For DON the analysis of this mycotoxin and its metabolite deepoxy-deoxynivalenol (DOM-1) in blood has been indicated as the most relevant end-point. On the other hand EFSA also pays importance to the safety testing of mycotoxin detoxifying agents. Possible interactions with respect to nutrients and/or veterinary drugs absorption should also be investigated (Anonymous, 2010).

The first aim of this present study was to test whether the guidelines published by EFSA can be applied for the *in vivo* efficacy testing of two commercially available mycotoxin detoxifying agents with respect to DON in broilers. We performed an *in vivo* trial with broilers according to these guidelines and tested the most relevant endpoints for the mycotoxin DON as stated by EFSA. A second aim of this study was to test if the two selected mycotoxin detoxifying agents can have an influence on the pharmacokinetics and tissue residues of commonly used antibiotics in poultry medicine.

MATERIALS AND METHODS

Materials

DON, used for artificial contamination of the feed, was produced with a reference strain obtained from the MUCL (Mycothèque de l'Université Catholique de Louvain, Belgium). Both antibiotics, oxytetracycline (Oxytetracycline 80%®) and amoxicillin (Amoxicilline 70%®), were obtained from Kela Veterinaria (Sint-Niklaas, Belgium). Blood was collected in heparinized tubes (Venoject®) obtained from Terumo Corp. (Tokyo, Japan). Standards used for analytical experiments (DON and DOM-1) were obtained from Sigma-Aldrich (Bornem, Belgium) and stored at ≤-15°C. The internal standard $^{13}C_{15}$-DON was purchased as a 25 µg/ml solution in acetonitrile from Biopure (Tulln, Austria). Water, methanol and acetonitrile were of HPLC grade, while ammonium acetate, ethyl acetate and glacial acetic acid were of analytical grade. All these reagents were obtained from VWR (Leuven, Belgium). Oasis® HLB solid-phase extraction (SPE) columns (60mg/3cc) were obtained from Waters (Zellik, Belgium). Millex®-GV PVDF filter units (0.22 µm) were obtained from Millipore (Brussels, Belgium).

Experimental design for Animals and Diets

Efficacy testing. Sixty-four one-day-old Ross broiler chicks, in an equal number of both sexes, were obtained from a commercial hatchery. During an acclimatization period of ten days, the animals were fed blank feed. Males and females were housed separately. Afterwards the chickens were randomly assigned to 1 of 8 dietary groups, each consisting of 8 animals (4♀ and 4♂). The animals were housed in 8 cages with a floor area of at least 1m^2. The concrete floor was covered with wood shavings. During the 3 weeks of experimental feeding the animals received different diets as illustrated in Table 1. The concentrations of the different mycotoxins as shown in Table 1 were determined by a multi-mycotoxin LC-MS/MS method (Monbaliu et al., 2010). The feed of group 3, 4 and 5 was the same feed as group 1 (blank feed) but it was artificially contaminated with DON in the laboratory. DON was produced with the reference strain MUCL 6131. This strain was grown in liquid GCY medium (glucose 10 g/L, yeast extract 1 g/L, peptone 1 g/L) together with 50 µM hydrogen peroxide (H_2O_2)

which induces an oxidative stress in the fungus that stimulates the production of DON (Audenaert et al., 2010). After 14 days of cultivation, with supplementation of H_2O_2 every 2 days, the culture was filtered and freeze-dried and mixed into the blank feed to have a final DON concentration of 2.44±0.70 mg/kg feed, thus below the maximum level of 5 mg/kg as recommended by the EFSA (Anonymous, 2006). For the last three groups (6, 7 and 8) a naturally contaminated feed was prepared out of contaminated maize. The concentration of DON in this feed (7.54±2.20 mg/kg feed) was higher than the recommended maximum level (Anonymous, 2006).

Two different kinds of commercially available mycotoxin detoxifying agents were supplemented to the diets. The adsorbing agent was a smectite type clay mineral (illite-ambrosite). The biotransforming agent, as classified by the EFSA (2010), was a bentonite-montmorillonite upgraded with a yeast. The yeast is claimed to be able to detoxify DON *in vitro* by opening the C-12,13 epoxide ring. Group 2 received blank feed supplemented with the adsorbing agent to test possible effects of the detoxifier. Water and feed were given *ad libitum* to all broilers. A light regime of 20 hours light and 4 hours darkness was applied. The experimental procedures conducted with the chickens were in accordance with the European guidelines for the care and use of animals for research purposes. The protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University) (EC 2010/064 and EC 2010/076).

Safety testing. Forty-eight one-day-old Ross broiler chicks, in an equal number of both sexes, were obtained from a commercial strain. During an acclimatization period of 7 days, the animals were fed blank feed. Males and females were housed separately. Afterwards the chickens were randomly assigned to 1 of 6 dietary groups, each consisting of 8 animals (4♀ and 4♂) and received experimental diets for 3 weeks. The animals were housed in 6 cages with a floor area of at least $1m^2$ covered with wood shavings. The composition of the dietary groups is shown in Table 2 and 3. The blank feed did not contain any mycotoxin above the limit of detection of a multi-mycotoxin LC-MS/MS method analyzing 23 mycotoxins (Monbaliu et al., 2010). This uncontaminated feed was useful for this study as an interaction with natural occurring mycotoxins had to be avoided. Both mycotoxin detoxifying agents, the same as used during the efficacy study, were added in a dosage of 1.5 kg/tonne feed.

Water and feed were given *ad libitum* to the broilers. A light regime of 20 hours light and 4 hours darkness was applied. From day 28 onwards, two different antibiotics, frequently used in poultry industry against bacterial diseases, were administered. Oxytetracycline is a broad-spectrum antibiotic that interacts with the ability of bacteria to produce proteins. On the other hand amoxicillin, a moderate-spectrum antibiotic with a bacteriolytic function, was tested. The antibiotics were administered to the animals as stated in Table 2 for oxytracycline and in Table 3 for amoxicillin. For the oral bolus study on day 28, the antibiotics were dissolved in tap water and administered directly in the crop with a tube, in order to conduct a pharmacokinetic study. Therefore blood was collected from the leg vein in heparinized tubes at following time points: 0' (before administration) and 20', 40', 60', 90', 2h, 2.5h, 3h, 5h, 8h, 12h p.a. for oxytetracycline and 0' (before administration), 5', 10', 20', 30', 40', 50', 1h, 1h15', 1h30', 1h45', 2h p.a. for amoxicillin. The blood samples were immediately centrifuged at 3000 rpm for 10 minutes at 4°C and plasma was stored at -70°C until analysis. Next, the antibiotics were added to the drinking water during 5 days for amoxicillin and 7 days for oxytetracycline, as stated by the leaflet. To be sure that every animal received the required dose, an extra oral bolus at half the dosage was administered every morning. On day 35 of the experiment with oxytetracycline, the animals were euthanized 16 hours after withdrawal of the antibiotic by cervical dislocation and liver and kidneys were collected. For amoxicillin, the animals were euthanized on day 33, 12 hours after the last antibiotic administration and the same organs as described for oxytetracycline were collected. The concentration of both antibiotics in plasma and tissues was determined by validated LC-MS/MS methods (based on Reyns et al., 2006; Reyns et al., 2008 for amoxicillin, based on Cherlet et al., 2003; Cherlet et al., 2006 for oxytetracycline). Plasma pharmacokinetic parameters such as area under the plasma concentration-time curve ($AUC_{0->\infty}$ and $AUC_{0->t}$), maximum plasma concentration (C_{max}), time to reach maximum plasma concentration (T_{max}) and elimination half-life ($T_{1/2el}$) were calculated using WinNonlin 6.2.0® (Pharsight Corporation, Mountain View, Ca, USA). The protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University) (EC 2010/063 and EC 2011/002).

Table 1. Composition of 8 dietary groups for the efficacy testing[1].

Group[2]	DON[3]	NIV[3]	3-aDON[3]	15-aDON[3]	FB1[3]	FB2[3]	FB3[3]	Detoxifying agent
1	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	No
2	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	adsorbing (1.5 kg/tonne)
3	2.439±0.70	0.086±0.030	0.571±0.22	2.051±0.80	<LOD	<LOD	<LOD	No
4	2.439±0.70	0.086±0.030	0.571±0.22	2.051±0.80	<LOD	<LOD	<LOD	adsorbing (1.5 kg/tonne)
5	2.439±0.70	0.086±0.030	0.571±0.22	2.051±0.80	<LOD	<LOD	<LOD	biotransforming (1.5 kg/tonne)
6	7.540±2.20	<LOD	1.481±0.57	<LOD	0.700±0.08	0.201±0.02	0.207±0.08	No
7	7.540±2.20	<LOD	1.481±0.57	<LOD	0.700±0.08	0.201±0.02	0.207±0.08	adsorbing (1.5 kg/tonne)
8	7.540±2.20	<LOD	1.481±0.57	<LOD	0.700±0.08	0.201±0.02	0.207±0.08	biotransforming (1.5 kg/tonne)

[1] Mycotoxin levels (mg/kg feed) in the diets fed to the broilers for three weeks after the acclimatization period.

[2] Group 3 to 5 received artificially DON-contaminated feed. Group 6 to 8 received naturally contaminated feed

[3] DON=deoxynivalenol (LOD = 11.09 µg/kg) , NIV= nivalenol (LOD = 66.26 µg/kg), 3-aDON= 3-acetyldeoxynivalenol (LOD = 8.96 µg/kg), 15-aDON= 15-acetyldeoxynivalenol (LOD = 5.62 µg/kg), FB1= fumonisin B1 (LOD = 58.24 µg/kg), FB2= fumonisin B2 (LOD = 44.57 µg/kg), FB3= fumonisin B3 (LOD = 42.40 µg/kg)

Table 2. Timetable of the critical events of the bird experiment with oxytetracycline.

Time	d 8 – d 35	d 28	d 29-d 33	d 34	d 35
8 a.m.	Administration of the feed to 3 different dietary groups: - Blank feed (n=8) - Blank feed supplemented with an adsorbing agent (n=8) - Blank feed supplemented with a biotransforming agent (n=8)	Oral bolus (100 mg/kg BW) followed by a kinetic study during 24 h	Oral bolus (50 mg/kg BW) Oxytetracycline in water (100 mg/kg BW)	Oral bolus (50 mg/kg BW) Refreshing of the medicated water (100 mg/kg BW)	Euthanasia 16 hours after withdrawal of medicated water
8 p.m.			Refreshing of the medicated water (100 mg/kg BW)	Unmedicated water	

BW= body weight; d= day

Table 3. Timetable of the critical events of the bird experiment with amoxicillin.

Time	d 8-d 33	d 28	d 29-d 31	d 32	d 33
8 a.m.	Administration of the feed to 3 different dietary groups: -Blank feed (n=8) -Blank feed supplemented with an adsorbing agent (n=8) -Blank feed supplemented with a biotransforming agent (n=8)	Oral bolus (20 mg/kg BW) followed by a kinetic study during 2 hours	Oral bolus (10 mg/kg BW) Refreshing of the medicated water (20 mg/kg BW)	Oral bolus (10 mg/kg BW) Refreshing of the medicated water (20 mg/kg BW)	Euthanasia 12 hours after withdrawal of medicated water
8 p.m.		Amoxicillin in water (20 mg/kg BW)	Refreshing of the medicated water (20 mg/kg BW)	Unmedicated water	

Experimental Parameters measured during the Efficacy Testing Study

Body weight (BW), Feed Consumption and Live Weight Gain. The chickens were weighed individually three times a week and feed consumption for each group was measured daily during the whole experiment. Live weight gain was calculated according to the formula: (BW d32-BW d11)/n, with n equal to the number of days of feeding the experimental diet.

Analysis of Mycotoxins in Plasma, Bile and Tissues. After the first week (day 18) of feeding the experimental diets, blood was taken from the leg vein in heparinized tubes. For animal welfare, it was not appropriate to take enough blood of all the birds to perform the LC-MS/MS analysis for DON and DOM-1 for this particular time point, so the blood of 2 times 4 birds was pooled to analyze plasma concentrations of DON and DOM-1 for each group. The second week (day 25) blood was taken from the leg vein of all the birds, and plasma was separated after centrifugation at 2,095 x *g* at 4°C for 10 minutes. The birds were euthanized after the third week (day 32). Blood, liver and kidneys were collected. The bile fluid was collected from each bird and pooled per group. All samples were frozen at ≤ -20°C until assayed. Prior to analysis, the tissue samples were minced and homogenized using a Moulinette mixer (Moulinex, Paris, France). The concentration of DON and deepoxy-deoxynivalenol (DOM-1) was determined in plasma and bile by a validated LC-MS/MS method (De Baere et al., 2011).

For the quantitative determination of DON and DOM-1 in liver and kidney samples the method of De Baere et al. (2011) was used with minor modifications: 112.5 µL of the internal standard working solution ($^{13}C_{15}$-DON, 1 µg/mL) was added to 2.0 g of tissue sample. After vortex mixing, the sample was left at room temperature for 5 min. Thereafter, 5 mL of a water/acetonitrile mixture (10/90, v/v) was added, followed by a vortex mixing step (15 sec). The sample was extracted on a homemade rotary apparatus for 20 min, followed by a centrifugation step (10 min, 2,095 x *g*). The supernatant was transferred to another extraction tube and 8 mL of a solution of 7 % acetic acid in ethyl acetate was added. After vortex mixing, the sample was extracted for 20 min, followed by a 10-min centrifugation step (2,095 x *g*). The organic phase was transferred to another extraction tube and evaporated using a gentle stream of N_2 (~ 45°C). The dry residue was reconstituted in 1 mL of a water/methanol (90/10, v/v) solution and vortex mixed for 15 sec. The sample was

further purified using an Oasis® HLB column (same procedure as for plasma and bile analysis). The dry residue was reconstituted in 150 µL of a 70/30 (v/v) mixture of mobile phase A/B (A: 5 mM ammonium acetate in water/methanol/acetic acid (94/5/1, v/v/v); B: 5mM ammonium acetate in methanol/water/acetic acid (97/2/1, v/v/v)) and filtered through a Millex®-GV PVDF filter unit (0.22 µm) (Millipore, Brussels, Belgium). An aliquot (10 µL) was injected onto the LC-MS/MS instrument. The HPLC settings were as followed: gradient elution: 0-2 min (70% A, 30% B), 6 min (linear gradient to 30% A), 6-14 min (30% A, 70% B), 15 min (linear gradient to 70% A), 15-20 min (70% A, 30% B), flow-rate: 200 µL/min, column temperature: 35°C, column oven temperature: 5°C. The LC-MS/MS instrument and the MS/MS conditions were the same as for plasma and bile analysis (De Baere et al., 2011).

Statistical Analysis

The data were analysed using ANOVA (SPSS® 17.0 software for Windows, IBM, USA) to address the significance of difference between the mean values, with significance level set at $p<0.05$.

RESULTS

Efficacy Testing

Zootechnical parameters: Body Weight, Feed Consumption and Live Weight Gain. Diets with(out) different concentrations of DON, with or without the supplementation of detoxifying agents, had no significant effect on the body weight and live weight gain of broilers after three weeks of feeding ($p>0.05$). The feed intake was also not affected ($p>0.05$).

EFSA end-points: DON and DOM-1 in Plasma. Plasma levels were measured above the limit of quantification (LOQ = 1.25 ng/mL) in the three groups which received feed naturally contaminated at a level of 7.54±2.20 mg DON/kg feed. This means that plasma concentrations could not be detected during feeding a contamination level of 2.44±0.70 mg DON/kg feed. The plasma levels of the animals that received naturally contaminated feed with or without supplementation of detoxifying agents are shown in Figure 1. For group 6 (naturally contaminated feed without supplementation) a trend was observed: the plasma concentrations were higher after one week of feeding than after two weeks. At the end of the experiment, after 3 weeks of feeding, even no plasma concentrations were detected for this group. For group 7, which received the same feed but with an adsorbing agent, the plasma concentrations were much higher than in group 6, but the same decreasing trend was observed and also after three weeks of feeding no plasma levels could be detected. The group supplemented with a biotransforming agent (group 8) also reached higher plasma concentrations than group 6, but the decreasing trend was not so obvious for this group within the first two weeks of feeding. After three weeks of feeding also for group 8 no plasma concentrations could be detected. The metabolite DOM-1 was also analyzed in the plasma samples, but no levels above the LOQ (LOQ = 1.25 ng/mL) could be measured in any of the groups.

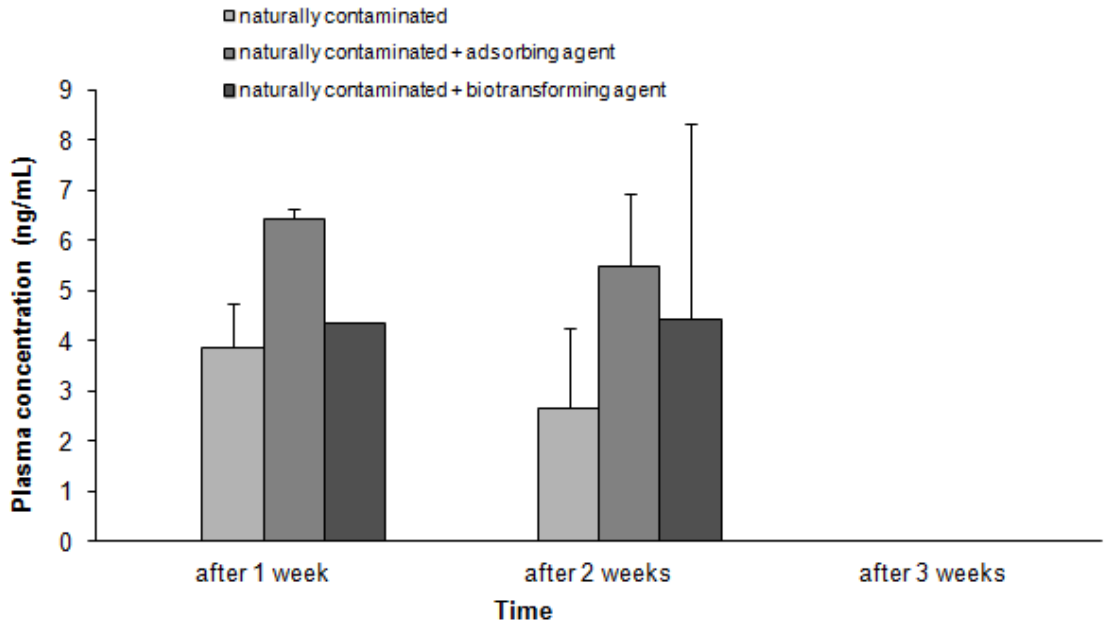

Figure 1. Plasma concentrations of deoxynivalenol (DON) in broilers after 1, 2 and 3 weeks of feeding naturally contaminated feed with DON in a concentration higher than the maximum guidance level of 5 ppm. The effect of the supplementation of an adsorbing or a biotransforming agent was evaluated. Results are presented as mean values + SD.

DON and DOM-1 in Tissues and Bile Fluid. No residues above the LOQ (LOQ=1.25 ng/g) could be detected in the liver and kidneys of the animals of the 8 different groups. The results of the analyses of bile fluid are shown in Table 4. DON was only detected in the bile fluid of the three groups that received naturally contaminated feed containing 7.54±2.20 mg DON/ kg feed. The metabolite DOM-1 was detected in all the groups that received contaminated feed. However, the concentration of DOM-1 in the groups that received artificially contaminated feed (group 3, 4 and 5) was significantly lower than the three groups fed naturally contaminated feed (group 6, 7 and 8) ($p<0.05$). Supplementation of a detoxifying agent did not have a significant influence on the concentration in the bile fluid of any measured analytes ($p>0.05$).

Table 4. Concentration of DON and DOM-1 in the pooled bile fluid of broilers (n= 8/ group) after three weeks of feeding (un)contaminated feed with DON.

	DON (ng/mL)	DOM-1 (ng/mL)
Group 1 (control)	ND	ND
Group 2 (control + adsorbing agent)	ND	ND
Group 3 (artificial DON contaminated)	ND	10.0
Group 4 (artificial DON contaminated + adsorbing agent)	ND	12.0
Group 5 (artificial DON contaminated + biotransforming agent)	ND	11.5
Group 6 (naturally contaminated)	7.5	51.6
Group 7 (naturally contaminated + adsorbing agent)	8.6	57.4
Group 8 (naturally contaminated + biotransforming agent)	5.7	52.7

(ND= not detected, LOQ DON= 1 ng/mL, LOQ DOM-1= 2.5 ng/mL)

Safety Testing

Influence of two different Detoxifying Agents on the Pharmacokinetics of oxytetracycline and amoxicillin. The plasma concentration-time profiles for oxytetracycline in the control and two detoxifying agents groups are shown in Figure 2. Remarkably, the area under the curve ($AUC_{0\rightarrow 12h}$) is significantly higher for the group fed a diet with a biotransforming agent (32.70 h.µg/mL) compared to the group without detoxifying agent (18.12 h.µg/mL) and the group fed an adsorbing agent (24.72 h.µg/mL) ($p<0.05$). The C_{max} of the group with the biotransforming agent (5.49 µg/mL) was also significantly higher than for the adsorbing agent and the control group (2.37 µg/mL and 3.04 µg/mL, respectively). No significant differences in T_{max} ($p=0.114$) and $T_{1/2el}$ ($p=0.305$) were noticed between the groups.

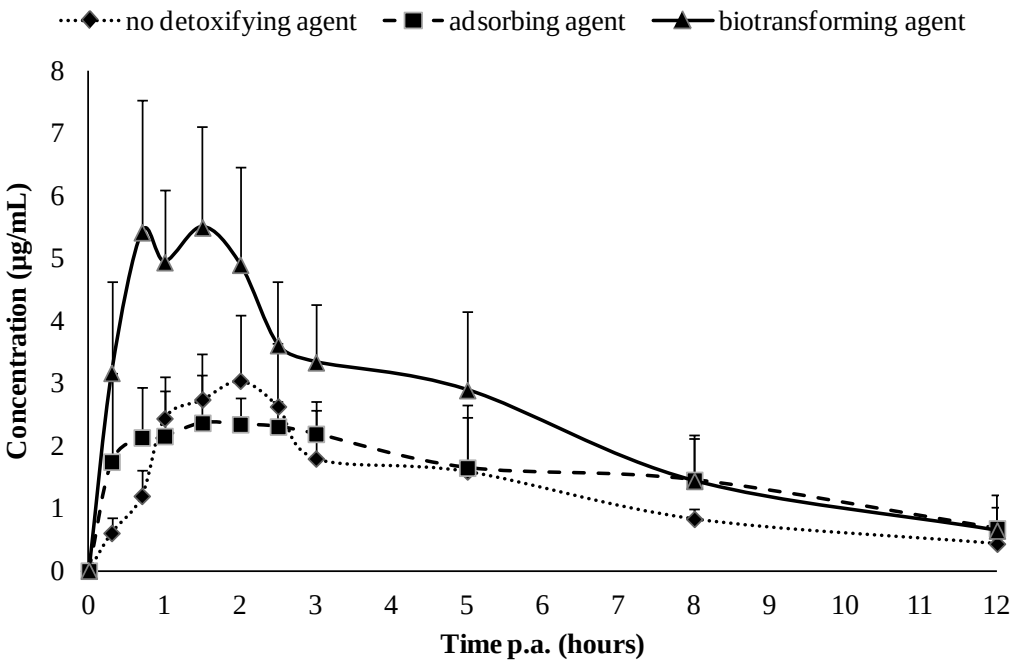

Figure 2. Effects of 3 weeks feeding of detoxifying agents on the plasma concentration versus time profile of oxytetracycline, administered as a single oral bolus of 100 mg/kg BW in broilers (n=8, mean + SD).

