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Influence of deoxynivalenol and T-2 toxin on the oral bioavailability of antibiotics in pigs

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List of abbreviations

ABC	ATP-binding cassette
ANOVA	analysis of variance
AP	apical
ATP	adenosine triphosphate
AUC	area under the plasma concentration time curve
BL	basolateral
b.w.	body weight
Caco-2	human colorectal adenocarcinoma cell
CTC	chlortetracycline
C_{max}	maximum plasma concentration
CYP	cytochrome P450
CYP1A	cytochrome P450, family 1, subfamily A
CYP3A	cytochrome P450, family 3, subfamily A
D	distribution coefficient
Da	Dalton
DAS	diacetoxyscirpenol
DMCTC	demethylchlortetracycline
DMEM	Dulbecco's modified Eagle's medium
DOM-1	de-epoxy-deoxynivalenol
DON	deoxynivalenol
DOX	doxycycline
EFSA	European Food Safety Authority
EU	European Union
FB1	fumonisin B1
FCS	fetal calf serum
FEEDAP	Panel on Additives and Products or Substances used in Animal Feed
FITC	fluorescein isothiocyanate

GIT	gastrointestinal tract
G	gauch
g	G-force
h	hours
HBSS	Hank's buffered salt solution
HCK	haematopoietic cell kinase
HT-2	HT-2 toxin
HPLC	high performance liquid chromatography
HPLC-UV	high performance liquid chromatography-ultraviolet
IPEC-1	intestinal porcine epithelial cells derived from the jejunum and ileum
IPEC-J2	intestinal porcine epithelial cells derived from the jejunum
IS	internal standard
IC50	inhibitory concentration 50%
kDa	kilodalton
kg	kilogram
k_{el}	elimination rate constant
KCl	potassium chloride
LC-MS/MS	liquid chromatography tandem mass spectrometry
LD50	median lethal dose
LDH	lactate dehydrogenase
LOD	limit of detection
LOQ	limit of quantification
MAPK	mitogen-activated protein kinase
M-cell	microfold cell
MOS	mannan-oligosaccharide
MS	mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUCL	Mycothèque de l'Université catholique de Louvain

m.w.	molecular weight
NADPH	nicotinamide adenine dinucleotide phosphate
NOAEL	no observed adverse effect level
OD	optical density
OTA	ochratoxin A
PAT	patulin
PBS	phosphate buffered saline
PFPA	pentafluoropropionic acid
PFTE	polytetrafluoroethylene
P-gp	permeability-glycoprotein