Results for the antibiotic amoxicillin are presented in Figure 3. For this antibiotic, the group that received the adsorbing agent reached higher plasma concentrations compared to the other groups. The area under the curve ($AUC_{0\rightarrow 2h}$) for the group with the adsorbing agent (608.26 min.µg/mL) was significantly different from the one with the biotransforming agent (414.26 min.µg/mL) ($p<0.05$), but not with the control group (507.52 min.µg/mL). The C_{max} of the group receiving the adsorbing agent (7.00 µg/mL) was significantly higher than for the group with the biotransforming agent (4.19 µg/mL), but no significant differences were noticed with the control group (5.20 µg/mL). No significant differences in T_{max} ($p=0.597$) and $T_{1/2el}$ ($p=0.915$) were observed between the groups.

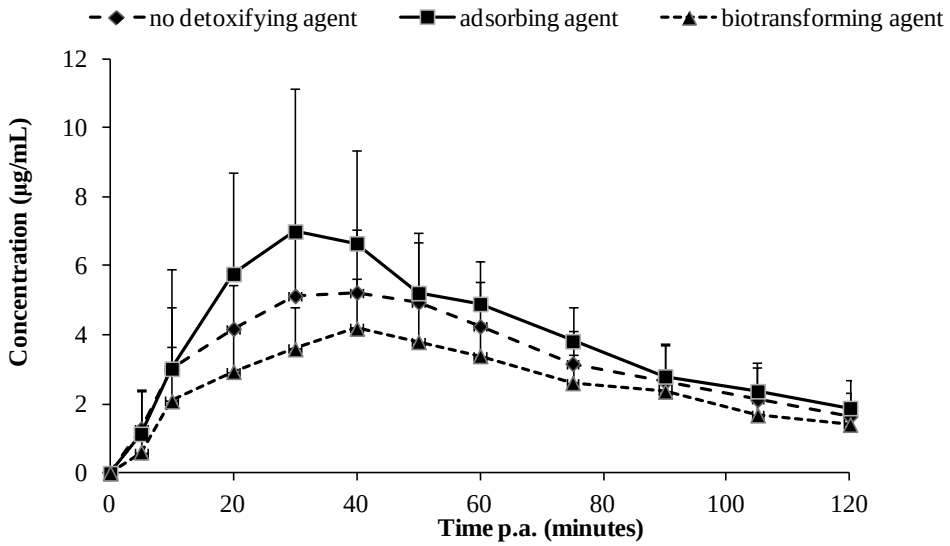

Figure 3. Influence of 3 weeks of feeding detoxifying agents on the plasma profile of amoxicillin, administered as a single oral bolus of 20 mg/kg BW in broilers (n=8, mean + SD).

Influence of two different Detoxifying Agents on the Tissue Residues of Oxytetracycline and Amoxicillin. The results of the analysis of oxytetracycline in liver and kidney tissues are shown in Figure 4. The residues of oxytetracycline in the liver were not significantly different between groups. For the kidneys, the concentration of oxytetracycline was much higher in the group that received feed supplemented with a biotransforming agent compared to the control group ($p<0.05$). This is in agreement with the results obtained in plasma (Fig. 2). For amoxicillin on the other hand, the residue levels in kidneys and liver were lower than the limit of quantification for all the animals (LOQ = 12.5 ng/g) (data not shown).

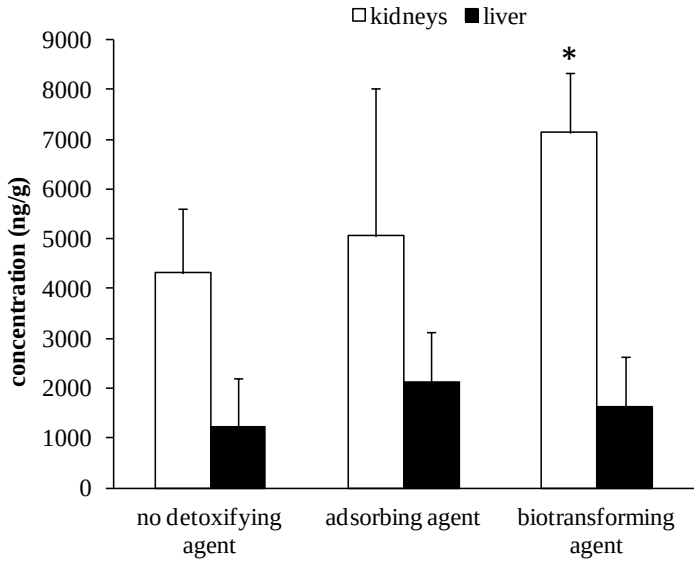

Figure 4. Influence of 3 weeks of feeding of detoxifying agents on residues of oxytetracycline in broiler tissues, obtained 16h after withdrawal medicated drinking water (n=8, mean + SD, * = significant difference).

DISCUSSION

This study is, to our knowledge, the first one which fully complies with recent EFSA guidelines for *in vivo* efficacy testing of mycotoxin detoxifying agents in broilers. Zootechnical parameters such as feed intake and live weight gain could not be used to demonstrate efficacy of the tested detoxifiers for DON in broilers since no significant differences were seen between groups. Nevertheless, the same biotransforming agent showed potential in diminishing deleterious effects of DON on growth performance and other non-specific parameters in pigs (Plank et al., 2009), and of T-2 toxin in broiler chickens (Diaz et al., 2005). Moreover, Dänicke et al. (2003) stated that this detoxifying agent even decreased the performance of broilers independently of the dietary mycotoxin concentration. Negative effects on the performance of the broilers however were not noticed during this trial.

Analysis of DON in plasma only resulted in quantifiable levels in those birds that received DON in a concentration of 7.54±2.20 mg/kg feed, which is higher than the guidance level of 5 mg/kg in feed, during a 3-week feeding period (Anonymous, 2006). It was remarkable that supplementation of the naturally contaminated feed with mycotoxin detoxifying agents even slightly increased the plasma levels of DON within the first two weeks of feeding. Devreese et al. (2012) performed *in vivo* efficacy testing of detoxifying agents in broilers with different oral bolus models. A single oral bolus of DON with or without a detoxifying agent in broilers also revealed a significant higher $AUC_{0\rightarrow\infty}$, C_{max} and relative oral bioavailability in the group with a detoxifying agent compared to the control group. Possible hypotheses for the increased oral bioavailability of DON include unspecific effects such as an increased ratio of villus height-crypt depth due to feeding detoxifying agents (de los Santos et al., 2007; Baurhoo et al., 2009; Star et al., 2009), but further investigations are needed to confirm this. Other nonspecific effects, such as increased protein digestibility, were also attributed to detoxifying agents and should be investigated, as they can have an influence on the bioavailability of essential feed constituents (Döll and Dänicke, 2004). Surprisingly, after three weeks of feeding, no plasma levels above the LOQ were observed in any of the experimental groups. After three weeks of feeding, the birds may have developed a kind of tolerance to the negative impact of deoxynivalenol. This resistance may be attributed to the

age of the animals or to a kind of adaptation process through a variety of metabolic and hormonal compensatory mechanisms (Moon et al., 2008; Kobayashi-Hattori et al, 2011). Very low plasma concentrations of DON were measured during this trial, which correspond with the literature, where ^{14}C-labeled DON was described to be poorly absorbed from the gastrointestinal tract of hens since peak plasma levels occurring 2-2.5h post-administration accounted for only 0.64% of the administered dose (Prelusky et al., 1986). Our group also demonstrated an absolute oral bioavailability of only 19.3% for DON in broilers after a single oral bolus of DON (0.75 mg/kg BW) (A. Osselaere, unpublished data). Other researchers reported concentrations of DON and its de-epoxidized metabolite DOM-1 below the LOQ of 2 ng/mL in plasma after 5 weeks of feeding DON in a concentration of 2.5 mg/kg (Dänicke et al., 2007). Because continuous feeding trials with plasma analysis do not seem to be promising to test detoxifiers for DON in poultry, bolus models to test the efficacy of detoxifying agents were developed at our department. Higher plasma concentrations were observed after a single oral administration of DON (0.75 mg/kg BW), which was more appropriate to test the efficacy of the detoxifiers compared with continuous feeding trials (Devreese et al. 2012). In an effort to look for other possible end-points, the concentrations of DON and DOM-1 were determined in liver, kidneys and bile fluid after three weeks of feeding. Prelusky et al. (1986) described the important role of biliary excretion in the elimination of DON from the body. Again, no residues above the LOQ were detected in the collected organs. On the other hand, DON was detectable in the bile fluid, but only for these groups which received a diet with an amount of DON higher than 5 mg/kg feed. DOM-1 was even found in the bile fluid of all the groups that received DON contaminated feed. This is in contrast with the results reported by Dänicke et al. (2007) where no concentrations of DOM-1 were detected in the bile fluid (LOQ = 4 ng/mL) of chickens after three weeks of feeding. Supplementation of the diets with an adsorbing or a biotransforming agent did not have significant effects on the concentration of DON and DOM-1 in the bile fluid.

The safety of mycotoxin detoxifying agents is an important issue that did not receive much attention until now (Phillips et al., 2009). Detoxifying agents are able to modify the chemical structure of mycotoxins or to exert their activity by binding mycotoxins in the gastro-intestinal tract. However, interactions with nutrients, feed additives or veterinary drugs, can possibly occur. Therefore we also investigated the possible interaction of detoxifying agents

with the pharmacokinetic profiles and tissue residues of the antibiotics oxytetracycline and amoxicillin, which are frequently used in poultry practice (Abo El-Sooud et al., 2004; Ismail and El-Kattan, 2004). To our knowledge, only the recent EFSA scientific opinion (Anonymous, 2011) and a report of The Bureau of Veterinary Drugs of Canada (Anonymous, 1992) discuss a possible interaction between bentonite and tylosin or with coccidiostats. After three weeks of feeding diets supplemented with a detoxifier, significant different plasma profiles depending on the type of antibiotic were observed. For oxytetracycline, the birds receiving feed supplemented with a biotransforming agent had a significantly higher C_{max} and AUC than the other birds ($p<0.05$). The results of the residue depletion study of oxytetracycline in tissues also showed higher concentrations of oxytetracycline in the kidneys of these birds compared to the control group. Kidneys play an important role in the elimination of oxytetracycline in poultry (Ismail and El-Kattan, 2004). In contrast, for amoxicillin, the group with the adsorbing agent reached significantly higher plasma concentrations and AUC, only when compared to the group with the biotransforming agent. For this antibiotic, no tissue residue levels above the limit of quantification were observed twelve hours after the last administration, which can be explained by the rapid elimination of amoxicillin in poultry (Anadon et al., 1996). In this study clay-based detoxifiers were used, which have a non-specific binding capacity. Moshtaghian et al. (1991) noted that clays can absorb micronutrients and thus can have a negative impact on the bioavailability of minerals and trace elements. Clays consist of a porous structure with electric elementary charges which can trap particles with a particular size and electric charge (Jouany, 2007). Surprisingly, we observed an enhancement in bioavailability for these antibiotics rather than a reduction. Although the mechanism is still unclear, the possible consequences of these findings are very important. Adaptation of the dosage of the antibiotic can be necessary; otherwise the higher plasma concentrations of the antibiotic can possibly lead to toxicity and to higher tissue residue levels. An advantage could be that the dosage of the antibiotic can be reduced and thus could lead to a reduced use of antibiotics. Influence of detoxifying agents on the pharmacokinetics of doxycycline has also been demonstrated in pigs by co-authors. Significantly higher plasma concentrations were observed in piglets that received T-2 toxin contaminated feed supplemented with a mycotoxin-detoxifying agent (Goossens et al., 2012).

In conclusion, we have shown that the analysis of DON and its metabolite *in vivo* is not a relevant end-point for the efficacy testing of mycotoxin detoxifying agents in plasma of broilers when fed in a continuous design. The safety testing of two commercially available detoxifiers revealed possible interactions with veterinary drugs, which can have important consequences and warrants further investigations to elucidate underlying mechanisms.

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Chapter 3.

The effects of deoxynivalenol and an adsorbing agent on the intestinal barrier and liver function

Adapted from: Osselaere, A., Santos, R., Hautekiet, V., De Backer, P., Chiers, K., Ducatelle, R., Croubels, S. (2013). Deoxynivalenol impairs hepatic and proximal intestinal gene expression of selected oxidative stress, tight junction and inflammation proteins in broiler chickens, but addition of an adsorbing agent shifts the effects to more distal parts of the small intestine**.** Submitted to PLOS One.

ABSTRACT

Broiler chickens are rather resistant to deoxynivalenol and thus, clinical signs are rarely seen. However, effects of subclinical concentrations of deoxynivalenol on both the intestine and the liver are less frequently studied at the molecular level. During our study, we investigated the effects of three weeks of feeding deoxynivalenol on the gut wall morphology, intestinal barrier function and inflammation in broiler chickens. In addition, oxidative stress was evaluated in both the liver and intestine. Besides, the effect of a clay-based mycotoxin adsorbing agent on these different aspects was also studied. Our results show that feeding deoxynivalenol affects the gut wall morphology both in duodenum and jejenum of broiler chickens. A qRT-PCR analysis revealed that deoxynivalenol acts in a very specific way on the intestinal barrier, since only an up-regulation in mRNA expression of claudin 5 in jejunum was observed, while no effects were seen on claudin 1, zona occludens 1 and 2. Addition of an adsorbing agent resulted in an up-regulation of all the investigated genes coding for the intestinal barrier in the ileum. Up-regulation of Toll-like receptor 4 and two markers of oxidative stress (heme-oxigenase or HMOX and xanthine oxidoreductase or XOR) were mainly seen in the jejunum and to a lesser extent in the ileum in response to deoxynivalenol, while in combination with an adsorbing agent main effect was seen in the ileum. These results suggest that an adsorbing agent may lead to higher concentrations of deoxynivalenol in the more distal parts of the small intestine. In the liver, XOR was up-regulated due to DON exposure. HMOX and HIF-1α (hypoxia-inducible factor 1α) were down-regulated due to feeding DON but also due to feeding the adsorbing agent alone or in combination with DON.

INTRODUCTION

Mycotoxin contamination can occur in all agricultural commodities in the field and/or during storage, if the conditions are favorable for fungi growth (CAST, 2003). Deoxynivalenol (DON), also called vomitoxin, is a trichothecene mycotoxin which is highly prevalent in Europe (Binder et al., 2007; Monbaliu et al., 2010; Streit et al., 2012). In poultry, DON rarely causes acute mycotoxicosis. However, chronic exposure to the toxin can lead to reduced production and an altered immune function (Awad et al., 2011). As poultry seems to be less susceptible to DON-mycotoxicosis compared to other animals, infected cereal batches are sometimes diverted to the poultry feed production (Awad et al., 2008). Mycotoxin detoxifying agents are frequently used feed additives to reduce the adverse effects of mycotoxins. Detoxifiers based on clay minerals are classified by the European Food Safety Authority (EFSA) as adsorbing agents (Anonymous, 2009).

Mycotoxins are food and feed contaminants and thus after ingestion the intestine can be exposed to high concentrations of the toxins (Maresca et al., 2002; Sergent et al., 2006). The epithelial surface of the intestine is characterized by a large contact area for absorption of nutrients and xenobiotics. This surface consists of a simple columnar epithelium, which is increased by the presence of villi (DeSesso and Jacobson, 2001). Both toxins and mycotoxin detoxifiers can interact with this surface area, resulting in altered extent and rate of absorption of xenobiotics such as drugs and mycotoxins. For example, we found in a previous study higher plasma concentrations of DON in animals fed contaminated feed in combination with a clay-based adsorbing agent compared to animals fed DON contaminated feed only (Goossens et al., 2012; Osselaere et al., 2012).

The absorbing epithelial cells (enterocytes) are connected strongly by tight junction proteins. These tight junctions seal off the luminal end of the intercellular space and so transport by this paracellular route is very limited (Arrieta et al., 2006). Claudins are transmembrane proteins which form the backbone of the tight junction strands. Claudin 1 and 5 are known to interact and are important to guarantee the intestinal barrier function. Both claudins have already been characterized in chickens (Furuse et al., 1998; Krause et al., 2008; Ozden et al.,

2010). The family of zona occludens, including zona occludens 1 (ZO 1) and zona occludens 2 (ZO 2), is a group of scaffolding proteins which is part of the cytoplasmic plaque of the tight junctions.

The intestinal epithelial cells also contribute to the regulation of inflammatory conditions and create a kind of barrier against invading pathogens. Toll-like receptors (TLR) in the intestinal epithelium, particularly TLR4, serve as rapid pathogen sensors.

After intestinal absorption of mycotoxins these compounds reach the liver as the gateway of the portal blood draining the gastrointestinal tract. Both intestine and liver consist of rapidly proliferating cells and have a high protein turnover rate. Therefore, we may suppose that these organs are more sensitive for the action of DON (Ueno, 1984).

The toxicity of DON is mediated by various mechanisms. Trichothecenes are potent inhibitors of the RNA, DNA and protein synthesis (Eriksen and Petterson, 2004). In addition, DON may induce the production of free radicals and cellular oxidative stress. It has been shown that oxidative stress causes up-regulation of hypoxia-inducible factor 1, subunit alpha (HIF-1α) (Wenger, 2000), a transcription factor which regulates genes involved in inflammation and cell death (Lee et al., 2007). Heme-oxigenase (HMOX) is another sensitive marker of oxidative injury, which affords protection against hepatocyte death (Carchman et al., 2011). Both HIF-1α and HMOX have already been characterized in chickens (Giusti and Fiszer de Plazas, 2012; Shan et al., 2004). Xanthine oxidoreductase (XOR) is an enzyme associated with the synthesis of reactive oxygen species and is part of the cellular defense enzyme systems (Harrison, 2002). In broilers, this enzyme is mainly expressed in the liver, but also in the intestine (60% of the amount in the liver) and other organs but in a lower amount (Carro et al., 2009). The intestine requires an efficient immune defense at the epithelial surface, and among other factors, XOR is secreted by the enterocytes of the small intestine (Van Den Munckhof et al., 1995).

The aim of our study was to assess the effects of three weeks dietary exposure to DON on the small intestine and liver in broiler chickens. To this end qRT-PCR analyses were conducted to study if genes coding for oxidative stress and inflammation response are influenced by DON, both in the liver and the small intestine. In addition, the effects of DON

on the intestinal morphology and intestinal barrier function were investigated with histopathology and qRT-PCR analysis, respectively. To our knowledge, this is the first *in vivo* study which observes these parameters in broiler chickens. Finally, the effects of a clay-based mycotoxin-detoxifying agent were also investigated during our trial.

MATERIALS AND METHODS

Ethics statement

The protocol was approved by the Ethical Committee of the Faculty of Veterinary Medicine (Ghent University) (EC 2010/064 and EC 2010/076). All husbandry practices and euthanasia were performed with full consideration of animal welfare.

Animals and diets

The animals and the experimental design have been described elsewhere (Osselaere et al., 2012). In brief, 32 1-day-old broiler chickens were fed uncontaminated feed during an acclimatization period of ten days. Afterwards, the animals were divided into four different dietary groups of 8 animals each: a control group receiving uncontaminated feed, a group receiving uncontaminated feed + adsorbing agent, a third group receiving naturally DON contaminated feed and a group fed naturally DON contaminated feed + adsorbing agent. Analyses of the feed were performed by a multi-mycotoxin LC-MS/MS method (Monbaliu et al., 2010). The naturally contaminated feed was contaminated as follows: DON (7.540 ± 2.20 mg/kg), 3-acetylDON (1.481 ± 0.57 mg/kg), fumonisin B1 (0.700 ± 0.08 mg/kg), fumonisin B2 (0.201 ± 0.02 mg/kg) and fumonisin B3 (0.207 ± 0.08 mg/kg). The adsorbing agent (illite-ambrosite clay) was added in a concentration of 1.5 kg/ton feed. After three weeks of feeding, the animals were euthanized and liver and intestinal samples were immediately collected. From the small intestine, samples were taken at three different locations: 2 cm after the gizzard (duodenum), just before Meckel's diverticulum (jejunum) and two cm before the ileo-cecal transition (ileum). Intestinal and liver samples were rinsed in phosphate buffered saline (PBS). Afterwards, the samples for qRT-PCR analysis were immediately frozen in liquid nitrogen and stored at -80 °C until analysis. Samples for morphological examination were also rinsed in PBS and then fixed in 4% (v/v) phosphate buffered formalin.

Quantitative RT-PCR method to analyze the intestinal barrier function, inflammation and oxidative stress

RNA from samples of liver and intestine (duodenum, jejunum and ileum) were isolated using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions, and total RNA was quantified by spectrophotometry (Nanodrop ND-1000, Thermo Scientific, Wilmington, NC, USA). Subsequently, 1 µg of extracted total RNA was reverse transcribed with the iScriptTM cDNA Synthesis kit (Biorad, Hercules, CA, USA). The obtained cDNA was diluted to a final concentration of 30 ng/µL. Primers were commercially produced (Eurogentec, Nijmegen, the Netherlands) (Table 1). The primers used were selected based on specificity and efficiency by qPCR analysis of a dilution series of pooled cDNA at a temperature gradient (55^{o}C to 65^{o}C) for primer-annealing and subsequent melting curve analysis. The reaction mixture for the qPCR containing 10 µL of the diluted cDNA was mixed with 15 µL iQSYBR Green Supermix (Biorad), forward and reverse primers (final concentration of 0.4 pmol/µL for each primer) and sterile water according to the manufacturer's instructions. qPCR was performed using the MyiQ single-colour real-time PCR detection system (Biorad) and MyiQ System Software Version 1.0.410 (Biorad). Amplification efficiency was determined per plate using linregPCR. Data were analyzed using the efficiency corrected Delta-Delta-Ct method (Pfaffl, 2001). Housekeeping genes were tested for all the test conditions after which most stable housekeeping genes for liver and intestinal samples were selected using the geNorm software (data not shown). The most stable housekeeping genes had a M-value between 0.2 and 0.5. To determine if the inclusion of an additional housekeeping gene was required, the cut-off value for variation was set at 0.2. The fold-change values of the genes of interest (GOIs) were normalized using two housekeeping genes: hypoxanthine-guanine phosphoribosyl transferase (HPRT) and hexose-6-phosphate dehydrogenase (H6PD). The mRNA expression of proteins involved in oxidative stress i.e. HMOX, HIF-1α and XOR were evaluated in the liver and intestine. Furthermore, mRNA expression of the tight junctions proteins claudin 1 and 5 (CLDN1 and CLDN5) and zona occludens 1 and 2 (ZO1 and ZO2) in sections from the duodenum, jejunum and ileum were measured. Two compounds of the immune system, namely Toll-like receptors (TLR) 2 and 4 were also investigated during our study. For the validation of the qPCR assays

following criteria were applied: slope between -3.6 and -3.1, efficiency between 90 and 110 %, $R^2 > 0.99$.

Morphological examination of the gut wall

Formalin-fixed intestinal samples were dehydrated in xylene and embedded in paraffin. With a microtome (Microm, Prosan, Merelbeke, Belgium), sections of 4 µm thickness were cut and mounted in glass slides. Afterwards, deparaffination occurred in xylene (2 times 5 min) and then rehydratation occurred in isopropylene (5 min), 95 % alcohol (5 min) and 50 % alcohol (5 min). Sections were stained with haematoxylin and eosin. Using light microscopy, villus height and crypt depth (10 villi per intestinal segment) from each of 8 chickens per treatment, were measured. For this, a Leica Camera DFC320 (Leica Microsystems Ltd, Wetzlar, Germany) coupled to a computer-based image analysis system LAS v.3.8. (Leica Microsystems Ltd) was used. Only intact villi were measured. Measurements were done on cross-sections of ring-shaped intestinal segments.

Data analysis

Results were compared by ANOVA after determination of normality and variance homogeneity. Multiple comparisons were performed using a LSD post-hoc test. Not normally distributed data were analyzed using the non-parametric Kruskal-Wallis analysis, followed by a Mann-Whitney test using SPSS 19.0 Software (SPSS Inc., Chicago, IL, USA). A P-value of <0.05 was considered statistically significant.

Table 1. Primers used for the quantification of housekeeping genes (HKG) and genes of interest (GOI).

	Gene	Accession N°	Primer	Sequence	Product size (pb)	Annealing T°
HKG						
	HPRT	NM_204848.1	Forward	5' CGTTGCTGTCTCTACTTAAGCAG 3'	90	65
			Reverse	5' GATATCCCACACTTCGAGGAG 3'		
	H6PD	XM_425746.2	Forward	5' GGAGAACCAGCACTTCTTAGAC 3'	84	64
			Reverse	5' GGGTTCAGCAATTCCACTG 3'		
GOI						
	CLDN1	NM_001013611	Forward	5' CTGATTGCTTCCAACCAG 3'	140	57-59
			Reverse	5' CAGGTCAAACAGAGGTACAAG 3'		
	CLDN5	NM_204201	Forward	5' CATCACTTCTCCTTCGTCAGC 3'	111	56-65
			Reverse	5' GCACAAAGATCTCCCAGGTC 3'		
	HIF-1α	NM_204297	Forward	5' CACCATTACCATACTTCAGCAG 3'	88	65
			Reverse	5' CTTCACATCATCCACACGTTC 3'		
	HMOX	NM_205344	Forward	5' CTTGGCACAAGGAGTGTTAAC 3'	78	61-63
			Reverse	5' CATCCTGCTTGTCCTCTCAC 3'		
	TLR2	NM_204278	Forward	5' CCTGCAACGGTCATCTCAG 3'	135	59
			Reverse	5' GTCTCAGGGCTTGTTCTTCAG 3'		
	TLR4	NM_001030693	Forward	5' CTGACCTACCCATCGGACAC 3'	111	59
			Reverse	5' GCCTGAGAGAGGTCAGGTTG 3'		
	XOR	NM_205127	Forward	5' GTGTCGGTGTACAGGATACAGAC 3'	110	61
			Reverse	5' CCTTACTATGACAGCATCCAGTG 3'		
	ZO1	XM_413773	Forward	5' CTTCAGGTGTTTCTCTTCCTCCTC 3'	131	59
			Reverse	5' CTGTGGTTTCATGGCTGGATC 3'		
	ZO2	NM_204918	Forward	5' CGGCAGCTATCAGACCACTC 3'	87	64-65
			Reverse	5' CACAGACCAGCAAGCCTACAG 3'		

RESULTS

Not only DON but also the adsorbing agent alters mRNA expression of oxidative stress markers in liver of broiler chickens

In the liver, both HIF-1α and HMOX mRNA were significantly down-regulated for all the broiler chickens receiving either DON, an adsorbing agent or DON and the adsorbing agent, when compared to the control group. Differently, XOR was significantly up-regulated in the group receiving DON. The group receiving an adsorbing agent, whether or not in combination with DON was not affect ed. Data are shown in Figure 1.

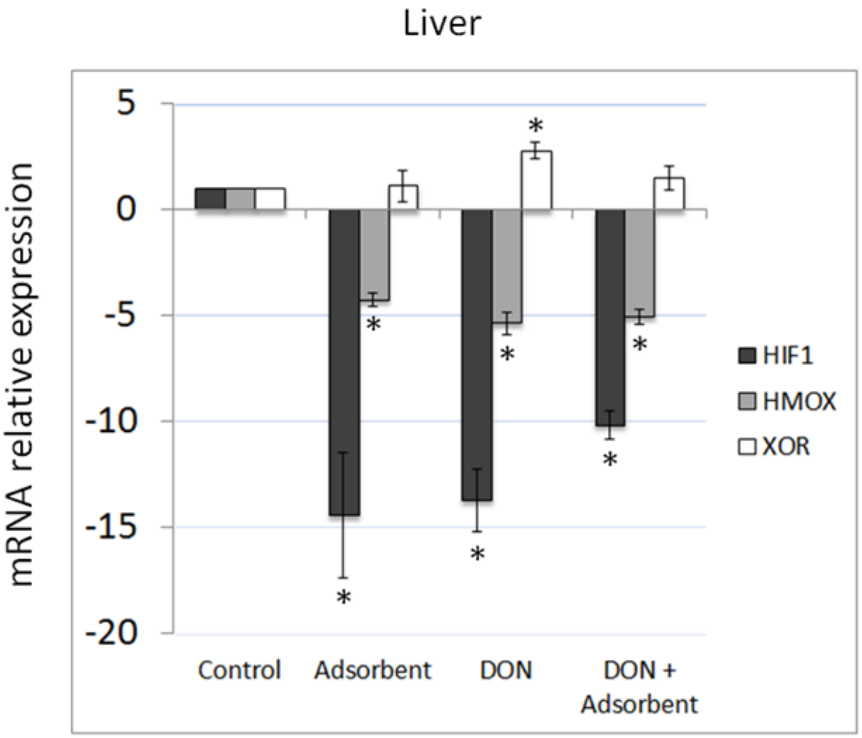

Figure 1. Effects of DON and an adsorbent on oxidative stress in the liver of broiler chickens. Results are presented as mean (± SEM) mRNA expression. Fold change in gene expression levels of the chicken liver relative to control group, which is considered 1. * Indicates significant differences between treated and control animals.

DON leads to oxidative stress in the jejunum and in the ileum of broiler chickens in combination with an adsorbing agent

For the small intestine, the expression of HIF-1α, HMOX and XOR mRNA was investigated in the duodenum, jejunum and ileum. Expression of HIF-1α was unaltered in the intestine, independently on the treatment or intestinal section. On the other hand, HMOX and XOR were significantly up-regulated in the jejunum of animals fed the DON contaminated feed, independently on the supplementation of an adsorbing agent. For the last part of the small intestine, the ileum, only XOR was up-regulated when animals were fed with feed containing DON and the adsorbing agent (Figure 2).

DON and adsorbent do not affect duodenal barrier function, but do so in jejunum and ileum

As observed for oxidative stress markers, barrier function of duodenum was unaffected by both DON and adsorbing agent, while jejunum presented a significant up-regulation of CLDN5 mRNA when animals were fed with DON contaminated feed. Feed supplementation with the adsorbing agent did significantly reduce the CLDN5 mRNA expression when compared to DON, but its expression remained significant higher than that observed in the control. The strongest effect on tight junctions was observed in the ileum when animals were fed with feed contaminated with DON and supplemented with the adsorbing agent, with a significant up-regulation of CLDN1, CLDN5, ZO1 and ZO2 mRNA (Figure 2).

DON leads to inflammatory reaction in duodenum and jejunum, but its negative effect in the ileum depends on the feed supplementation with an adsorbing agent

A significant up-regulation of TLR4 mRNA was observed in the duodenum and jejunum of animals fed with DON contaminated feed. Although feed supplementation with an adsorbing agent was efficient to decrease the TLR4 expression, it was efficient to recover control levels only in the duodenum and not in the jejunum. Finally, as observed with tight junctions' analysis, ileum exposure to DON and adsorbing agent resulted in the significant up-regulation of TLR4 mRNA (Figure 2).

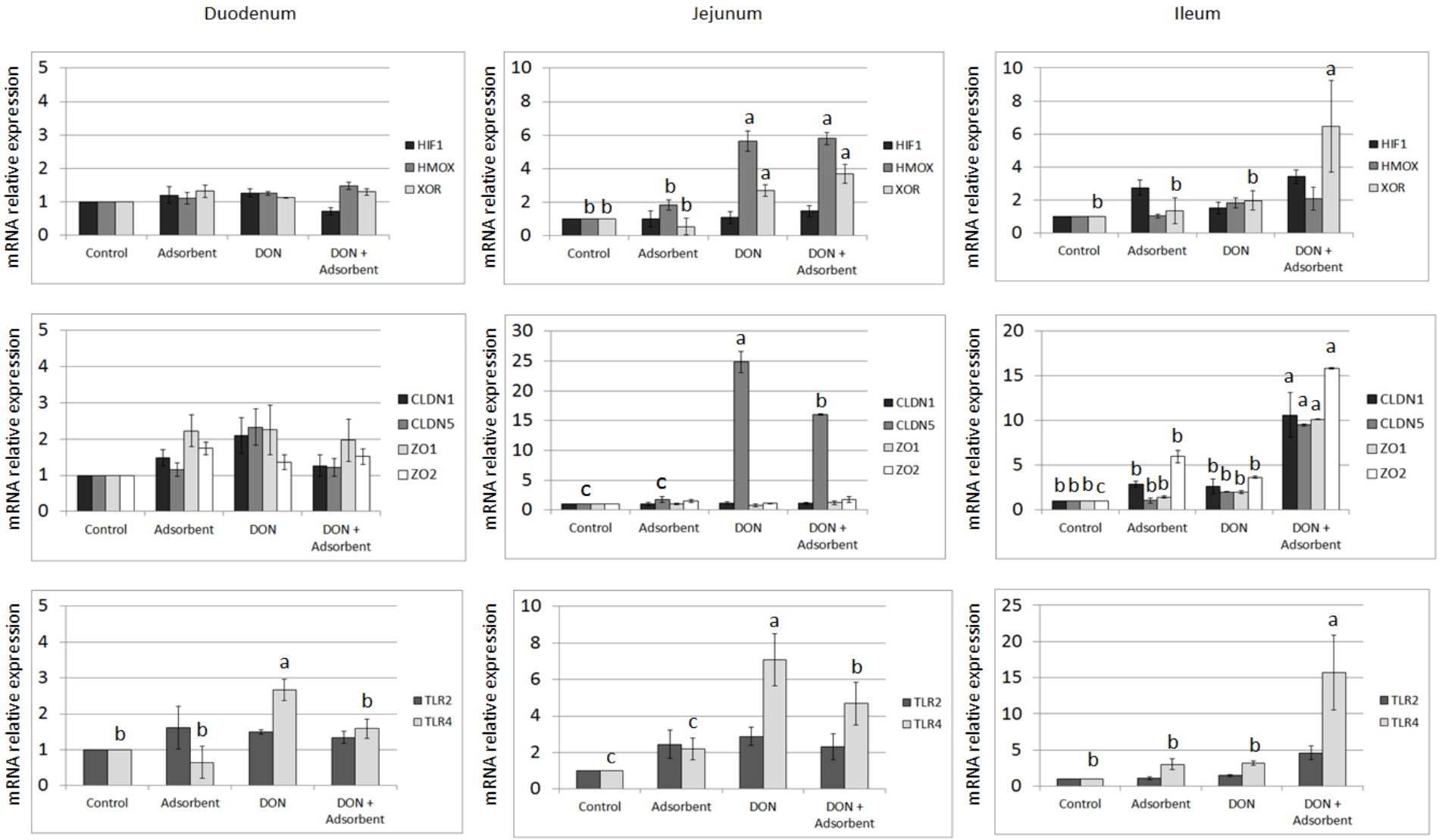

Figure 2. Effects of DON and an adsorbent on intestinal barrier in broiler chickens. Results are presented as mean (± SEM) mRNA expression. Fold change in gene expression levels of the chicken intestines relative to control group, which is considered 1. [a-c] Different lower-case letters indicate significant differences between groups.

DON alters the gut wall morphology in duodenum and jejunum of broiler chickens, but addition of an adsorbing agent counteracts these effects

Feeding DON contaminated feed resulted in a decreased villus length and crypt depth both in duodenum and jejunum of the broiler chickens. Addition of an adsorbing agent resulted in longer villi, even in combination with DON and this over the entire length of the small intestine. The crypt depth however, was not influenced by the addition of an adsorbing agent to control feed in the duodenum and jejunum, when compared to the control group. On the other hand, the adsorbing agent had a positive effect on the crypt depth in these intestinal parts, when added to DON contaminated feed. In the ileum, no effect of feeding DON contaminated feed without an adsorbing agent was observed. In this part of the small intestine, it was the adsorbing agent in combination with DON or not which resulted in higher villi and deeper crypts (Table 2).

Table 2. Length of villi (µm) and crypt depth (µm) in duodenum, jejunum and ileum after 3 weeks feeding a control diet or feed contaminated with DON, either or not supplemented with an adsorbing agent. Results are presented as mean values and standard deviations of fifteen villi or crypts measured from 8 chickens per treatment group.

	Control	Adsorbing agent	DON	DON + adsorbing agent
Duodenum *Villus height* *Crypt depth*	1734 ± 26 ^{a} 131 ± 7 ^{a}	1773 ± 43 ^{c} 134 ± 7 ^{a}	1449 ± 31 ^{b} 114 ± 9 ^{b}	1789 ± 39 ^{c} 128 ± 8 ^{a}
Jejunum *Villus height* *Crypt depth*	1343 ± 37 ^{a} 120 ± 8 ^{a}	1521 ± 39 ^{c} 116 ± 10 ^{a}	1184 ± 48 ^{b} 101 ± 8 ^{b}	1509 ± 43 ^{c} 109 ± 7 ^{c}
Ileum *Villus height* *Crypt depth*	596 ± 30 ^{a} 113 ± 6 ^{a}	773 ± 63 ^{b} 124 ± 17 ^{b}	616 ± 38 ^{a} 110 ± 15 ^{a}	744 ± 63 ^{b} 119 ± 18 ^{ab}

a,b mean values within a row with unlike superscript letters are significantly different ($p \leq 0.05$)

DISCUSSION

Being an interface between the outside world and the inside body, the gastro-intestinal tract (GIT) is a dynamic barrier (Oswald, 2006). This barrier is responsible for two major processes, which are on the one hand uptake of nutrients and fluids and on the other hand defense mechanism against xenobiotics. We performed a study with broiler chickens fed with naturally contaminated feed to investigate the effects of DON on the intestinal barrier and hepatic function. Co-contamination of different mycotoxins in naturally contaminated feed is common and this was also the case for the experimental feed used in this study. DON was the most prevalent mycotoxin and was even present in a concentration higher than the European recommended maximum level of 5 mg/kg (Anonymous et al., 2006). The other contaminant present, 3-acetylDON, is considered a masked mycotoxin, i.e. a conjugated form of DON also produced by *Fusarium* fungi. It is hypothesized that this conjugated form may be hydrolysed and release DON in vivo, but the question remains whether this occurs in every animal species and if this occurs already in the GIT and/or liver and/or systemic circulation. The sum of the concentration of the co-contaminants fumonisin B1 and B2 of 0.901 mg/kg was much lower than the European guidance value of 20 mg/kg in poultry feed (Anonymous et al., 2006). Thus, the co-contamination with fumonisins can be considered as negligible. In our study, three weeks feeding DON at 7.54 mg/kg feed reduced the villus height and the crypt depth both in the duodenum and the jejunum. Reduced villi in the duodenal and jejunal segment of the small intestine were also observed in broiler chickens after 6 weeks feeding a diet of 10 mg/kg DON (Awad et al., 2006). Yunus et al. (2012) observed a linear correlation between increasing levels of DON and the decrease in villus height in both the mid-duodenum and mid-jejunum (Yunus et al., 2012). Possible explanation for these histological changes can be a direct irritant effect of the mycotoxin or suppression of mitosis or protein synthesis (Eriksen and Petterson, 2004; Yunus et al., 2012). In order to maintain an effective barrier function, the intestinal epithelium needs to regenerate continuously. Mature cells migrate along the crypt-villus axis towards the villus-top, in the mean time these cells become differentiated cells (Booth and Potten, 2000). DON can be responsible for a reduced cell proliferation (Bensassi et al., 2009; Kasuga et al., 1998; Yang et al., 2008). This can be an explanation for the reduced crypt depth observed during

our trial. A decreased crypt depth in the mid-duodenum in broiler after chronic exposure to DON (12 mg/kg) has been reported earlier (Yunus et al., 2012). Interestingly, the adsorbing agent resulted in longer villi over the entire length of the small intestine. These longer villi seen in our study in the chickens receiving the adsorbing agent can be responsible for the higher oral bioavailabilities of xenobiotics as observed in our previous study (Osselaere et al., 2012).

Several studies both *in vitro* and *in vivo* already reported that DON is able to alter intestinal permeability. Intestinal physiology can even be affected by DON in the absence of clinical signs (Awad et al., 2004). The function of the tight junctions can be evaluated by measurements of the trans-epithelial electrical resistance (TEER) and of the paracellular efflux of macromolecules (Madara, 1998). These techniques, however, do not give information which specific protein of the tight junctions is affected (Van Itallie et al., 2009). Therefore, a qRT-PCR method was applied in our study to evaluate the effects of DON on the different specific proteins of the tight junctions, namely CLDN1, CLDN5, ZO1 and ZO2. An important advantage of this technique is the generation of quantitative results, which makes it possible to detect small differences which could otherwise be missed when using immunofluorescence. Moreover, due to the lack of suitable commercial avian antibodies, no effects at the protein level could be studied. This general lack in anti-chicken antibodies for use in Western blot and immunofluorescence is well known in poultry research.

Major effects of feeding DON without an adsorbing agent on the intestinal barrier were observed in the jejunum. A significant up-regulation of CLDN5 was observed in the jejunum of the groups fed contaminated feed with or without an adsorbing agent. No significant differences were noticed in the jejunum for the mRNA expression of the other genes coding for the intestinal barrier function. The ileum on the other hand, is less susceptible to DON due to the fact that the majority of ingested DON is absorbed in the proximal parts of the small intestine (Awad et al., 2007). However, in the group receiving DON in combination with an adsorbing agent, detrimental effects were seen in the ileum. This indicates that addition of the adsorbing agent results in a sustained presence of DON in the intestine.

The results of our study suggest that DON selectively acts on the different parts of the tight junction complex as only an up-regulation of CLDN5 was observed. A selective effect of DON has been observed *in vitro* in intestinal porcine epithelial cells and human Caco-2 cells. After 48h exposure to DON at a concentration of 9000 ng/mL both claudin 3 and 4 showed reduced protein expression, but ZO1 and occludin were not affected (Pinton et al., 2009). The same authors also described a reduced claudin 4 expression in growing pigs after *in vivo* exposure to DON (2.85 mg DON/kg feed) for 5 weeks, using Western blot analysis and immunohistochemistry. Immunohistochemistry results showing no changes in the overall morphology of the cells, but only a decreased staining for the claudins strengthens our hypothesis of a selective action of DON (Pinton et al., 2009). Selective action of DON on claudin-isoforms was confirmed in other more recent *in vitro* studies (Diesing et al., 2011; Van de Walle et al., 2010). Our study is, to our knowledge, the first one showing the effects of DON on the intestinal barrier in poultry after *in vivo* exposure to DON.

Different authors also suggest that trichothecenes may be responsible for the production of free radicals, causing damage to DNA and membranes and thus suggesting that oxidative stress may play an important role in their toxicity (Atroshi et al., 1997; Leal et al., 1999; Minervini et al., 2005; Rizzo et al., 1994; Vila et al., 2002). Up-regulation of HIF-1α often occurs in the first hours of hypoxia and, thereafter, returns to basal levels. This can be an explanation for the basal levels of HIF-1α found in the small intestine during this study. However, instead of basal expression of HIF-1α, we have observed its down-regulation in the liver of chicken, after exposure to DON or the adsorbing agent alone or in combination. As shown recently by Sparkenbaugh et al. (2011), HIF-1α is up-regulated during liver injury in the initial phase of inflammation and oxidative stress, and should guarantee cell protection when the stress becomes chronic, which was not observed in our study. Furthermore, protection against hepatocyte death is related to the up-regulation of HMOX (Carchman et al., 2011). In our present study, however, hepatic HMOX was also significantly down-regulated in animals fed with adsorbent supplemented feed, contaminated with DON, or with a combination of both. In contrast, in the jejunum a significant up-regulation of HMOX was observed in the animals receiving DON contaminated feed with or without the adsorbing agent. XOR, which responds more in the chronic phase, was significantly up-

regulated in the jejunum in all the animals receiving DON, but in the liver an up-regulation was observed only in the group receiving DON without an adsorbing agent. In summary, DON caused oxidative stress in the small intestine. This has previously been reported in Caco-2 cells, where DON caused a significantly increased production of malondialdehyde, a biomarker of lipid peroxidation (Kouadio et al., 2005). The hepatic effects of *in vivo* exposure to 10 mg/kg DON in broiler chickens have previously been reported by Frankic et al. (2006). They observed no differences in liver content of malondialdehyde, glutathione peroxidase and total antioxidant status, which are all markers for lipid peroxidation (Frankic et al., 2006). These findings suggest a more direct genotoxic effect of DON, rather than via the oxidative pathway (Awad et al., 2012; El Golli-Bennour and Bacha, 2011).

Due to the damage to the intestinal barrier, an increased passage of non-invasive commensal bacteria may occur (Maresca et al., 2008). Both in duodenum and jejunum a significant up-regulation of TLR4 was observed during our study, which suggests inflammation, more specific due to the presence of Gram-negative bacteria (Reynolds et al., 2012). In contrast, no effects on TLR2 were observed. TLR2 is more affected by the presence of Gram-positive bacteria (Takeuchi et al., 1999).

In the last part of the small intestine, the ileum, inflammation was caused by the presence of DON in combination with the adsorbing agent. In addition, in this group all the genes coding for the tight junction complex were also up-regulated and the same trend was observed for the gene XOR, coding for oxidative stress. Along the entire length of the small intestine administration of the adsorbing agent resulted in longer villi. From our qRT-PCR results, we can conclude that it is not the adsorbing agent that causes damage as no significant differences in gene expression were seen in the group receiving control feed in combination with the adsorbing agent. The adsorbing agent is a mineral clay and seems to protect DON from degradation by the gastric fluids and intestinal enzymes in the proximal part. This may result in a higher concentration of the mycotoxin in the distal part of the small intestine when an adsorbing agent is used. Thus the binding or interaction of DON with the adsorbing agent results in a longer exposure time of the intestine to DON.

From our *in vivo* study, we can conclude that DON acts in a very specific way on the intestinal barrier in broiler chickens. Increased intestinal barrier permeability after chronic exposure to DON may lead to intestinal inflammation. The mechanism of action of DON can be different depending on the investigated target organ. The investigated mycotoxin adsorbing agent does not cause direct damage or irritation. However, feeding this clay mineral in combination with DON may result in higher concentrations of the mycotoxin in more distal parts of the small intestine, resulting in damage of the intestinal barrier there.

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COMPETING INTEREST SECTION

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Chapter 4.

CYP3A in the liver and the small intestine of healthy broiler chickens

Adapted from: Osselaere, A., De Bock, L., Eeckhaut, V., De Backer, P., Van Bocxlaer, J., Boussery, K., Croubels, S. (2013). Hepatic and intestinal CYP3A expression and activity in broilers. Journal of Veterinary Pharmacology and Therapeutics, doi:10.1111/jvp.12034.

ABSTRACT

Cytochrome P450 is involved in drug metabolism. Subfamily CYP3A shows a degree of similarity across different animal species. However, little information is available about its expression and activity in broiler chickens. A RT-PCR method was developed for the quantification of CYP3A37 expression in the liver and small intestine of broilers. A higher expression in the jejunum was observed compared with that in the ileum. In the liver, a significantly lower expression compared witht that in the jejunum was noticed. Thus, the role of the small bowel in drug metabolism cannot be neglected in broilers. CYP3A activity was studied *in vitro* using midazolam as a substrate. Two protocols for the preparation of intestinal microsomes were compared. Mincing of the tissues before ultracentrifugation seemed to be more appropriate than a protocol based on ethylenediaminetetra-acetic acid separation. CYP3A activity revealed to be the highest in the duodenum with a decreasing trend towards the ileum. Activity in liver was comparable to duodenal activity.

INTRODUCTION

Cytochrome P450 (CYP450) enzymes play a dominant role in the process of oxidative biotransformation (Hollenberg, 1992). These heam proteins are classified in different families, with CYP1 to CYP4 being the most important ones. Members of the CYP1, CYP2 and CYP3 group are responsible for phase I metabolism of xenobiotics. On the other hand, the CYP4 family is responsible for the catalytic functions in the metabolism of fatty acids and prostaglandins (Bains et al., 1985) and also for the oxidation of many drugs with an acidic function (Moody, 1992).

The CYP enzymes have mainly been studied in humans and laboratory animals, but little is known about hepatic and especially intestinal CYP-enzymes in domestic animals (Antonovic & Martinez, 2011). To date, several subtypes of CYP enzymes have already been characterized and some have even been cloned in chicken. For the CYP1A subfamily, homology between mammalian CYP1A1 and CYP 1A2 and avian CYP1A4 and CYP 1A5, respectively, has been described (Kubota et al., 2006). Both CYP1A4 and CYP1A5 have also been cloned in chickens (Gilday et al., 1996). CYP1B is another member of the CYP1 family in chicken with a homology of 72% with the human CYP1B1 (Antonovic & Martinez, 2011). For the CYP2 family, many iso-enzymes have already been described in chickens. In chickens, CYP2G is a homologue of human CYP2G (Antonovic & Martinez, 2011). The chicken CYP2C45 has a homology of 56% with human CYP2H1 (Baader et al., 2002). Similarity was proven between human CYP2C and chicken CYP2H1 and CYP2H2 and between human CYP2J2 and chicken CYP2J19 (Antonovic & Martinez, 2011). The CYP3A subfamily is the last family important for phase I metabolism. In humans, approximately 50% of the therapeutic drugs are metabolized by the CYP3A subfamily. CYP3A4 has even been proven to be the most dominant CYP3A isoenzyme in the human intestine (Koch et al., 2002). The presence of CYP3A has been confirmed in chickens as an isoform, designated as CYP3A37, which is 60% homologous to human CYP3A4 (Ourlin et al., 2000). On the other hand, the existence of other CYP3A isoforms cannot be excluded (Antonovic and Martinez, 2011; Cortright and Craigmill, 2006; Murcia et al., 2011; Ourlin et al., 2000). In the evolutionary history of CYP3A gene family, it is described that the CYP3A80-like genes were lost, except in reptiles and aves. In all Eutheria CYP3A genes are the descendants of the CYP3A37 gene (Qiu et al.,

2008). The presence of CYP3A80 has already been described in turkeys, but some authors suggest that it may be a pseudo-gene without activity. For example, for the metabolisation of aflatoxin B1 in turkeys, CYP3A37 was the only investigated CYP3A isoform (Rawal and Coulombe, 2011).

Midazolam (MDZ) is known to be a marker substrate in humans and rats for CYP3A4 (Kobayashi et al., 2002; Kronbach et al., 1989; Watkins, 1994). This drug belongs to the group of the benzodiazepines and is metabolized via CYP4503A members in the liver to 1-hydroxy, 4-hydroxy or 1,4-dihydroxy metabolites. Chickens form 1-hydroxy (1-OH)-midazolam as the major metabolite, which is also reported for humans (Cortright and Craigmill, 2006).

In our study, we investigated the hepatic and intestinal gene expression of CYP3A37 in broilers using RT-PCR. A comparative study was performed between two different microsomal preparation methods for chicken small intestines. In addition, we also evaluated the CYP3A activity using midazolam as a substrate.

MATERIALS AND METHODS

Chemicals

Gibco® Dulbecco's phosphate buffered saline (PBS) was obtained from Life Technologies (Ghent, Belgium). Potassium chloride, potassium dihydrogenphosphate, sodium chloride, disodium hydrogenphosphate, dipotassium hydrogenphosphate, glycerol and ethylenediaminetetra-acetic acid (EDTA) were purchased from VWR (Leuven, Belgium). Chlorpropamide, trisodium citrate dihydrate, dithiothreitol, histidine and sucrose were purchased from Sigma-Aldrich (St-Louis, MO, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) was obtained at Biopredic International (Rennes, France). Midazolam and 1-OH-midazolam were kindly donated by Roche (Basel, Switzerland). Protease Inhibitor Cocktail Tablets Complete Mini were obtained from Roche (Vilvoorde, Belgium). These Protease Inhibitor Cocktail Tablets consist of pancreatic extract (0.02 mg/ml), pronase (0.005 mg/ml), thermolysin (0.0005 mg/ml), chymotrypsin (0.0015 mg/ml) and papain (0.33 mg/ml).

Animals and sample collection

The experimental procedures conducted with the broiler chickens were in accordance with the European guidelines for the care and use of animals for research purposes.

Sampling for intestinal microsome preparation. Healthy broiler Ross chicks (n=6, 3 ♀ and 3 ♂) aged 4 weeks used in this study were of a commercial strain. After cervical dislocation, various parts of 12 cm of the small intestines were collected. The different sampling sites along the digestive tract of the broilers were as follows: 2 cm posterior to the gizzard (intestine 1), immediately anterior to the Meckel's diverticulum (intestine 2) and 2 cm anterior to the ileo-caecal transition (intestine 3). Parts were divided in three different parts of 4 cm each and were opened longitudinally with a pair of scissors. The tissues were rinsed with PBS to remove partially digested feed residues and put in separate vials. No scraping of the mucosa was performed. After snap-freezing, the tissues were kept at -80°C until analysis. To try to reduce variability, random combinations of samples were used to test the protocols. Samples were pooled as follows: 1 tissue set consisted of 3 pieces of 4 cm from

the same intestinal part but from three random chickens. Consequently, every intestinal tissue sample had a total length of 12 cm. Three tissue sets per region were tested for each protocol.

Sampling for CYP3A37 gene expression. Fourteen broiler chickens (7 ♀ and 7 ♂) ages 4 weeks were euthanized by cervical dislocation. Samples of the liver (100 mg) and various parts of 1 to 2 cm of the small intestines were collected. The sampling sites along the digestive tract were as described above. The samples were rinsed in PBS, immediately frozen in liquid nitrogen and kept at -80°C until analysis.

Sampling for CYP3A activity measurements. The same number of animals as described for gene expression, were euthanized. A segment of 4 cm was taken from each intestinal part (intestine 1, 2 and 3 as described previously). The segments were opened and rinsed with PBS. Liver samples of 8 g were also washed carefully with PBS. All the samples were collected in separate cryovials, immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Microsomal preparation for CYP3A activity measurements

Hepatic microsomes were prepared using a process of differential ultracentrifugation (Wilson et al., 2003). All processing of tissue was performed on a bed of ice. Liver tissue was minced and homogenized in 4 ml homogenization buffer (pH 7.25, 0.25 M phosphate buffer, 1.15% KCl) per g tissue. After centrifugation at 10,000 *x g* (15 minutes, 4°C), the resulting supernatant was centrifuged at 100,000 *x g* for 75 minutes (4°C). In order to wash the formed microsomal pellet, it was resuspended in homogenization buffer (4 ml/g tissue) and centrifuged again following the last conditions. The final microsomal pellet was resuspended in 1 ml/g tissue of resuspension buffer (pH 7.25, 0.25 M phosphate buffer, 1.15% KCl, 30% glycerol). The resuspended microsomes were immediately frozen in liquid nitrogen and stored at -80°C until activity measurements.

Intestinal microsomes were prepared using two different methods, described by Mohri and Uesawa (2001) for rat small intestinal epithelial cells. The first method was based on mincing

in a Potter system, the other method also used the Potter system but was preceded by an EDTA-based separation of the enterocytes.

To start both methods, samples were immersed in Solution A (pH 7.3, 1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4 and 1 tablet protease inhibitor cocktail/10 ml) while thawing for approximately 3 minutes at 41°C in an incubation oven, which corresponds with the avian body temperature. The thawed sample was blot dried and weighed.

Mincing method. The thawed sample was minced with scissors and homogenized using an automated Potter-Elvehjem system (500 rpm, 7 up and down movements) in 4 ml homogenization buffer (pH 7.4, 50 mM KH_2PO_4, 50 mM K_2HPO_4, 1 mM EDTA and 1 tablet protease inhibitor cocktail/ 10 ml) per g sample. The homogenate was centrifuged at 18,000*xg* for 15 minutes (4°C), and the resulting supernatant was centrifuged at 100,000 *x g* for 60 minutes (4°C). The resulting microsomal pellet was washed with resuspension buffer (pH 7.5, 125 mM KH_2PO_4, 125 mM K_2HPO_4, 1.25 mM EDTA, 20% glycerol and 1 tablet protease inhibitor cocktail/10 ml), centrifuged again at 100,000 *x g* for 60 minutes (4°C) and resuspended in 1 ml resuspension buffer per g tissue.

EDTA-separation method. The thawed sample was immersed in 4 ml Solution B for 2 minutes (pH 7.4, 2.7 mM KCl, 137 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 1.5 mM EDTA, 0.5 mM dithiothreitol and 1 tablet protease inhibitor cocktail/10 ml) while gently squeezing the tissue and swirling the dish. The separated enterocytes were collected, and the immersion in Solution B was repeated 3 times in total. The cells were rinsed with 12 ml Solution C (pH 7.0, 5 mM histidine, 0.25 mM sucrose, 0.5 mM EDTA and 1 tablet protease inhibitor cocktail/10 ml) and centrifuged at 800*xg* for 15 minutes (4°C). Subsequently, the cells were homogenized with Solution C (Potter-Elvehjem at 500 rpm) and centrifuged at 15,000 *x g* for 15 minutes (4°C). The supernatant was then centrifuged at 100,000 *x g* for 45 minutes (4°C), and the microsomal pellet was resuspended as described above in the mincing method.

The obtained microsomal suspensions, after applying one of the 2 above mentioned methods, were snap frozen in liquid nitrogen and stored at -80°C. Protein concentration of all microsomes was measured using the method described by Bradford (1976).

Sample processing

CYP3A37 gene expression. According to the manufacturer's instructions, total RNA was isolated from the liver and intestinal samples using RNAzol® RT (MRC Inc., Cincinnati, USA). An Experion RNA StdSens Analysis kit (Biorad Laboratories, Hercules, CA, USA) was used to check the purity of the RNA samples. RNA concentrations were measured using a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) at an absorbance of 260 nm. Reverse transcription was carried out in a 20 µl final volume that included 4 µl of 5 x iScript Reaction Mix, 1 µl of iScript Reverse Transcriptase, 1 µl of RNA template (1 µg) and nuclease-free water to complete the final volume. Different components described above are part of the iScript cDNA Synthesis Kit (Biorad Laboratories). The mix was incubated at 25°C for 5 min, heated to 42°C for 30 min and inactivated at 85°C for 5 min. The obtained cDNA was stored at -20°C until analysis. Absolute quantification was carried out using the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers used for CYP3A37 amplification were adopted from Zhang *et al.* and were as follows: CYP3A37 forward, 5'-CGAATCCCAGAAATCAGA–3'; CYP3A37 reverse, 5'-AGCCAGGTAACCAAGTGT-3' (GenBank ID: NM 001001751.1) (Zhang et al., 2010). The annealing temperature was set at 60°C for the primers. The quantitative RT-PCR reaction was carried out in 96-well plates with 500nM of the forward and reverse primers, 5 µl iQ™ SYBR® Green Supermix (Biorad) and 1 µl of the fivefold diluted cDNA template, in a total reaction volume of 10µl. The thermal cycler conditions were 15 min at 95°C, followed by 40 cycles of 20s at 95°C, 30s at 60°C, 30s at 72°C. The melt curve analysis showed that the RT-PCR generated PCR-products had identical melt-points for all tissues, indicating that the PCR-products amplified are the same in the various tissues. For construction of the standard curve, a PCR product of 396 bp was generated using DNA from chicken liver and the following primers: forward, 5'-CCCCAGTGATGCTGTAGATT-3'; reverse, 5'-TAGAGCCGGAGGGTTTCATT-3'. After purification with an Invitek kit (Germany) and measuring the DNA concentration with a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies), the volume of the linear dsDNA standard was adjusted to 1 x 10^8 copies/µl. This stock solution was serially diluted to obtain a standard series with each step differing by 10-fold. The copy numbers of samples were determined by reading off the standard series with the C_t values of the samples. Three amplifications were performed for each CYP mRNA

and the standard curve was repeated on each plate in duplicate. For the validation of the qPCR assays following criteria were applied: slope between -3.6 and -3.1, efficiency between 90 and 110 %, $R^2 > 0.99$.

CYP3A activity measurements. Microsomal incubations were performed at 41°C in a shaking heat block. For each broiler and tissue two separate incubations were run in duplicate. Mixtures of 1.15% KCl, 0.05 M phosphate buffer (pH 7.4) and midazolam (final concentration of 25 µM according to the study of Cortright and Craigmill (2006) our chosen concentration corresponds with Vmax) were pre-heated. After addition of NADPH (final concentration in incubation medium of 1 mM), the mixture was pre-incubated for 3 minutes, after which the incubation reaction was initiated by the addition of the microsomal dilution. Incubations contained 0.275 mg protein/ml. After exactly 20 minutes, the enzymatic reactions were terminated through the addition of the stop reagent (water/acetonitrile/formic acid (42/55/3, (v/v/v)) containing the internal standard (IS) chlorpropamide (final concentration in terminated incubation mixture of 0.072 µM). The terminated samples were vortex mixed and cooled on ice, after which they were centrifuged at 20,000 *x g* for 10 minutes (4°C). The supernatants were collected and frozen at -20°C until analysis.

The amounts of 1-OH-midazolam that were formed during the incubations were quantified using a validated UPLC-MS/MS method published elsewhere (De Bock et al., 2012). In short, 20 µl of the supernatant was injected onto a Waters Acquity UPLC BEH C18 column (50 mm x 2.1mm, 1.7 µm particle size) with a VanGuard pre-column (5 mm x 2.1 mm, 1.7 µm particle size). 1-OH-MDZ eluted at 2.08 min, and the IS at 2.75 min, after gradient elution, using water with 0.1% formic acid and acetonitrile with 0.1% formic acid at a flow rate of 400 µl/min. Detection was performed using a quadrupole mass spectrometer in the multiple reaction monitoring mode (*m/z* 1-OH-MDZ 342.04> 323.70, *m/z* IS 276.86> 174.69). A linear calibration curve with weighting factor $1/x^2$ was calculated within 4.29-1929.60 nM range. The method accuracy was 93.52 – 102.61%, and the between-day and within-day precision were <4.82% RSD and <4.61% RSD, respectively. Data were analysed with MassLynx software (v 4.1).

Data analysis

Results from the expression and activity measurements were compared between the different sections by ANOVA after determination of normality and variance homogeneity. Multiple comparisons were performed using a LSD post-hoc test. Not normally distributed data were analyzed using the non-parametric Kruskal-Wallis analysis, followed by a Mann-Whitney test using SPSS 19.0 Software (SPSS Inc., Chicago, IL, USA). A *P*-value of <0.05 was considered statistically significant.

RESULTS

MINCING preparation method results in higher CYP3A activity per gram of intestinal tissue compared to the EDTA protocol

Mean total protein content of the microsomes prepared using the mincing method was higher (mean= 7.063 mg/ml; SD= 1.61 mg/ml) than with the EDTA-separation method (mean= 2.196 mg/ml; SD= 0.56 mg/ml). Normalization of the CYP3A activity to gram tissue confirmed that the mincing protocol had a higher yield than the EDTA-based method (data not shown). The EDTA preparation resulted in a lower protein content, which makes it more difficult to evaluate the activity of CYP3A in the small intestines of broiler chickens. Therefore, the mincing protocol was applied to test intestinal CYP3A activity in the group with 14 animals (see results presented below).

Quantification of CYP3A37 mRNA expression in liver and along the entire length of small intestines of broiler chickens

Analysis of the data shown in Figure 1 indicates that the CYP3A37 gene expression in the intestines is substantially higher immediately anterior to the Meckel's diverticulum (intestine 2), even significantly higher than in the ileum (intestine 3) ($P<0.05$). The expression in the proximal part (intestine 1) is comparable to the most distal part of the small intestine (intestine 3). Remarkably, the expression level is the lowest in the liver of the broilers with a significant difference with intestine 2 ($P<0.001$). A similar trend was observed for both male and female animals.

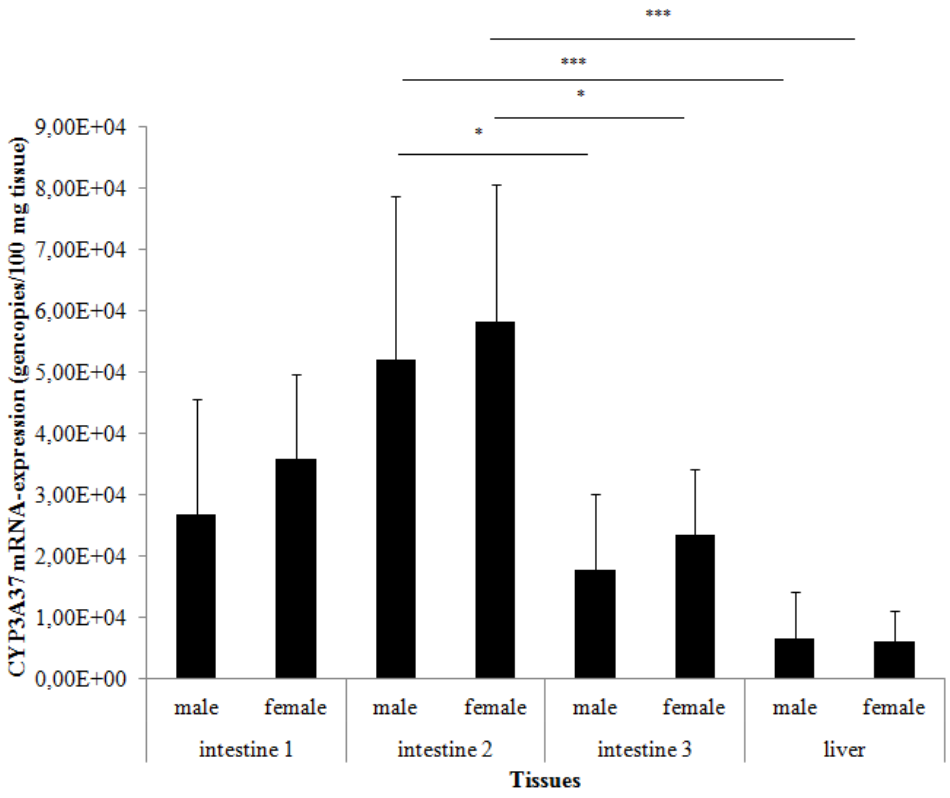

Figure 1. Expression of CYP3A37 mRNA in the small intestines and liver of broilers (n= 14, 7 ♂ and 7 ♀). Each expression level represents the mean + standard deviation of the seven broilers based on three independent amplifications. (*: P<0.05; *** : P<0.001).

CYP3A activity decreases from proximal to distal in the chicken small intestine

Figure 2 shows the CYP3A activity after applying the mincing method for the preparation of intestinal microsomes. The highest metabolic activity was observed in the proximal part, with a clearly decreasing trend towards the distal part of the small intestines (P<0.001). The mean hepatic midazolam biotransformation activity is similar as the level observed in the proximal part of the small intestines. Again, a similar trend was observed for both male and female birds.

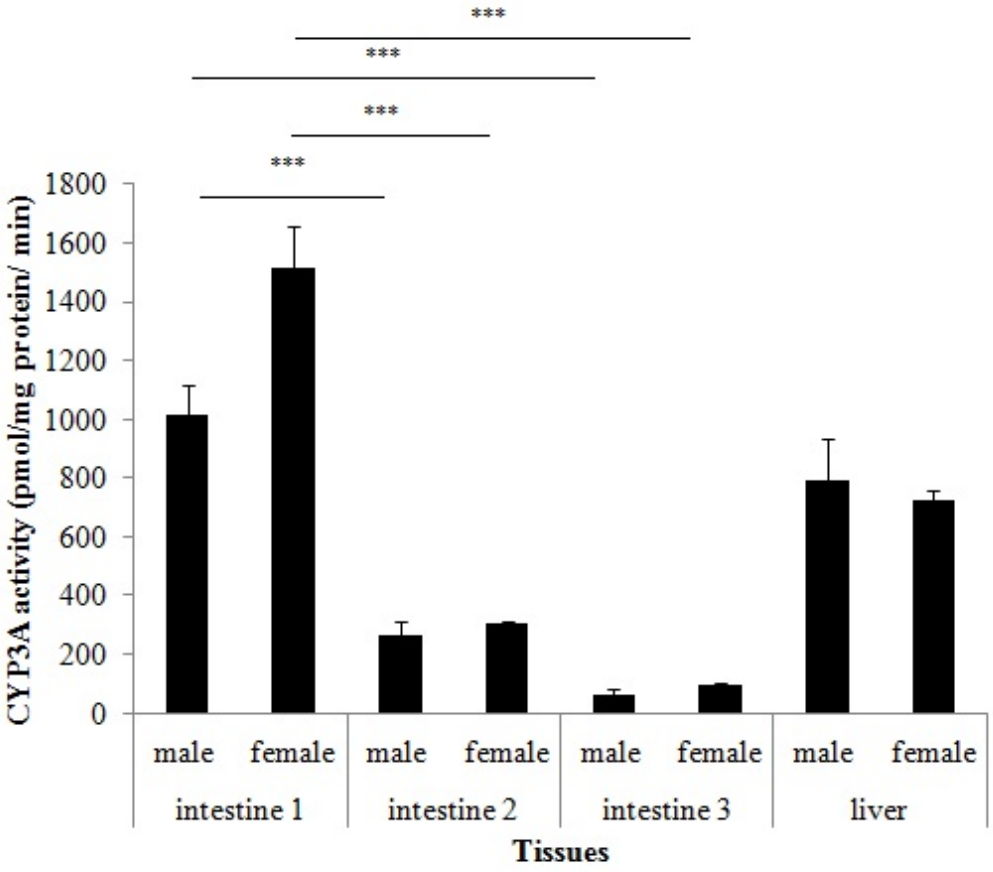

Figure 2. CYP3A subfamily activity in the small intestines and liver of broilers (n= 14, 7 ♂ and 7 ♀). For each broiler and tissue, two separate incubations were run in duplicate. Data are represented as mean activity + standard deviation.

DISCUSSION

In this study, we analyzed the CYP3A expression and activity in liver and small intestines of broilers. Discordant regulation of these enzymes in liver and small intestine has been observed in man (Lown et al., 1994) and rats (Hakkak et al., 1993).

A newly developed RT-PCR method showed that CYP3A37 mRNA in broilers is expressed in a much higher extent immediately anterior to Meckel's diverticulum. From these results, it can be concluded that the CYP3A37 expression in the jejunum of broilers is substantially higher than in the duodenum or ileum of these animals. In cynomolgus monkeys, mRNA of CYP3A4 was abundantly expressed in the distal part of the duodenum and the upper jejunum. Again a decreasing trend was observed towards the distal ileum (Nakanishi et al., 2010). A similar trend was also observed in rats (Mitschke et al., 2008). However, our results are in contrast with the findings in horses, where the CYP3A4 expression was noticed to be the highest in the proximal intestine with a decreasing trend towards the distal parts (Tyden et al., 2004). Unfortunately, there is no further literature available to compare with, especially not for poultry species. Our study also showed that the expression of CYP3A37 mRNA in the livers of broilers is lower in comparison to the intestines of these animals. Previous studies in horses and humans also resulted in a higher CYP3A4 mRNA expression in the proximal intestine compared to the liver (Kolars et al., 1992; Tyden et al., 2004).

In addition, the expression of CYP3A37 mRNA showed high inter-individual differences, in both liver and intestines. Interpatient CYP3A4 heterogeneity in humans was previously described by Lown *et al.* (1994). Moreover, existence of large inter-breed differences in CYP450 expression patterns has been reported by Fink-Gremmels (2008). Ozdemir *et al.* reported that at least 60% or more of the variability in CYP3A4 activity is under genetic control (Ozdemir et al., 2000). It is known that CYP450 isoforms are also influenced by many other factors including physiological and environmental ones (Watkins, 1994; Antonovic & Martinez, 2011). However, gender differences in CYP3A37 mRNA expression were not seen in our study, both sexes revealed similar variation in expression. CYP3A activity, on the other hand, was observed using midazolam as substrate. The protocols that were evaluated in this study to prepare intestinal microsomes, were based on the paper of Mohri and Uesawa (2001). These authors compared different preparation methods of rat intestine, based on

either scraping or EDTA-separation of the enterocytes, followed by Ca-aggregation or ultracentrifugation in order to obtain the microsomal fraction. Other useful information was obtained from the study by Tyden et al. (2004), which describes a procedure for the preparation of horse intestinal microsomes after scraping of the mucosal cells with a scalpel, as also done by Paine et al. (1997) for human intestinal microsomes. The subsequent procedure was similar to the ultracentrifugation procedure described by Mohri and Uesawa. Results of our study revealed that the protocol based on mincing of the tissues leads to a higher total protein content compared to the method with EDTA-separation. Different preparation procedures can indeed result in different enzymatic activities. Mohri and Uesawa (2001) concluded that the separation of enterocytes with EDTA-treatment is more appropriate than a method that involves scraping of the enterocytes with a microscope slide. In our study, the EDTA-separation seemed to be difficult to standardize with a lower reproducibility as consequence. This method also did not provide a guarantee about the quantity of enterocytes that is separated in the supernatant, which is further used for homogenization. In the mincing protocol, the whole tissue is homogenized after mincing. For the investigation of the *in vitro* biotransformation of drugs, high enzymatic activities are necessary to guarantee accurate and representative measurements, and therefore the mincing method is considered superior. Moreover, high protein levels are necessary for chickens, due to the low hepatic CYP content reported in these animals compared to other species, such as mice, rats, horse and ruminants (Khalil et al., 2001).

Watkins (1994) described different substrates to test CYP3A enzymes such as erythromycin, midazolam, cortisol, nifedipine, dapsone and lignocaine. From all these substrates midazolam seemed to be the most appropriate as substrate for CYP3A (Watkins, 1994). In addition, MDZ has already been used in different avian species as a substrate for CYP3A (Cortright and Craigmill, 2006). In chicken the production of MDZ metabolites in liver microsomes demonstrated Michaelis-Menten kinetics with K_m equal to 2.1±0.8 µM for 1-OH-MDZ (Cortright and Craigmill, 2006). Therefore, MDZ was also used in our study to evaluate the CYP3A activity in liver and intestines of the broilers. Both liver and duodenum are involved in the first pass metabolism, which explains the high CYP3A activity in the duodenum and the liver.

The discordance between the CYP3A mRNA expression and the activity of these enzymes may suggest that transcriptional or posttranscriptional factors may be involved in the regulation of CYP3A in the small intestines of chickens. A poor correlation between CYP3A mRNA expression and protein activity has also been seen in humans. However, the protein concentration and activity were in accordance and thus, posttranscriptional regulation of CYP was supposed. (Lown et al., 1994). On the other hand, it can't be excluded that the results for CYP3A activity may be influenced by the presence of other CYP3A isoforms for which midazolam can be a substrate in chickens (Murcia et al., 2011).

From our findings, we can conclude that the cytochrome P450 enzymes in the mucosal villi of the small intestine in broilers can have an equal or even a higher importance for the first-pass metabolism of some drugs and xenobiotics than the liver. The inter-species variations can lead to large differences in oral bioavailability of drugs which are subject to first-pass metabolism. A variable susceptibility to diseases and environmental factors of the animals can also be explained. From the differences between the various intestinal sections, we can conclude that the sample sites have to be carefully described and selected for the measurement of the CYP mRNA expression levels and activity.

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Chapter 5.

Effects of T-2 toxin on intestinal and hepatic biotransformation mechanisms and transporter systems

Adapted from: Osselaere, A., Li, S.J., De Bock, L., Devreese, M., Goossens, J., Vandenbroucke, V., Van Bocxlaer, J., Boussery, K., Pasmans, F., Martel, A., De Backer, P., Croubels, S. (2013). Toxic effects of dietary exposure to T-2 toxin on intestinal and hepatic biotransformation enzymes and drug transporter systems in broiler chickens. Food and Chemical Toxicology 55, 150-155..

ABSTRACT

The effects of the mycotoxin T-2 on hepatic and intestinal drug-metabolizing enzymes (cytochrome P450) and drug transporter systems (MDR1 and MRP2) in poultry were investigated during this study. Broiler chickens received either uncontaminated feed, feed contaminated with 68 µg/kg or 752 µg/kg T-2 toxin. After three weeks, the animals were euthanized and MDR1, MRP2, CYP1A4, CYP1A5 and CYP3A37 mRNA expression were analyzed using qRT-PCR. Along the entire length of the small intestine no significant differences were observed. In the liver, genes coding for CYP1A4, CYP1A5 and CYP3A37 were significantly down-regulated in the group exposed to 752 µg/kg T-2. For CYP1A4, even a contamination level of 68 µg/kg T-2 caused a significant decrease in mRNA expression. Expression of MDR1 was not significantly decreased in the liver. In contrast, hepatic MRP2 expression was significantly down-regulated after exposure to 752 µg/kg T-2. Hepatic and intestinal microsomes were prepared to test the enzymatic activity of CYP3A. In the ileum and liver CYP3A activity was significantly increased in the group receiving 752 µg/kg T-2 compared to the control group. The results of this study show that drug metabolizing enzymes and drug transporter mechanisms can be influenced due to prolonged exposure to relevant doses of T-2.

INTRODUCTION

Mycotoxins are widely distributed contaminants of different crops intended for human and animal consumption (Fink-Gremmels, 1999; Placinta et al., 1999). T-2 toxin (T-2) is a trichothecene mycotoxin, produced by fungi belonging to the *Fusarium* species. Among the trichothecenes, T-2 is the most acutely toxic (Hussein and Brasel, 2001). In broiler chickens, T-2 causes reduced feed intake and body weight gain, but also severe oral lesions and immunological dysfunction (Devegowda and Murthy, 2005). Clinical signs depend on the exposure time and on the dose of the toxin. In addition, T-2 is a potent inhibitor of the protein synthesis and tissues with a high cell division rate, like the intestinal mucosa and the liver, are the most susceptible (Sokolovic et al., 2008). Moreover, this toxin causes oxidative stress that alters the cell cycle and induces apoptosis *in vitro* and *in vivo* (Chen et al., 2008; Islam et al., 1998). Although T-2 is known to cause important economic losses, no maximum guidance limits for T-2 in food and feedstuff have been pointed out by the European Union until now (Anonymous, 2011). However, T-2 can be considered as an emerging contaminant since concentrations up to 1810 µg T-2 toxin per kg wheat have been reported in Germany (Schollenberger et al., 2006). In poultry the generally accepted no observed adverse effect level (NOAEL) value is set at 0.5 mg T-2 /kg feed (Eriksen and Pettersson, 2004).

Drugs and feed contaminants are both xenobiotics. Being considered foreign products, they are both eliminated from the animal body by comparable processes. Before their excretion in the urine or faeces, biotransformation of these compounds can take place. Biotransformation is also possible before absorption of the compound into the systemic circulation. Both liver and intestine are involved in this first pass metabolism of orally ingested xenobiotics. Cytochrome P450 (CYP450) enzymes play a dominant role in the process of oxidative biotransformation (Hollenberg, 1992). In humans, most drugs are metabolized by the CYP3A4 isoform (Hardman, 2001). Presence of CYP3A subfamily has been confirmed in chickens, as an isoform CYP3A37 has been found with a homology of 60% to human CYP3A4 (Ourlin et al., 2000).

Beside the superfamily of cytochrome P450, active transport-systems also play a role in the protection against xenobiotics. These transporters are called adenosine-tri-phosphate (ATP)-binding cassette (ABC) carrier proteins and are able to remove xenobiotics from cells using

ATP hydrolysis as energy source. Multiple drug resistance 1-gene (*MDR1*, which encodes P-glycoprotein (P-gp)) and multidrug resistance-associated protein 2 (*MRP2*) gene are genes coding for membrane proteins belonging to the ABC superfamily (Cherrington et al., 2002; Tusnady et al., 1997).

Drug-food interactions often occur through effects on gastro-intestinal motility or by drug binding. On the other hand, drug metabolism and drug transporter can also be influenced by food or feed and their contaminants. Grapefruit for example, is a very well-known inhibitor of CYP3A4 in humans and is responsible for increased drug levels (Ameer and Weintraub, 1997). Grapefruit compounds such as flavonoids, furocoumarins and furanocoumarins have been identified as inhibitors of the intestinal CYP3A4 in different *in vitro* studies (Fukuda et al., 1997; Ho et al., 2001). These grapefruit compounds bind irreversibly to CYP3A4 and permanently inactivate the isoenzyme. The mode of action is called mechanism-based inhibition and the duration of this inhibition may be longer than competitive inhibition since new CYP3A4 isoenzymes must be synthesized for activity to be restored. Inhibition of intestinal CYP3A4 enzymes leads to an increased maximum plasma concentration and total amount of the drug (Bressler, 2006). Different studies already reported the negative influence of T-2 on the normal metabolism of xenobiotics in the liver (Kravchenko et al., 1986; Suneja et al., 1989). Negative effects of short-term exposure to high doses of T-2 on CYP1A expression have been reported in rats and rabbits (Galtier et al., 1989; Guerre et al., 2000). One study reports the impact of chronic exposure to T-2 on CYP1A protein expression in pigs (Meissonnier et al., 2008). Studies mentioned above mainly focused on the CYP1A subfamily expression and only in the liver. We focused on the effects of long-term exposure to T-2 on CYP1A4, CYP1A5 but also on CYP3A-expression in broilers. In addition, the influence of prolonged exposure to T-2 on the enzymatic activity of CYP3A was studied, using midazolam as substrate. Both liver and small intestines were included in our study, since both organs play a dominant role in the first pass effect. The last aim of our study was to investigate the possible impact of prolonged T-2 exposure on the mRNA expression of MDR1 and MRP2 transporter-mechanisms in broilers.

MATERIALS AND METHODS

Chemicals

Dulbecco's phosphate buffered saline (PBS) was obtained from Gibco® Life Technologies (Ghent, Belgium). Potassium chloride, potassium dihydrogenphosphate, sodium chloride, disodium hydrogenphosphate, dipotassium hydrogenphosphate, glycerol and ethylenediaminetetra-acetic acid (EDTA) were purchased from VWR (Leuven, Belgium). Chlorpropamide and trisodium citrate dihydrate were purchased from Sigma-Aldrich (St-Louis, MO, USA). Nicotinamide adenine dinucleotide phosphate (NADPH) was obtained at Biopredic International (Rennes, France). Midazolam and 1-OH-midazolam were kindly donated by Roche (Basel, Switzerland). Protease inhibitor Cocktail Tablets Complete Mini were obtained from Roche (Vilvoorde, Belgium).

Animal experiments

Animals and tissue sampling. Twenty-four one-day old broiler chickens (12 ♀ and 12 ♂) were obtained from a commercial strain (Belgabroed, Hoogstraten, Belgium) and housed together according to the requirements of the European Union (Anonymous, 2007). The experiments started after 10 days of acclimatization, allowing the animals to adapt to the environment. For the study, the animals were divided in three experimental groups, each consisting of 8 animals. Three different experimental diets were fed during three weeks: the first group received control feed, the second and third group received feed contaminated at 68 and 752 µg/kg T-2, respectively. Feed and water were given *ad libitum*. The applied light cycle was the same as on the commercial farm (20 hours light/ 4 hours dark). Animals were weighed before feeding the different diets and at the end of the experiment, in order to calculate the body weight (BW) gain over the experimental period (BW d 33 – BW d 11). After three weeks of feeding, the animals were killed by cervical dislocation. Organs (liver, heart, kidneys, proventriculus and ventriculus) were weighed and the lengths of the duodenum, jejunum and ileum were measured. Afterwards, tissue samples were collected. Intestinal sections, 1 cm long, were collected for the mRNA expression measurements (qRT-PCR analysis). The different sampling sites along the digestive tract of the broilers were as follows: 2 cm posterior to the gizzard (duodenum), immediately anterior to the Meckel's

diverticulum (jejunum) and 2 cm anterior to the ileo-cecal transition (ileum). After rinsing in PBS and freezing in liquid nitrogen, the tissues were kept at -80°C until analysis. For the investigation of CYP activity the same sampling sites were applied, but parts of 2 cm were taken. From the liver, samples of approximately 1 cm^3 (qRT-PCR analysis) and 2 cm^3 (activity measurements) were taken and rinsed in PBS. The samples were immediately frozen in liquid nitrogen and kept at -80°C until analysis. All animal experiments were approved by the ethics committee of the Faculty of Veterinary Medicine, Ghent University (EC 2011/033).

Feed preparation and experimental diets. Conventional broiler feed (Bromix Plus®) was purchased from Versele-Laga (Deinze, Belgium). It was considered as uncontaminated feed after analysis for the presence of mycotoxins by a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry (LC-MS/MS) method (Fytolab, Zwijnaarde, Belgium). This feed is further referred to as control feed and was used to feed the animals during the acclimatization period as well as to prepare the T-2 contaminated feeds needed for the experiment.

To produce feed of a contamination level of approximately 100 µg T-2/kg feed, a stock solution of 0.55 mg/mL was prepared by dissolving 55 mg T-2 (Fermentek, Jerusalem, Israel) in 100 mL ethanol (Merck, Darmstadt, Germany). The contaminated feed was produced by adding 9.1 mL of the stock solution to 500 g of control feed. This premix was then mixed with 5 kg control feed to assure homogeneous distribution of the toxin. The premix was finally mixed for 15 min in the total amount of feed (50 kg) needed for the experiment. To test T-2 homogeneity in the feed, a sample was taken at three different locations in the batch and analysed with LC-MS/MS to determine the concentration of T-2. A mean concentration of 68 µg/kg feed was found in this T-2 contaminated feed. To produce feed with a contamination level of approximately 1000 µg T-2/kg feed, a similar procedure as described above was applied but 90.9 mL of the stock solution was used instead of 9.1 mL. After the production of the contaminated feed, again analysis of the presence of T-2 was investigated at three different sampling sites. The analysis resulted in a mean contamination level of 752 µg/kg feed.

Analysis of CYP1A4, CYP1A5, CYP3A37, MDR1 and MRP2 mRNA expression using qRT-PCR

Samples of the liver and different intestinal segments were collected as described above. Total RNA from the tissue samples was isolated using RNAzol®RT (MRC Inc., Cincinnati, USA) according to the manufacturer's instructions. The RNA concentration was measured by absorbance at 260 nm with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). An Experion RNA StdSens Analysis kit (Biorad Laboratories, Hercules, CA, USA) was applied to test the purity of the RNA samples. Reverse transcription was carried out using the iScript cDNA Synthesis kit of Biorad Laboratories. This reaction was carried out in a 20 µL final volume that included 4 µL of 5x iScript Reaction Mix, 1 µL of iScript Reverse Transcriptase, 1 µL of RNA template (1 µg), and nuclease-free water to complete the final volume. The reverse transcription mix was incubated at 25°C for 5 min, heated to 42°C for 30 min, and inactivated at 85°C for 5 min. The obtained cDNA was stored (-20°C) until further analysis. The primers for CYP1A4, CYP1A5 and CYP3A37 were adopted from Zhang *et al.* (Zhang et al., 2010) and for the transporter carriers MDR1 and MRP2 from Haritova *et al.* (Haritova et al., 2010). The specificity of the primers was tested by performing a BLAST search against the genomic NCBI database. A list of the genes and their sequences used for quantitative PCR analysis is given in Table 1. Housekeeping genes were tested for all the test conditions and all the samples after which most stable housekeeping genes for liver and intestinal samples were selected using the geNorm software (data not shown). The most stable housekeeping genes had a M-value between 0.2 and 0.5. To determine if the inclusion of an additional housekeeping gene was required, the cut-off value for variation was set at 0.2. GAPDH and β-actin were used as housekeeping genes for the hepatic samples; HPRT and RPL7 were the most stable housekeeping genes for the intestine. The genes of interest (CYP1A4, CYP1A5, CYP3A37, MDR1 and MRP2) were quantified using real-time quantitative PCR. PCR reactions were carried out in 96-well plates with the appropriate forward and reverse primers (500 nM), 5 µL of iQ™ SYBR® Green Supermix (Biorad Laboratories) and 1 µL of the fivefold diluted cDNA template. Thermocycling parameters were used according to the manufacturer's instructions and included 40 cycles of 20 s at 95°C, 30 s at 60°C, 30 s at 72°C. For the validation of the qPCR assays following criteria were applied: slope between -3.6 and -3.1, efficiency between 90 and 110 %, R^2 > 0.99. The threshold cycle values (Ct) were first normalized to the geometric means of appropriate reference mRNAs and the

normalized mRNA levels were calculated according to $2^{-\Delta\Delta}$Ct method (Livak and Schmittgen, 2001).

Table 1. List of genes and sequences of the primers used for quantitative PCR analysis.

Gene name	Forward primer (5'→3')	Reverse primer (5'→3')	Accession number
β-actin	CACAGATCATGTTTGAGACCTT	CATCACAATACCAGTGGTACG	NM_205518
GAPDH	GGCACGCCATCACTATC	CCTGCATCTGCCCATTT	NM_204305
HPRT	CCCAAACATTATGCAGACGA	TGTCCTGTCCATGATGAGC	NM_204848
RPL7	ACATGTACAGACAGGAGATCCGCA	AAGCTGTAACACCTTCCGGACCTT	NM_001006345
CYP1A4	AGGACGGAGGCTGACAAG	CAGGATGGTGGTGAGGAAGA	NW_003763853
CYP1A5	TCACCATCCCGCACAGCA	AAGTCATCACCTTCTCCGCATC	NW_003763826
CYP3A37	CGAATCCCAGAAATCAGA	AGCCAGGTAACCAAGTGT	NW_003763931
MDR1	GCTGTTGTATTTCCTGCTATGG	ACAAACAAGTGGGCTGCTG	NM_204894
MRP2	CTGCAGCAAAATGAGAGGACAATG	CAGAAGCGCAGAGAAGAAGACCAC	XM_421698

Analysis of CYP3A activity

For the preparation of hepatic microsomes, a process of differential ultracentrifugation was used (Wilson et al., 2003). Liver tissues were first minced and afterwards homogenized in homogenization buffer (4 mL/gram tissue: pH 7.25, 0.25 M phosphate buffer, 1.15% KCl). The supernatant recovered after an initial centrifugation step (10000 *g*, 15 minutes, 4°C), was subsequently centrifuged at 100000 *g* for 75 minutes (4°C). Between centrifugation steps, tissue was held on ice. The microsomal pellet was washed in homogenization buffer and again centrifuged following the last conditions. The final pellet was then resuspended in resuspension buffer (1 mL/gram tissue: pH 7.25, 0.25 M phosphate buffer, 1.15% KCl, 30% glycerol). Finally, the microsomes were quickly frozen in liquid nitrogen and stored at -80°C until further analysis.

For the preparation of intestinal microsomes the mincing method was applied as published in a previous paper (Osselaere et al., 2012) In brief, the sample was minced with scissors and homogenized using an automated Potter-Elvehjem system (500 rpm, 7 up and down movements) in homogenization buffer (4 mL/gram tissue: pH 7.4, 50 mM KH_2PO_4, 50 mM K_2HPO_4, 1 mM EDTA and 1 tablet protease inhibitor cocktail /10 mL). The homogenate was centrifuged (18000 *g*, 15 minutes, 4°C) and the resulting supernatant was centrifuged (100000 *g*, 60 minutes, 4°C). Afterwards the microsomal pellet was washed with resuspension buffer (pH 7.5, 125 mM KH_2PO_4, 125 mM K_2HPO_4, 1.25 mM EDTA, 20% glycerol and 1 tablet protease inhibitor cocktail /10 mL) and centrifuged (100000 *g*, 60 minutes, 4°C). Finally, the pellet was resuspended in 1 mL resuspension buffer per gram tissue. The microsomes were immediately frozen in liquid nitrogen and stored at -80°C until activity measurements.

Protein concentration of all microsomes was measured using the method described by Bradford(Bradford, 1976). Microsomes were incubated in a heating block at 41°C, corresponding to the body temperature of poultry. A preheated mixture (41°C) with 1.15% KCl, 0.05 M phosphate buffer (pH 7.4) and midazolam (final concentration of 25 µM) and NADPH (1 mM) was pre-incubated for 3 minutes. The incubation reaction started after the addition of the microsomal dilution. The incubations were performed with 0.3 mg protein / mL. The enzymatic reactions were terminated after exactly 20 minutes by the addition of a stop reagent (water/acetonitrile/formic acid (42/55/3, (v/v/v)) containing the internal standard chlorpropamide (final concentration 0.072 µM). Afterwards, the samples were vortexed, cooled on ice and centrifuged at 20000 *g* for 10 minutes (4°C). Supernatants were stored at -20°C until analysis.

Amounts of 1-OH midazolam formed during the incubations were quantified using a validated UPLC-MS/MS method (De Bock et al., 2012). Data were analyzed with MassLynx software (v4.1).

Data analysis

The differences in mRNA expression and enzymatic activity among groups were assessed by performing ANOVA (SPSS® 19.0 software for Windows, IBM, USA) after determination of normality and variance homogeneity. Multiple comparisons were performed using LSD post-hoc test. Significance level was set at 0.05.

RESULTS

A reduced body weight gain in broilers was observed after 3 weeks feeding T-2 at 752 µg T-2/kg feed

During the whole experiment animals were observed for possible clinical signs. No symptoms of T-2 mycotoxicosis were observed. One animal died in the group with the highest concentration of T-2, already after 4h feeding. No lesions were observed during autopsy. The group receiving 752 µg T-2/kg feed, showed a significantly decreased BW gain compared to the control group and the group receiving T-2 at a lower level (68 µg T-2/kg feed) (Table 2). No significant differences were observed in organ weights and length of the small intestine between the different dietary groups (data not shown).

Table 2. Mean (± SD) body weight (BW) before and after prolonged exposure to T-2 and body weight gain.

	control	68 µg T-2 /kg feed	752 µg T-2 /kg feed
BW d 11 (g)	287 ± 64	307 ± 24	312 ± 31
BW d 33 (g)	2037 ± 295	2088 ± 136	1774 ± 142*
BW gain (g)	1750 ± 248	1780 ± 147	1464 ± 127*

After an acclimatization period of ten days the animals were divided in three different groups each receiving different diets (control or T-2 contaminated feed). Broilers were exposed to T-2 at two different concentration level (68 µg/kg and 752 µg/kg). The animals were weighed at the end of the acclimatization period (d 11) and on the day of the euthanasia (d 33). BW gain was measured as the difference between BW day 33 and BW day 11. * refers to a significantly lower BW or BW gain compared to the other dietary groups ($p<0.05$).

T-2 down-regulates CYP3A37, CYP1A4, CYP1A5 and MRP2 mRNA expression in liver, but not in the small intestine of broilers

In the intestine no significant differences between the dietary groups could be found regarding the expression of the different genes in the broilers (data not shown). However, in the liver the three CYP-genes were significantly down-regulated in the group exposed to 752

µg T-2/kg feed. For CYP1A4 also a down-regulation was observed in the animals receiving feed contaminated at 68 µg T-2/kg feed (Figure 1). In the liver also a significant down-regulation in the mRNA expression of MRP2 was noticed as illustrated in Figure 1. On the other hand, for MDR1 mRNA expression level, only a decreasing trend could be observed in the liver.

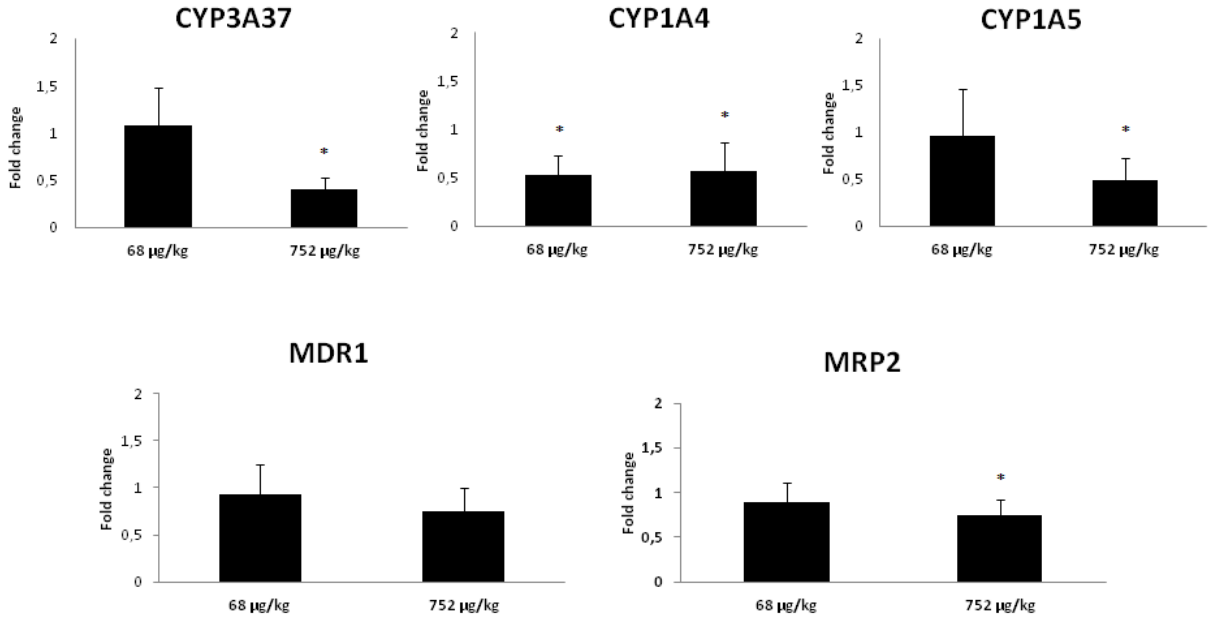

Figure 1. Fold change in CYP, MDR1 and MRP2-genes expression levels in broilers' livers after dietary T-2 exposure relative to the control group. Broilers were exposed to T-2 at two different concentration levels (68 µg/kg and 752 µg/kg). After three weeks of feeding the experimental diets, mRNA expression levels in liver were examined. Data represent the normalized target gene amount relative to control which is considered 1. Data are presented as mean + standard deviation for a total of 8 animals per dietary group, except for the group receiving 752 µg T-2/kg feed (n=7). Bars marked with * indicate significantly different responses compared to the control group ($p<0.05$).

CYP3A enzymatic activity is up-regulated in the liver and ileum of broilers after three weeks exposure to T-2 at 752 µg T-2/kg feed

T-2 exposure during three weeks at a contamination level of 752 µg/kg feed resulted in a significant increased activity of CYP3A in the liver of broilers. Results are shown in Figure 2. Feed contaminated at a lower level of T-2 (68 µg/kg feed) didn't cause significant up- or

down-regulation of CYP3A activity compared to the control group. T-2 influence on CYP3A activity was also investigated at three different sampling sites in the small intestines of the animals. CYP3A activity was measured in duodenum and jejunum, but no effects of T-2 exposure were detected at these sites. However, in the ileum CYP3A activity was significantly increased, but again only in the group receiving 752 µg/kg feed compared to the group fed an uncontaminated diet. Results are shown in Figure 3.

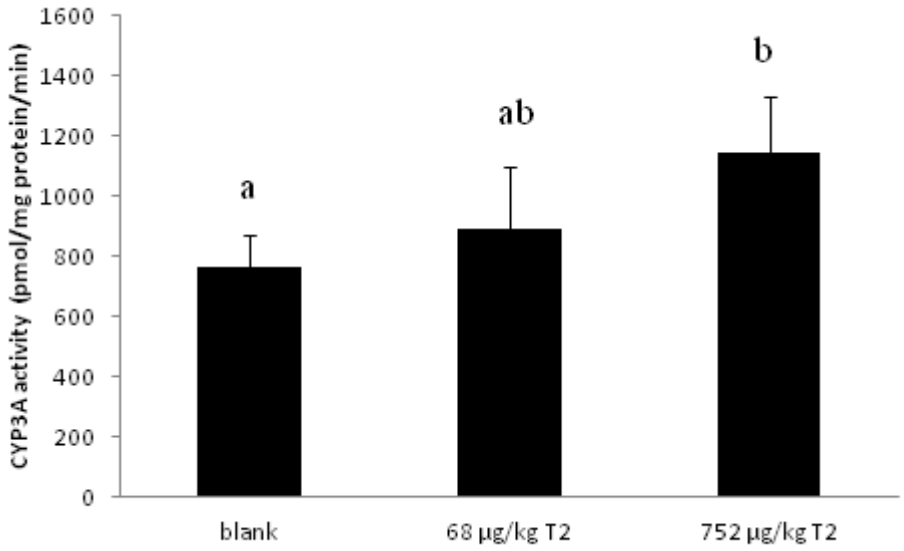

Figure 2. Effect of dietary exposure to T-2 on hepatic CYP3A activity in broilers. After three weeks of feeding the experimental diets, liver microsomes were prepared and incubated with midazolam to measure CYP3A activity. Results are presented as the mean + standard deviation of the hepatic activity for a total of 8 animals per dietary group, except for the group receiving 752 µg T-2/kg feed (n=7). Bars marked with different letters indicate significantly different activity ($p < 0.05$).

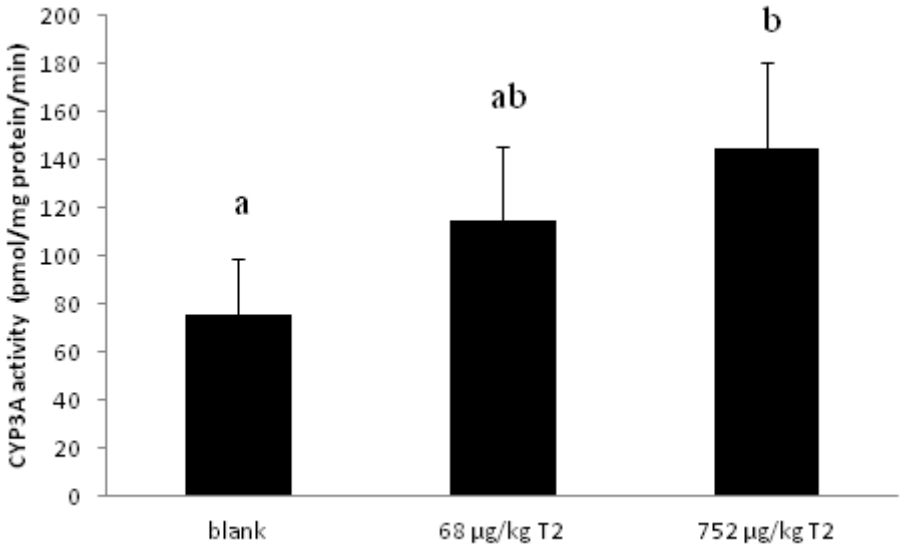

Figure 3. Effect of dietary exposure to T-2 toxin on CYP3A activity in the ileum of broilers. After three weeks of feeding the experimental diets, intestinal microsomes were prepared and incubated with midazolam to measure CYP3A activity. Results are presented as the mean + standard deviation of the intestinal activity for a total of 8 animals per dietary group, except for the group receiving 752 µg T-2/kg feed (n=7). Bars marked with different letters indicate significantly different activity ($p < 0.05$).

DISCUSSION

The aim of this study was to characterize the effects of T-2 toxin on hepatic and intestinal drug metabolizing enzymes (CYP450) when fed during three weeks to broilers. In addition, the effects of T-2 on drug transporter systems (MDR1 and MRP2) were also studied in the same organs. The lower dose of T-2 contaminated feed (68 µg/kg feed) is situated between the minimum (10 µg/kg feed) and maximum (112 µg/kg feed) levels observed in the survey of Monbaliu *et al.* (2010). In this study 67 contaminated feed samples were analyzed with a multi-mycotoxin LC-MS/MS method (Monbaliu et al., 2010). The higher dose (752 µg/kg feed) can also be considered of practical relevance, as even higher levels have been reported in a study in Germany (Schollenberger et al., 2006). However, the generally accepted NOAEL value for T-2 in poultry is 0.5 mg/kg feed (Eriksen and Pettersson, 2004).

At the higher contamination level (752 µg/kg feed) CYP1A4, CYP1A5 and CYP3A37 mRNA expression was significantly down-regulated in the liver. Hepatic CYP1A4 was even down-regulated at a contamination of 68 µg T-2/kg feed. To our knowledge, no other studies were performed in poultry to investigate the effect of T-2 on CYP mRNA expression. Most studies investigated the effect of T-2 toxin *in vitro* in man or pigs (Verbrugghe et al., 2012). The results of these *in vitro* experiments are in contrast with our results. T-2 was noticed to be a CYP1A1 inducer in human intestinal epithelial cells from the colon after acute exposure to a dose of 0.03 µg/mL (Kruber et al., 2011). In pigs, hepatocyte CYP3A22 mRNA expression was induced 2.5-fold after 48h exposure to T-2 toxin at a concentration of 0.1 µg/mL (Ge et al., 2010). In a recent study, mRNA levels of CYP1A were similar in the control group and T-2 treatment groups in porcine primary hepatocytes, but porcine CYP3As were induced in the T-2 treatment groups. Also in the latter study, primary hepatocytes were exposed during only 48h at doses of 0.02, 0.05 and 0.1 µg/mL (Wang et al., 2011). The discrepancy between our study and the *in vitro* studies can be explained by variance among the different experimental models. *In vitro* cell response of a given trichothecene is not always an accurate predictor of toxicity in whole animals (Rocha et al., 2005). The toxicity of T-2 is known to be affected by factors such as administration route, time of exposure and dosage (Sokolovic et al., 2008). On the other hand, CYP3A is highly conserved in eutheria, but not in chickens and reptiles (Antonovic and Martinez, 2011; Qiu et al., 2008.

Effect of chronic *in vivo* exposure of T-2 on CYP enzymes was studied in the livers of rat, rabbits and pigs. Results of these studies are in accordance with our results. Guerre *et al.* reported a dose-dependent decrease of CYP1A1, CYP1A2, CYP2A1 and CYP2B4 protein expression in white rabbits after 5-days oral treatment with T-2 at doses of 0.5, 0.25 and 0.1 mg T-2/kg feed (Guerre et al., 2000). A similar decreasing trend was observed in young rats after 1, 4 or 8 days exposure to 1.0 mg/kg body weight (Galtier et al., 1989). The longest exposure experiment was performed by Meissonnier *et al.* in pigs. After 28-days of feeding T-2 (2102 µg/kg feed), hepatic CYP1A showed a significantly lower expression compared to the control group. For CYP2B, CYP2C and CYP3A protein expression a decreasing trend was observed in the pigs, but no significant differences were noticed (Meissonnier et al., 2008). A possible explanation could be the sesquiterpenoid structure of the trichothecenes. Some sesquiterpenoid molecules are substrates of CYP450 sub-families and are responsible for the up- or down-regulation of the expression or biotransformation activities in both rodent and human models (Usia et al., 2004). In addition, T-2 is a lipophilic molecule, which can explain the sensitivity of CYP enzymes to T-2 toxicosis. CYP450 enzymes are embedded in the bilayer of the smooth endoplasmatic reticulum and are thus easily accessible for the toxin (Meissonnier et al., 2008). Moreover, T-2 is known to be a potent inhibitor of the RNA synthesis, which can explain the reduced mRNA expression (Rocha et al., 2005; Sokolovic et al., 2008).

The effect of dietary exposure to T-2 on MDR1 and MRP2 genes was also analyzed during our study. MRP2 was reduced significantly but hepatic MDR1 not, maybe due to the fact that MRP2 is more present in the liver of healthy broilers compared to MDR1 (Haritova et al., 2010). MRP2 has a protective role against accumulation of drugs and toxins (Elias and Mills, 2007).

Both CYP and drug transporter systems are regulated at the transcriptional level by nuclear receptors, such as the Pregnane X receptor (PXR), which is highly expressed in the liver (Beigneux et al., 2002; Honkakoski and Negishi, 2000). Expression of PXR can be influenced by different factors such as drugs and toxins, but also several diseases can have an influence. This can be a possible pathway to explain the reduction of both CYP and MRP2 expression after prolonged exposure to T-2.

In our study, the effect of T-2 on CYP, MDR1 and MRP2 expression in the small intestine was also investigated. However, no significant differences were observed along the different parts of the digestive tract. A possible explanation could be that the liver plays a major role in the metabolism of T-2 before biliary excretion in the intestine (Chi et al., 1978; Wu et al., 2010).

Besides mRNA expression, the CYP3A enzymatic activity was also studied using midazolam as a substrate. Midazolam has already been reported to be a good substrate for CYP3A in human (Watkins, 1994), but also in poultry (Cortright and Craigmill, 2006). Another important characteristic of midazolam is that it is not transported by P-gp and relatively few drugs have been reported in this regard (Schmiedlin-Ren et al., 1997). Remarkably, both liver and ileum showed a higher activity of CYP3A in the T-2 treatment group with the higher contamination level (752 µg/kg feed) compared to the control group. A possible explanation for the higher CYP3A activity can be the presence of compensation mechanisms for the reduced expression of CYP3A. Another possibility is post-transcriptional or even post-translational regulation of CYP3A activity. It is essential to distinguish between mRNA and protein expression, and understand that mRNA expression does not necessarily correlate to activity. This has been observed after administration of ionophores in broilers (Zhang et al., 2010). Another possible explanation for the discrepancy between the CYP3A37 mRNA expression and the CYP3A activity, is that the existence of the other CYP3A isoforms in chickens can't be excluded for which midazolam can be a substrate (Ourlin et al., 2000).

Lown *et al.* (1994) also observed a poor correlation between the intestinal CYP3A mRNA expression and activity in humans. However, a good correlation was obtained between the protein expression and the protein activity of CYP3A (Lown et al., 1994). In our study, the protein expression could not be studied. Immunoblot and ELISA analyses were performed with a polyclonal rabbit anti-human antibody, but no signal could be obtained despite several assays. A possibility for the lack of CYP3A detection is that the rabbit anti-human antibody does not cross-react with chicken proteins due to differences in the antigenic determinants of avian species.

In conclusion, prolonged exposure to practically relevant doses of T-2 toxin could interfere with the normal CYP-mediated metabolism of endogenous or xenobiotic substances. In

addition, drug transporter mechanisms, with substrates such as tetracyclines antibiotics (Mealey, 2004), could also be influenced by the presence of the mycotoxin T-2. This may be of importance for the animal, for the pharmacokinetics and efficacy of therapeutic drug substrates used, and consequently for the withdrawal time of these drugs.

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GENERAL DISCUSSION

The occurrence of mycotoxins is a worldwide problem which can affect both human and animal health. The main difficulty in assessing the risk of exposure is the multiplicity of factors affecting the production or presence of mycotoxins. Good agricultural practice is necessary to minimize crop contamination (Paterson and Lima, 2010). It is generally accepted that although actions are undertaken to reduce contamination, the presence of mycotoxins can not totally be excluded. In European moderate climate regions, three *Fusarium* mycotoxins are important, namely the trichothecenes deoxynivalenol (DON) and T-2 toxin (T-2), and zearalenone (ZEN). Different studies already reported DON being the mycotoxin with the highest prevalence in European feed samples (Binder 2007; Monbaliu et al., 2010). T-2, on the other hand looks to be the most acute toxic mycotoxin (Edwards et al., 2009). Zearalenone is known to have a particular affinity for the oestrogen receptor (Bennett and Klich, 2003) and therefore, it is generally accepted that ZEN mainly influences the fertility which is of greater importance for the broiler breeders industry or laying hens than for broiler chickens raised exclusively for the production of meat.

Several studies reported the effects of chronic feeding of trichothecenes on technical parameters in farm animals, and poultry has been reported to be one of the most resistant species. On the other hand, it is not because clinical signs are rarely observed in broiler chickens that the mycotoxins do not act at the molecular level (Pestka, 2008). Trichothecenes are inhibitors of the RNA, DNA and protein synthesis. Cells with high proliferation rate and high turn-over are known to be the main targets of DON and T-2. Both liver and intestine have these characteristics and in addition, these organs are the first to be encountered after intake of contaminated feed. Therefore, the principal aim of this thesis was to investigate if DON and/or T-2 can cause detrimental effects on the intestinal barrier and liver function.

In practice, mycotoxin detoxifiers are frequently used feed additives to reduce the risk of mycotoxicosis. Based on their mode of action, mycotoxin detoxifying agents can be divided in two different classes: adsorbing and biotransforming agents. However, there is a lack of knowledge concerning their efficacy and safety *in vivo*. Therefore, another goal of this thesis was to evaluate the efficacy of detoxifiers using the European Food Safety Authority (EFSA) guidelines and proposed end-points (Anonymous, 2009).

What are good biomarkers to evaluate the exposure to mycotoxins in broiler chickens?

Literature reports different *in vivo* studies mentioning the effects of DON or T-2 on the feed intake and growth of broiler chickens. For DON the European Commission recommends 5 mg/kg feed as a maximum level for complementary and complete feedstuffs such as poultry feed (Anonymous, 2006). In older studies mainly the effects of higher doses were investigated, while more recent experiments respect this guidance level (Awad et al., 2006b; Awad et al., 2011; Dänicke et al., 2007; Yunus et al., 2012b). However, some disagreement can be observed between the results of these different *in vivo* experiments and in addition, no clear dose-response relationship can be observed. In our study, no detrimental effects of DON on feed intake and body weight could be observed in broiler chickens exposed to 7.5 mg DON/kg feed for three weeks. These results are in contrast with other studies, which demonstrated negative effects of DON on the animal body condition after exposure to lower concentrations of the toxin compared to our study (Dänicke et al., 2007; Yunus et al., 2012b). Experimental studies to observe the effects of T-2 on the condition of the animals were also conducted in a wide concentration range, again without a clear dose-response relationship. During our study with 68 and 752 µg T-2/kg feed, no clinical symptoms of T-2 mycotoxicosis such as oral lesions and feather alterations were observed. However, the broilers fed 752 µg T-2/kg feed showed a decreased weight gain compared to the control group. The group receiving a lower dose of 68 µg T-2/kg feed did not present significant differences concerning B.W. and feed intake with the control group. Our results are in discrepancy with the study of Sklan et al. (2001) who did not observe decreased body weights in broiler chickens after chronic feeding T-2 at a concentration of 1050 µg T-2/kg feed. Therefore, we suggest that performance parameters are not suitable to evaluate the negative effects of exposure to mycotoxins. Both feed intake and body weight gain are also always influenced by other factors such as environmental and genetic factors. Naturally contaminated feed generally causes more pronounced effects, mainly due to the presence of other mycotoxins (Rotter et al., 1996; Smith et al., 1997). In addition, the presence of masked mycotoxins can not be neglected. The effects of mycotoxins are also influenced by the exposure time and the age of the animals. All these influencing factors make it difficult to standardize studies and thus to compare different studies.

In this thesis we investigated if the plasma levels of DON and its metabolites can be used to determine the dietary exposure to DON. However, our results revealed that plasma levels above the limit of quantification of 1.25 ng/ml only could be observed when feeding DON at a concentration higher than the maximum recommended level of 5 mg/kg feed. The metabolite deepoxy-deoxynivalenol (DOM-1) was not detected in any of the samples, using also a limit of quantification of 1.25 ng/ml. During our toxicokinetic study of DON a low oral bioavailability of 19.3% was observed, which can explain the low plasma levels observed during our feeding trial. Also for T-2 and ZEN, toxicokinetic studies were conducted which reported even lower oral bioavailabilities for these toxins as no plasma levels of T-2 or ZEN could be observed after a single oral bolus. From these results, we can conclude that plasma levels of the tested toxins are not good biomarkers for poultry and thus they can also not be considered as suitable end-points to evaluate the efficacy of mycotoxin detoxifiers *in vivo* in broiler chickens. Nevertheless, EFSA recommends that plasma samples of DON or ZEN and their main metabolites have to be analyzed to determine the efficacy of detoxifiers (Anonymous, 2010). However, the EFSA recommendations may be suitable for other animal species, such as pigs.

The question remains which other biomarkers can be used in practice to evaluate dietary exposure to trichothecenes? A possibility is the analysis of faeces or urine for the presence of the toxin and its metabolites. In humans, urine has already been reported to be a good biomarker for DON exposure (Turner et al., 2008). However, in chicken no difference can be made between urine and faeces since urine flows from the ureters into the coprodeum, rectum and ceca (Goldstein and Skadhauge, 2000). In addition, a recent study reported that only 6% of the ingested DON is recovered in the excreta of poultry (Awad et al., 2011). DON is namely converted to DOM-1 in the proximal part of the small intestines, mostly before absorption (Rotter et al., 1996; Swanson et al., 1988). In poultry, the intestinal flora is also able to convert DON to DOM-1 which makes it even more difficult to find DON in the excreta of chickens (He et al., 1992; Lun et al., 1988; Lun et al., 1986). Recovery of DOM-1 in the faeces was also reported to be poor (Awad et al., 2009), suggesting intestinal absorption and/or that DOM-1 may be further transformed to other compounds by the microbial flora of the gut. T-2 on the other hand is characterized by a very short half-life as reported in our toxicokinetic study. In addition, T-2 is rapidly metabolized to more polar metabolites, but

there are still derivates of T-2 which have not been identified and the toxicity of all these metabolites is still not well understood (Li et al., 2011). Also in humans, biomarkers for T-2 are not well developed (Anonymous, 2011a). These facts confirm that it still remains difficult to diagnose DON or T-2 mycotoxicosis in poultry flocks *in vivo*.

Diagnosis after necropsy is also an option. Beside macroscopic evaluation of possible lesions, histological examination of the intestines and the liver can be done. Evaluation of the length of intestinal villi and crypt after feeding DON was studied in this thesis and revealed that exposure to DON is characterized by shorter villi and reduced crypt depths. For the liver, both a macroscopic and microscopic evaluation can be performed in order to look for necrosis. However, caution is needed to be sure that the observed lesions are solely due to the exposure to mycotoxins. The analysis of the bile fluid as a possible diagnostic tool for DON has also been investigated in this thesis. Results revealed that levels of DON above the limit of quantification of 1.25 ng/ml could only be observed in the bile when feeding DON at concentrations higher than the maximum recommended level of 5 mg/kg. DOM-1, on the other hand, could be observed in all the groups receiving DON even at concentrations lower than 5 mg/kg feed suggesting DOM-1 in bile could be a valuable post-mortem biomarker in broilers. However, nor guidance levels for bile fluid nor 'background' contamination levels are available up till now. Analyses of residues in organs such as liver and kidneys were also performed in the same study, but no levels above the limit of quantification could be detected. Furthermore, it is also important to mention that all these analyses are expensive and that diagnosis is thereby mostly confirmed at a stage that the animals may already have suffered damage.

Generally, it can be concluded that the diagnosis of mycotoxicosis is still based on suspicions or by exclusion of other pathologies. Therefore, the monitoring of feed before administration to the animals still remains a very important aspect of risk assessment in combination with Good Agricultural Practices.

Are broiler chickens really as resistant to mycotoxins as claimed?

It is generally considered that chickens are one of the most resistant farm animal species to mycotoxins (Devegowda and Murthy, 2005). Symptoms mainly occur after exposure to much higher mycotoxin levels compared to other animals. Different hypotheses have already been reported in the literature concerning the resistance of poultry. The differential capacity among species to transform trichothecenes in the intestine is probably the factor of greatest importance concerning the detoxification of these mycotoxins in the different species. For example, poultry are known to transform DON to DOM-1 in the proximal part of the intestine, before absorption (Rotter et al., 1996; Swanson et al., 1988). This is confirmed in our study. We demonstrated that DON causes damage in the proximal parts of the small intestine, but not in the ileum. Hereby, poultry are more resistant to DON compared to pigs that transform DON to DOM-1 in the distal parts of the small intestine. In addition, we found in our toxicokinetic study an absolute oral bioavailability of 19.3% for DON in broiler chickens, while in pigs the oral bioavailability is around 80% (Prelusky et al., 1988).

Besides, we observed during our studies a possible adaptation mechanism to the negative effects of DON in broiler chickens. After three weeks of feeding DON, no plasma levels could be observed above the limit of quantification in none of the experimental groups. These observations were confirmed by a recent study which describes that the plasma level of DON linearly decreased with the level of previous exposure (Yunus et al., 2012a). The main mechanism responsible for this kind of adaptation or tolerance still needs to be elucidated.

The molecular mode of action of DON in broiler chickens has been investigated poorly until now. Probably due to the fact that chickens are considered to be relatively tolerant. However, during our studies we proved that DON is able to damage the intestinal barrier without clinical symptoms and without influence on the feed intake or body weight gain. T-2 could also reduce the mRNA expression of CYP1A4 at a concentration that does not affect the feed intake or the body weight gain of the broiler chickens. These results underline that the action of trichothecenes at the molecular level is frequently underestimated as the effects on the hepatic and intestinal barrier are not always associated with clearly reduced performance.

What are the possible consequences of the effects of DON on the intestinal barrier function?

The intestinal barrier is responsible for two major processes. The first one is the absorption of nutrients, xenobiotics and fluids. Second, the intestinal epithelial monolayer is responsible for the protection against harmful pathogens and xenobiotics (Oswald, 2006). The integrity of the intestinal epithelium is critical in maintaining a physical selective barrier between external and internal environments.

Histological examination of the duodenal and jejunal segments revealed shortened villi and crypts in the broiler chickens exposed to 7.5 mg/kg DON. These results indicate that DON is able to reduce the absorption surface of the intestinal monolayer and thus may lead to a reduced absorption of nutrients. However, no effects on the body weight gain were observed during our investigations with DON. The reduced crypt depth can be indicative of a reduced regeneration of the barrier with possible negative effects on the barrier function.

Tight junctions are the backbone of the paracellular barrier. Both chemical and physical factors can damage the structure and function of the tight junctions. We demonstrated the ability of DON to interact selectively with claudin 5, which may suggest a higher permeability of the intestinal barrier. Loss of the tight junctions integrity can lead to invasion of pathogens via the paracellular pathway. This was illustrated in our research by a higher mRNA expression of TLR4, which acts as a rapid pathogen sensor, in broiler chickens after three weeks of feeding DON. Beside barrier disruption as an etiological factor, the ribotoxic stress-induced cellular response as was seen in our experiments after intake of DON contaminated feed can also be related to the induction of epithelial inflammation. This has previously also been reported in pigs (Vandenbroucke et al., 2011). In humans a correlation has already been pointed out between the presence of DON as a food contaminant and different chronic intestinal inflammatory diseases, such as coeliac and Crohn's disease or ulcerative colitis (Maresca and Fantini, 2010).

The regulation of the uptake of xenobiotics is also an important function. In pigs DON was able to increase the paracellular flux of the tracer [(3)H]-mannitol along with a reduced protein expression of claudin 4 (Van de Walle et al., 2010). Of particular interest is the effect

of the higher permeability of the gut wall on the absorption of frequently used drugs and coccidiostats in the poultry industry. In pigs, intestinal barrier disruption caused by DON and T-2 was shown to promote the transepithelial passage of antibiotics such as doxycycline and paromomycin (Goossens et al., 2012). Major concern is the question whether dose-adaptation is necessary for these drugs? As a consequence of the higher permeability, a greater oral bioavailability of drugs may be observed with the possibility to reach higher levels, which could have consequences for the treated animal or even for the consumer of the edible tissues due to higher residues and prolonged withdrawal time. Special attention has to be paid to the coccidiostats belonging to the class of the ionophores. Toxic syndromes such as anorexia, diarrhea, ataxia, depression, recumbency and death have been reported due to the uptake of too high doses of monensin in poultry (Novilla, 1992). Beside a negative effect for the animal, human health could also be affected due to residues of the drugs in tissues and organs for human consumption. In order to fully understand these consequences, appropriate residue depletion studies should be conducted in the target animal species.

What are the possible consequences of the effects of T-2 toxin on the liver and intestinal barrier function?

Once xenobiotics are absorbed from the intestine after oral administration, metabolism of xenobiotics in various organs plays an important role in their disposition in the animal. The main organ for drug metabolism is generally considered to be the liver (Wang and Tompkins, 2011). However, our study concerning the expression and activity of CYP3A proved that the role of the small intestine could be equally or even more important compared to the liver in broiler chickens.

When xenobiotics reach the intestinal or liver cells, they are converted by various phase I and/or phase II enzymes to more hydrophilic metabolites, which can be excreted via kidney and other excretion routes. If a molecule is still taken up by the intestinal cells, transporter proteins can efflux it back to the intestinal lumen to increase the chances that the xenobiotic encounters one of the metabolizing enzymes. T-2 is able to alter the activities of several

hepatic enzymes such as glucuronyltransferase and glutathione S-transferase (Galtier et al., 1989; Suneja et al., 1989). CYP450 enzymes play a dominant role in the biotransformation of xenobiotics. T-2 may also affect them. The effect of T-2 on hepatic CYP has already been described in rats, rabbits and pigs *in vivo* (Galtier et al., 1989; Guerre et al., 2000; Meissonnier et al., 2008; Goossens et al., 2013). However, extrapolation between species can not be made for CYP proteins (Nebbia et al., 2001). We demonstrated T-2 to be able to influence both the mRNA expression and activity of different hepatic CYP proteins and the activity of CYP proteins in the ileum. This fact underlines the role of the intestine in detoxification. In addition, we also investigated the impact of T-2 on two efflux transporters. However, a significant effect was only seen on hepatic MRP2 and not on P-gp which suggests that not all the transporter systems may be as sensitive to mycotoxins and that not all the efflux transporters react in the same way. This emphasizes that more research is needed concerning this issue. A reduced expression of efflux pumps is associated with higher levels of xenobiotics in the animal body, which can be a possible explanation for the reduced body weight gain observed in our study.

At the mRNA level we demonstrated a down-regulation of CYP genes, but the activity of the enzymes was enhanced. We conclude that the effect of T-2 at the different levels, i.e. gene expression, protein expression and protein activity is not the same. Discrepancies between the results of different techniques might be explained by post-transcriptional or post-translational regulation of CYP proteins and differences in sensitivity of the techniques. In addition, low correlations between mRNA expression, protein concentration and activity have been reported in different animal species. A possible explanation could be the substrate change or loss of selectivity toward the drug metabolizing enzymes. In animal species there is also a lack for suitable antibodies to perform immunoblotting or immunochemistry and only a few substrates have been investigated to establish their usefulness by the measurement of Km and Vmax.

Role of mycotoxin binders?

The ubiquitous distribution of mycotoxins, together with their resistance to processing, enables toxins to persist in the human and animal food and feed supply. In practice the use of mycotoxin detoxifying agents has therefore become common. Two important factors have to be evaluated concerning these feed additives, namely their safety and their efficacy *in vivo*.

In the literature different studies reported the beneficial effects of these detoxifiers on production parameters of broiler chickens (Awad et al., 2006a; Dänicke et al., 2003). However, according to EFSA the end-point to test their efficacy is to measure the plasma levels of DON and ZEN and their main metabolites (Anonymous, 2009). In this thesis, we demonstrated that blood is not a useful sample to test the efficacy of detoxifiers against DON in broilers when the maximum recommended levels are respected. This is in line with the low absolute oral bioavailability of DON observed during a toxicokinetic study. In addition, the observed tolerance or adaptation to DON may also reduce the plasma levels after prolonged exposure. As we reported even lower oral bioavailabilities for T-2 and ZEN in our toxicokinetic studies, we can question if plasma levels of these parent toxins are a suitable end-point for DON, T-2 or ZEN in poultry as stated by EFSA. However, after feeding three weeks DON at a concentration of 7.5 mg/kg feed, which is higher than the recommended level of 5 mg/kg feed, remarkably higher plasma concentrations of DON were observed in the groups receiving an adsorbing or a biotransforming agent. No positive effects were thus demonstrated concerning the detoxifiers on plasma levels, questioning their efficacy. Nevertheless, when looking at the molecular level a positive effect of the adsorbing agent could be observed in the jejunum. The observed up-regulation of claudin 5 due to three weeks feeding DON-contaminated feed to broiler chickens was not so high in the group receiving an adsorbing agent in combination with DON. Although this is a significant positive effect of the adsorbing agent, the levels could not be reduced to the levels of the control group in the jejunum. Furthermore, we demonstrated that the adsorbing agents shifted the negative effects of DON to the more distal ileum.

Regarding the possible interaction of mycotoxin detoxifiers with veterinary drugs, we demonstrated that the plasma concentration of amoxicillin and oxytetracycline can be

increased by the presence of detoxifiers. As a possible explanation, histological examination revealed that the use of an adsorbing agent leads to longer villi even in combination with DON. The biotransforming agent even resulted in significant higher residues of oxytetracycline in the kidneys compared to the control group. These observations underline the need for the evaluation of the safety of frequently used drugs in poultry in combination with mycotoxin detoxifiers. For bentonite the interactions with tylosin or with coccidiostats have already been reported (Anonymous, 1992; Anonymous, 2011b, Devreese et al., 2012). The investigated adsorbing agent in this thesis was composed of a smectite-type clay mineral (illite-ambrosite) and the biotransforming agent was a bentonite-montmorillonite upgraded with a yeast. We may thus conclude that bentonite can interact with more components than actually officially reported, and on the other hand other detoxifiers can apparently also influence the oral bioavailability of antibiotics. In this thesis we only focused on the effects of detoxifiers on orally administered drugs, but attention also has to be paid in the future to the interactions with nutrients, vitamins and other feed additives.

Future research perspectives

Evaluation of possible effect of trichothecenes on transporter proteins and biotransformation mechanisms at the protein level

Our results indicate that chronic exposure to T-2 can reduce the mRNA expression of CYP genes in broiler chickens. On the other hand, we also reported that the activity of the enzyme CYP3A is enhanced in the same animals. We only observed the effects of T-2 at the mRNA level by qRT-PCR and at the functional level by *ex vivo* studies with the substrate drug midazolam. However, we were not able to evaluate the effects at the protein level as no suitable antibodies are available to perform western blot analyses or ELISA. Therefore, an immunohistochemistry method could be developed for the evaluation of CYP expression in broiler chickens. In this way, one could distinguish between post-transcriptional or post-translational regulation of CYP proteins in broiler chickens. For the efflux pumps MDR1 and MRP2 on the other hand, the effects of T-2 were only evaluated at the mRNA level. In the

future, immunohistochemical demonstration of the effects of T-2 would be useful in combination with an *in vivo* study using a substrate to evaluate their protein expression and activity, respectively. Beside T-2, the effects of DON on transporter proteins and biotransformation enzymes should also be investigated in broiler chickens.

Development of a rapid screening method for the detection of possible interactions between mycotoxin detoxifying agents and veterinary drugs or other feed additives

The results of this thesis indicate that mycotoxin detoxifying agents can influence the pharmacokinetic profiles of oxytetracycline and amoxicillin in broiler chickens. We demonstrated that this interaction could lead to a higher oral bioavailability of these drugs, which indicates that dosage adaptation could be necessary when antibiotics and detoxifiers are administered simultaneously. However, in this thesis only two different detoxifiers were tested, while in practice several types of detoxifying agents are frequently used in the poultry industry (Anonymous, 2009). In addition, interactions with other antibiotics but also with other feed additives such as coccidiostats may be possible. *In vivo* testing of all the detoxifying agents in combination with different frequently applied drugs in poultry is impossible and not acceptable from an ethical point of view. Therefore, we suggest the need for the development of suitable *in vitro* methods for the rapid screening of possible interactions. These methods should not only be applicable for poultry, but also for other species as interspecies extrapolation is difficult. A method using Transwell® cell culture inserts in combination with species specific intestinal epithelial cell lines could be used to mimic the animal intestines. If from the results of the screening method possible interactions are suggested, *in vivo* studies can be done to confirm the findings, e.g. by studying the pharmacokinetic parameters of the compound in the particular species.

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SUMMARY

The production of mycotoxins by fungi may depend on a lot of factors and thus good management comprises all stages from 'farm to fork'. Despite different prevention measures, animal nutritionists have to cope with a given level of contamination. In the European moderate climate regions *Fusarium* mycotoxins are the main contaminants. For deoxynivalenol (DON) the European Union recommends a concentration of 5 mg/kg feed as a maximum guidance value for poultry feed. For zearalenone (ZEN), maximum levels were set at 2 mg/kg feed for cereals and cereal products and at 3 mg/kg feed for maize by-products. For T-2 toxin (T-2) in Belgium, the Federal Agency for the Safety of the Food Chain currently imposes a limit of 0.4 mg/kg feed for T-2 and HT-2 in poultry feed. Only very recently, the European Commission Recommendation of 27 March 2013 sets a level of 0.25 mg/kg feed for the sum of T-2 and HT-2 in compound feed.

Poultry is generally accepted to be one of the most resistant species to mycotoxicosis as symptoms mainly occur after exposure to very high mycotoxin concentrations. However, little data is available about the toxicokinetic profiles of *Fusarium* mycotoxins in poultry. The diagnosis of mycotoxicosis in the poultry industry is frequently based on suspicions or by exclusion of other pathologies, mainly due to a lack of suitable biomarkers. In practice, mycotoxin detoxifying agents are frequently used feed additives to reduce the risk of mycotoxicosis. Following their mode of action, detoxifiers can be divided in two groups, namely adsorbing and biotransforming agents. Adsorbing agents bind mycotoxins, while biotransforming agents are responsible for the degradation of mycotoxins into less or non-toxic metabolites in the gastrointestinal tract of the animal. The European Food Safety Authority (EFSA) implements the investigation of the safety and efficacy of these detoxifiers.

Therefore, in **Chapter 1**, toxicokinetic studies of DON, T-2 and ZEN were described in order to evaluate their absolute oral bioavailability and toxicokinetic parameters in broiler chickens. Toxins were administered intravenously and orally in a two-way cross-over design. For DON a bolus of 0.75 mg/kg body weight (BW) was administered, for T-2 0.02 mg/kg BW and for ZEN 0.3 mg/kg BW. The plasma levels of the toxins and their main metabolites were quantified using validated LC-MS/MS methods. An absolute oral bioavailability of 19.3% was observed for DON in broiler chickens, while for T-2 and ZEN even no plasma levels above the limit of quantification of 2.5 ng/ml and 1 ng/ml respectively, could be observed after a single

oral bolus. The shortest elimination half-life after intravenous administration was detected for T-2 (3.9 min), followed by DON (27.9 min) and ZEN (31.8 min).

In **Chapter 2**, an animal trial was described in order to evaluate different parameters as suitable biomarkers to assess DON exposure. Broiler chickens were exposed to a concentration of DON below the guidance level of 5 mg/kg feed, namely 2.4 mg/kg feed, or to a higher concentration of 7.5 mg/kg feed for three weeks. In addition, adsorbing and biotransforming agents were evaluated concerning their efficacy. No detrimental effects of DON on the feed intake or the body weight gain of the animals were observed. Analyses of plasma levels of DON and its main metabolite deepoxy-deoxynivalenol (DOM-1) were performed with a validated LC-MS/MS method. DON could only be detected in the plasma of the animals receiving the highest DON concentration of 7.5 mg/kg feed. DOM-1 could not be detected in plasma of the animals. These results indicate that plasma concentrations of DON and DOM-1, as stated by the EFSA, are not reliable end-points to evaluate the efficacy of mycotoxin detoxifying agents against DON in broiler chickens. In an effort to look for possible other biomarkers, the concentrations of DON and DOM-1 were also determined in liver, kidneys and bile fluid. No residues of DON or DOM-1 could be detected in the organs above the limit of quantification of 1.2 ng/g. However, in the bile fluid DON and DOM-1 were detected in the animals receiving DON at a concentration of 7.5 mg/kg feed and DOM-1 was even detected in the animals fed DON at 2.4 mg/kg feed. From these findings, we can conclude that analysis of the bile fluid can be a suitable tool for post-mortem diagnosis in broiler chickens. However, no correlation with in feed concentrations could be recorded and nor guidance levels in bile nor 'background' contamination levels are available up till now.

For the evaluation of possible interaction of the administered adsorbing and biotransforming agents with other components such as drugs, another *in vivo* trial was performed with broiler chickens. The animals received blank feed as such or blank feed supplemented with an adsorbing or a biotransforming agent. The adsorbing agent was a smectite-type clay mineral (illite-ambrosite) and the biotransforming agent was a bentonite-montmorillonite clay upgraded with a yeast. After three weeks of feeding the experimental diets, two different frequently used antibiotics in the poultry industry were administered to the animals. Both a pharmacokinetic study and a residue study were performed for

oxytetracycline and amoxicillin, separately. For amoxicillin, the plasma concentrations were significantly higher for broilers receiving an adsorbing agent in comparison to birds receiving the biotransformation agent, but both groups with detoxifiers were not significantly different to the control group. The residue levels of amoxicillin in the liver and the kidneys were lower than the limit of quantification of 12.5 ng/g in all the birds. The plasma and kidney tissue concentrations of oxytetracycline were significantly higher in broilers receiving a biotransforming agent in the feed compared with control birds and the birds receiving an adsorbing agent. We can conclude that detoxifiers can interact with the oral bioavailability of antibiotics depending on the type of detoxifier and antibiotics.

Although poultry may be more resistant to mycotoxicosis, effects of trichothecenes such as DON and T-2 at the molecular level may not be underestimated. The inhibition of the RNA, DNA and protein synthesis and the activation of mitogen-activated protein kinases, are underlying mechanisms of their toxicity which can affect numerous organs and tissues. Especially the intestinal and hepatic cells are the first to be exposed to DON or T-2. Therefore, an important aim of this research was to evaluate if DON and T-2 damage the intestinal barrier and/or liver function in broiler chickens.

The anatomy and histological structure of the digestive tract are constituted to guarantee its barrier function. The surface of the intestines is characterized by a large area for absorption of nutrients and xenobiotics. But, intestinal cells also create a barrier against invading pathogens and xenobiotics. These absorbing epithelial cells are strongly joined together by tight junctions. In pigs, DON was demonstrated to be able to interact with the gut wall morphology and to act selectively on components of these tight junctions. After intestinal absorption occurred, mycotoxins may reach the liver as the gate-way of the portal blood draining of the gastrointestinal tract and thus provoke hepatic lesions.

In another study, described in **Chapter 3**, we thus investigated the possible effects of DON on the intestinal and hepatic barrier functions. Tissue samples of the animals of the study described in Chapter 2 were used, i.e. the group receiving blank feed, the group receiving DON contaminated feed at a level of 7.5 mg/kg feed, the group receiving DON contaminated feed in combination with an adsorbing agent and a fourth group receiving the control feed

supplemented with an adsorbing agent. Histological examination of the gut wall morphology in these different groups showed shorter villi and smaller crypts due to chronic DON exposure. These observations were reported both in the duodenum and jejunum. The use of an adsorbing agent resulted in longer villi over the entire length of the intestines even in combination with DON, which can be an explanation for the higher plasma concentrations of xenobiotics observed in Chapter 2. The crypt depth was partially restored when DON contaminated feed was fed in combination with an adsorbing agent. No effects of DON on the gut wall morphology were observed in the ileum. A qRT-PCR method was used to study the effects of DON on different tight junction components, more precisely on the mRNA expression of claudin 1, claudin 5, zona occludens 1 and zona occludens 2. Remarkably, DON caused a selective up-regulation of claudin 5 mRNA and this only at the level of the jejunum. When DON and an adsorbing agent were administered simultaneously, the effect of DON in the jejunum was less pronounced but mRNA expression of claudin 5 was still significantly up-regulated compared to the control group. However, in the animals receiving DON in combination with an adsorbing agent, DON caused damage in the ileum and not specific on one gene, but the mRNA expression of all the investigated genes was up-regulated in this segment. We can therefore conclude that the adsorbing agent is able to shift the effects of DON to more distal parts of the small intestines. DON was also able to induce inflammatory reactions in both the duodenum and jejunum, but when DON was fed in combination with the adsorbing agent, up-regulation of pro-inflammatory Toll-like receptor 4 was only observed in the ileum. A shift towards distal parts could also be observed concerning the genes encoding for oxidative stress. This study also showed that not only DON but also the adsorbing agent alone were able to alter the mRNA expression of oxidative stress markers in hepatocytes of broiler chickens.

Cytochrome P450 (CYP450) enzymes play a dominant role in the process of oxidative biotransformation of xenobiotics. Both the small intestines and the liver can be involved in these processes. CYP enzymes have mainly been studied in humans and laboratory animals. In humans, mainly CYP3A4 is responsible for drug metabolism. The presence of CYP3A4 has been confirmed in chickens since an isoform, CYP3A37, which is 60% homologous to human,

has been cloned. No information was available at the start of this PhD research concerning the gene expression of CYP3A37 and the activity of CYP3A enzymes in broilers.

Consequently, in **Chapter 4,** hepatic and intestinal CYP3A expression and activity in healthy broiler chickens were investigated. A qRT-PCR method was developed for the absolute quantification of CYP3A37 at the mRNA level in broiler chickens. For this study, livers and small intestines of healthy chickens were collected. These chickens were fed with uncontaminated feed. A significant higher expression was observed in the jejunum compared to the ileum and the liver. Prior CYP3A activity measurements, a method for preparation of intestinal microsomes was developed. Activity of CYP3A was determined in hepatic and intestinal microsomes using midazolam as a substrate drug. The highest CYP3A activity was noticed in the proximal part of the small intestines with a decreasing trend towards distal. Duodenal and hepatic activities were comparable. We can conclude that the intestinal CYP3A proteins can have an equal or maybe even higher importance for the substrate drug metabolism than the hepatic ones.

Beside the superfamily of CYP450, active efflux transporter systems also play a role in the protection against xenobiotics. Most of these drug transporters are members of the ABC family or the adenosine-tri-phosphate (ATP)-binding cassette protein family. Members of this family are present in both the liver and small intestines, where they are able to remove xenobiotics from intestinal and hepatic cells.

Both drug metabolism and drug efflux transport can be influenced by feed, nutrients, drugs and contaminants. Therefore, in **Chapter 5**, an *in vivo* experiment was performed to evaluate the toxic effects of chronic dietary exposure to T-2 in broiler chickens. Beside the study of animal performance, principal aims were to investigate the effects of this toxin on intestinal and hepatic biotransformation enzymes (CYP450), but also on drug transporter systems such as P-glycoprotein or MDR1 and multidrug resistance-associated protein 2 (MRP2). One group of birds received uncontaminated feed while the other animals received two different concentrations of T-2, namely 68 µg T-2/kg feed and 752 µg T-2/kg feed. The animals receiving the highest concentration of T-2 had a lower body weight gain compared to the other groups after three weeks of feeding. A qRT-PCR method was developed to analyze

mRNA expression of CYP1A4, CYP1A5, CYP3A37, MDR1 and MRP2 genes. Results revealed that T-2 did not have a significant effect on the expression of the different genes in the small intestines. In the liver on the other hand, exposure to 752 µg T-2/kg feed led to down-regulation of CYP1A4, CYP1A5 and CYP3A37. For CYP1A4 even a down-regulation could be observed in the animals receiving the lowest T-2 concentration of 68 µg T-2/kg feed. CYP3A enzymatic activity was up-regulated in the liver and ileum of broiler chickens after three weeks exposure to T-2 at a concentration of 752 µg T-2/kg feed compared to the control group and the animals receiving the lower T-2 dose. No effects of T-2 on CYP3A activity were observed in duodenum or jejunum. In conclusion, prolonged exposure to practical relevant doses of T-2 toxin could thus interfere with CYP-mediated metabolism of xenobiotics and with drug transporter systems which can have an impact on the pharmacokinetic parameters of substrate drugs.

The general conclusion may be that the effects of trichothecenes in poultry may be underestimated, mainly due to the absence of clear visible clinical signs. Although, we demonstrated that DON and T-2 are characterized by low absolute oral bioavailabilities in broiler chickens, serious effects at the molecular level do occur. Both mycotoxins are indeed able to affect the intestinal and hepatic barrier function which can have consequences for the animal health. In addition, we also demonstrated that more research is needed concerning the efficacy and safety of mycotoxin detoxifying agents with respect to interactions with the oral absorption of veterinary drugs.

SAMENVATTING

De productie van mycotoxines door schimmels wordt beïnvloed door diverse factoren. Goed management bij het principe van 'riek tot vork' is daarom onontbeerlijk. Ondanks allerlei voorzorgsmaatregelen moet men toch altijd rekening houden met een mogelijke contaminatiegraad. Gezien het gematigd klimaat in Europa, zijn voornamelijk *Fusarium* mycotoxines belangrijk. Zo voorziet de Europese Unie maximum toegelaten concentraties voor deoxynivalenol (DON) in kippenvoeder, namelijk 5 mg/kg voeder. Voor zearalenone (ZEN) is het maximum gehalte voor granen en graanproducten vastgelegd op 2 mg/kg voeder, terwijl in maïs gehaltes tot 3 mg/kg voeder toegelaten zijn. Voor het mycotoxine T-2 adviseert het Belgisch Federaal Agentschap voor de veiligheid van de voedselketen (FAVV) een grenswaarde van 0.4 mg/kg voeder voor de som van T-2 en HT-2 in kippenvoeder te respecteren. Sinds 27 maart 2013 adviseert de Europese Commissie een indicatieve waarde voor de som van T-2 en HT-2 van 0.25 mg/kg voor mengvoeders.

Kippen worden algemeen beschouwd als zijnde één van de meest resistente diersoorten aangezien ze slechts klinische symptomen vertonen na blootstelling aan zeer hoge mycotoxine concentraties. Er zijn echter weinig gegevens beschikbaar over de toxicokinetische eigenschappen van *Fusarium* mycotoxines in pluimvee. De diagnose van mycotoxicoses in de pluimveeindustrie is tot op heden voornamelijk gebaseerd op een vermoeden van intoxicatie ofwel door uitsluiting van andere pathologieën. Dit is het gevolg van een gebrek aan geschikte biomerkers voor het stellen van de diagnose. In de praktijk worden mycotoxine binders of modifiers frequent aangewend om het risico op mycotoxicoses te reduceren. Mycotoxine binders binden de mycotoxines in het spijsverteringsstelsel van het dier, terwijl modifiers de mycotoxines in het dier omzetten tot minder of niet-toxische metabolieten. De Europese Autoriteit voor Voedselveiligheid (EFSA) benadrukt dat de efficaciteit en veiligheid van deze voederadditieven moeten onderzocht worden.

In **Hoofdstuk 1** werden daarom toxicokinetische studies voor DON, T-2 en ZEN beschreven met als doel de absolute orale biologische beschikbaarheid en de toxicokinetische parameters voor deze mycotoxines te bepalen in vleeskippen. De mycotoxines werden oraal en intraveneus toegediend in een two-way cross-over design. Voor DON werd een bolus van 0.75 mg/kg lichaamsgewicht (LG) toegediend, voor T-2 0.02 mg/kg LG en voor ZEN 0.3 mg/kg

LG. Vervolgens werden de plasmaconcentraties van de mycotoxines en hun belangrijkste metabolieten bepaald aan de hand van gevalideerde LC-MS/MS methodes. Er werd een absolute orale biologische beschikbaarheid van 19.3% vastgesteld voor DON, terwijl voor T-2 en ZEN zelfs geen plasmaconcentraties boven de kwantificatielimiet van respectievelijk 2.5 ng/ml en 2 ng/ml, konden worden teruggevonden na toediening van een éénmalige orale bolus. De kortste eliminatie halfwaardetijd na intraveneuze toediening werd berekend voor T-2 (3.9 min), gevolgd door DON (27.9 min) en ZEN (31.8 min).

In **Hoofdstuk 2** werd een dierproef beschreven met als doel verschillende biomerkers te evalueren om DON blootstelling te diagnosticeren. Hiervoor werden vleeskippen gedurende drie weken gevoederd met enerzijds 2.4 mg DON/kg voeder, wat lager is dan het maximum toegelaten gehalte (5 mg/kg voeder), en anderzijds met een hogere concentratie van 7.5 mg DON/kg voeder. Daarnaast werden ook een mycotoxine binder en een modifier ingemengd in het voeder om hun efficaciteit te beoordelen. DON had geen invloed op de groei of de voederopname van de dieren. De plasmaconcentraties van DON en zijn voornaamste metaboliet deepoxy-deoxynivalenol (DOM-1) werden gekwantificeerd met behulp van een gevalideerde LC-MS/MS methode. Er werd enkel DON teruggevonden in het plasma van de dieren die DON kregen aan een concentratie van 7.5 mg/kg voeder. DOM-1 kon niet worden gedetecteerd in het plasma van de kippen. Hieruit kunnen we besluiten dat de bepalingen van DON en DOM-1 in plasma van vleeskippen, zoals opgelegd door EFSA, geen goede merkers zijn om de efficaciteit van mycotoxine detoxifiers na te gaan. DON en DOM-1 werden ook gekwantificeerd in de lever, de nieren en de gal van de dieren. In de organen werd noch DON noch DOM-1 gedetecteerd boven de kwantificatielimiet van 1.2 ng/g. Zowel DON als DOM-1 werden echter wel teruggevonden in de gal van de dieren die DON verstrekt kregen aan een concentratie van 7.5 mg/kg voeder. DOM-1 werd zelfs teruggevonden in de gal van de kippen die 2.4 mg DON/kg voeder kregen. Uit deze resultaten kunnen we besluiten dat analyse van DON en DOM-1 kan gebruikt worden voor post-mortem diagnose in vleeskippen. Een correlatie met de gehaltes in het voeder kan evenwel niet worden vastgelegd. Bovendien zijn er geen richtwaarden voor gehaltes in gal, noch voor ‘achtergrond’contaminatie gehaltes beschikbaar.

Er werd een andere dierproef met vleeskippen uitgevoerd teneinde na te gaan of de toegediende mycotoxine binder en modifier mogelijks interageren met andere componenten zoals bijvoorbeeld geneesmiddelen. De dieren kregen ofwel niet gecontamineerd voeder ofwel niet gecontamineerd voeder gesupplementeerd met de binder of modifier. De mycotoxine binder die gebruikt werd was een klei mineraal, meer bepaald een smectiet (illiet-ambrosiet). De modifier was een bentoniet-montmorilloniet kleimineraal in combinatie met een gist. De verschillende voeders werden gedurende drie weken aan de kippen toegediend. Daarna werden twee frequent aangewende antibiotica in de pluimveesector verstrekt aan de kippen. Voor zowel amoxicilline als oxytetracycline werden twee onafhankelijke farmacokinetiek en residustudies uitgevoerd. De plasmaconcentraties van amoxicilline waren significant hoger in de dieren die de binder toegediend kregen in vergelijking met de modifier, maar beide niet significant verschillend ten opzichte van de controlegroep. De residuen van amoxicillin in de lever en de nieren waren voor alle kippen lager dan de kwantificatielimiet van 12.5 ng/g weefsel. Voor oxytetracycline waren zowel de plasmaconcentraties als de gehaltes in de nieren significant hoger in de kippen die de modifier kregen in vergelijking met de controledieren en de dieren die de binder gevoederd kregen. Hieruit kunnen we dus besluiten dat er interactie mogelijk is tussen antibiotica en mycotoxine detoxifiers, maar dat de effecten verschillend zijn afhankelijk van het type antibioticum en de detoxifier.

Ondanks het feit dat pluimvee als relatief resistent beschouwd wordt, mogen de effecten van DON en T-2 op moleculair niveau toch niet onderschat worden. Zowel DON als T-2 zijn potente inhibitoren van de RNA, DNA en eiwitsynthese. Daarnaast kunnen ze mitogeen-geactiveerde proteine kinasen activeren. Het is dus duidelijk dat verschillende organen hun doelwit kunnen zijn, maar het zijn vooral de dunne darm en de lever die het eerst in contact komen met mycotoxines. Daarom was een ander belangrijk doel van dit onderzoek om na te gaan of DON of T-2 mogelijk schade kunnen toebrengen aan de darmbarrière en/of de leverfunctie van vleeskippen.

De anatomie en de histologische structuur van het spijsverteringsstelsel is dusdanig dat ook een belangrijke barrièrefunctie van de darm kan gegarandeerd worden. Het darmoppervlak is gekenmerkt door een groot contactoppervlak voor de absorptie van nutrienten en

xenobiotica, maar tegelijkertijd moet de invasie van mogelijke darmpathogenen en schadelijke componenten worden tegengegaan. Darmcellen zijn met elkaar verbonden door tight junctions. Onderzoek in varkens toonde eerder aan dat DON in staat is om selectief in te werken op de verschillende bouwstenen van deze tight junctions. Nadat intestinale absorptie zich heeft voorgedaan, bereiken mycotoxines de lever via de portale vene en kunnen mycotoxines mogelijks ook hepatische beschadiging uitlokken.

In **Hoofdstuk 3**, werden daarom mogelijke effecten van DON op de darm- en leverbarrière onderzocht. Hiervoor werden stalen gebruikt van dieren uit de studie beschreven in Hoofdstuk 2, met name de blanco groep, de groep met DON gecontamineerd voeder aan een concentratie van 7.5 mg DON/kg voeder, hetzelfde DON gecontamineerd voeder gesupplementeerd met een mycotoxine binder en blanco voeder gesupplementeerd met de mycotoxine binder. Histologisch onderzoek van de darmpreparaten van deze verschillende diergroepen toonde duidelijk aan dat chronische DON blootstelling leidt tot kortere villi en kleinere cryptes. Deze bevindingen werden zowel voor het duodenum als het jejunum opgemerkt. Het gebruik van een mycotoxine binder leidde daarentegen tot langere villi over de hele darmlengte, zelfs in combinatie met DON, welke mogelijks een verklaring kan zijn voor de hogere plasmaconcentraties van xenobiotica beschreven in Hoofdstuk 2. De schade toegebracht door DON aan de crypten werd gedeeltelijk beperkt door de mycotoxine binder. Er werd histologisch geen schade door DON vastgesteld in het ileum. Aan de hand van een qRT-PCR methode werd het effect van DON gemeten op verschillende tight junction bouwstenen, meer bepaald de mRNA expressie van claudine 1, claudine 5, zona occludens 1 en zona occludens 2 werden gemeten. Opmerkelijk was de significante up-regulatie van claudine 5 mRNA na DON blootstelling en dit enkel ter hoogte van het jejunum. Dit werd uitsluitend voor dit eiwit geregistreerd. Na het voederen van DON in combinatie met de mycotoxine binder, werd er ook een significante up-regulatie van claudine 5 opgemerkt in het jejunum, maar echter niet zo uitgesproken als in de dieren die enkel DON kregen. Daarentegen gaf DON in combinatie met de binder wel aanleiding tot schade in het ileum, gekenmerkt door niet alleen een up-regulatie van claudine 5 maar ook van alle andere bestudeerde genen, met name claudine 1 en zona occludens 1 en 2. Dit in tegenstelling tot DON alleen. De resultaten wijzen erop dat de binder het effect van DON naar meer distaal

gelegen darmgedeeltes kan verschuiven. Daarnaast kan ook opgemerkt worden dat DON een inflammatoire respons veroorzaakte in zowel het duodenum als het jejunum, aangezien DON een up-regulatie van de pro-inflammatoire Toll-like receptor 4 veroorzaakte. Ook hier leidde de combinatie DON en mycotoxine binder eerder tot schade in het ileum, wat ook aantoont dat het effect van DON opgeschoven wordt naar meer distaal door de aanwezigheid van de mycotoxine binder. Dezelfde trend werd ook gezien voor de genen die coderen voor oxidatieve stress. In dezelfde studie werd ook aangetoond dat niet alleen DON, maar ook de binder in staat is om de mRNA expressie van merkers voor oxidatieve stress te beïnvloeden in de lever van kippen.

Cytochroom P450 (CYP450) enzymes zijn belangrijk voor de oxidatieve biotransformatie van xenobiotica. Zowel de darm als de lever spelen hierin een rol. CYP eiwitten werden hoofdzakelijk bestudeerd bij de mens en in laboratoriumdieren. De humane CYP3A4 is het belangrijkste eiwit voor geneesmiddel metabolisatie. In kippen werd CYP3A37, zijnde een 60% homoloog van het humane CYP3A4, gekloond. Bij de start van dit doctoraatsonderzoek was er echter geen informatie voorhanden betreffende de expressie van CYP3A37 en de activiteit van CYP3A enzymes in vleeskippen.

Er werd bijgevolg, zoals beschreven in **Hoofdstuk 4**, een studie uitgevoerd om de expressie en de activiteit van CYP3A na te gaan in vleeskippen. Een qRT-PCR methode werd ontwikkeld voor de absolute kwantificatie van CYP3A37 op mRNA niveau in vleeskippen. Levers en darmen van gezonde en met een niet-gecontamineerd dieet gevoederde vleeskippen werden hiervoor verzameld. Uit de resultaten van deze studie bleek dat de CYP3A37 mRNA expressie significant hoger is in het jejunum in vergelijking met het ileum en de lever. Teneinde de CYP3A activiteit na te gaan in de verschillende darmsegmenten, werd er eerst een methode op punt gesteld om darmmicrosomen aan te maken. Zowel de hepatische als de intestinale CYP3A activiteit werden vervolgens gemeten met behulp van midazolam als substraat. De hoogste CYP3A activiteit werd teruggevonden in de proximale delen van de dunne darm met een dalende trend naar de distale gedeeltes. De hepatische activiteit van CYP3A was vergelijkbaar met de activiteit in het duodenum. Het is dus duidelijk dat intestinale CYP3A eiwitten minstens even of misschien zelfs belangrijker kunnen zijn dan hepatische voor de metabolisatie van substraten van CYP3A.

Naast CYP450 eiwitten, bieden ook efflux transporter systemen een bescherming tegen opname van xenobiotica. De meeste van deze efflux transporters zijn lid van de ABC familie of adenosine-trifosfaat (ATP)-binding cassette eiwitfamilie. Leden van deze familie zijn zowel in de lever als darm aanwezig, waar zij verantwoordelijk zijn voor het terugpompen van xenobiotica uit de intestinale en hepatische cellen.

Zowel metabolisatie als efflux transport van farmaca kan beïnvloed worden door voeder, nutriënten, geneesmiddelen en contaminanten. Daarom werd een *in vivo* proef uitgevoerd om de toxische effecten van chronisch voederen van T-2 te evalueren in vleeskippen, welke wordt beschreven in **Hoofdstuk 5**. Naast de studie van de productieparameters, was het hoofddoel van de studie om de effecten van het T-2 toxine op intestinale en hepatische biotransformatie enzymes (CYP450) na te gaan, alsook op transporter eiwitten zoals P-glycoproteine of MDR1 en multidrug resistance-associated protein 2 (MRP2). De dieren werden onderverdeeld in verschillende groepen volgens het type experimenteel voeder, namelijk niet gecontamineerd voeder, gecontamineerd voeder met 68 µg T-2/kg voeder of 752 µg T-2/kg voeder. De dieren die gevoederd werden met de hoogste T-2 concentratie hadden een significant lagere gewichtstoename in vergelijking met de andere twee groepen na drie weken voederen. Een qRT-PCR methode werd ontwikkeld om de mRNA expressie na te gaan van de volgende genen: CYP1A4, CYP1A5, CYP3A37, MDR1 en MRP2. Uit de resultaten bleek dat T-2 geen significant effect had op de expressie van deze verschillende genen in de dunne darm. In de lever daarentegen, gaf het voederen van 752 µg T-2/kg voeder aanleiding tot een significante down-regulatie van CYP1A4, CYP1A5 en CYP3A37 mRNA. Een significante down-regulatie van CYP1A4 mRNA werd zelfs vastgesteld in de dieren die de laagste T-2 concentratie (68 µg T-2/kg voeder) toegediend kregen. De enzymatische activiteit van CYP3A was significant up-gereguleerd in het ileum en de lever van de vleeskippen die drie weken gecontamineerd voeder kregen aan een concentratie van 752 µg T-2/kg voeder, en dit in vergelijking met de dieren die de lagere T-2 dosis of niet gecontamineerd voeder kregen. Er konden geen effecten van T-2 op de CYP3A activiteit worden opgemerkt in het duodenum of het jejunum. Als besluit kunnen we stellen dat langdurige blootstelling aan relevante dosissen van T-2 een invloed kan hebben op de CYP-gemedieerde metabolisatie van xenobiotica en op de werking van efflux eiwitten. Dit kan

mogelijks een invloed hebben op de farmacokinetische parameters van substraatgeneesmiddelen.

Het algemeen besluit zou kunnen zijn dat het negatief effect van trichothecenen in vleeskippen onderschat wordt, ten gevolge van de afwezigheid van een duidelijk ziektebeeld. Ondanks het feit dat zowel DON en T-2 gekenmerkt zijn door een lage absolute orale biologische beschikbaarheid, konden we aantonen dat deze mycotoxinen in staat zijn om ernstige schade te veroorzaken op moleculair niveau. Beide mycotoxines zijn in staat om de darm- en leverbarrière te beschadigen met negatieve gevolgen voor de gezondheid van het dier. Daarnaast kunnen we uit de resultaten van deze thesis ook concluderen dat meer onderzoek nodig is om de efficaciteit en veiligheid van mycotoxine detoxifiers na te gaan. Ook hun interactie met de absorptie van oraal toegediende farmaca dient te worden bestudeerd.

CURRICULUM VITAE

Ann Osselaere werd geboren op 9 januari 1985 te Schaarbeek.

Na het behalen van het diploma hoger secundair onderwijs aan het Maria Assumptalyceum te Laken (Wetenschappen-Wiskunde), begon zij in 2003 de studie Diergeneeskunde aan de Universiteit Gent en behaalde het diploma van Dierenarts in 2009.

In september 2009 trad zij in dienst als doctoraatsstudente bij de vakgroep Farmacologie, Toxicologie en Biochemie van de faculteit Diergeneeskunde. Zij verrichtte er onderzoek naar de invloed van de mycotoxines deoxynivalenol en T-2 toxine op de darmbarrière en de leverfunctie van vleeskippen. Verder begeleidde zij als promotor verschillende studenten in het behalen van hun masterproef en volgde zij verschillende cursussen van de doctoral schools.

In het kader van haar onderzoek is ze auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Tevens nam zij ook deel aan meerdere nationale en internationale congressen.

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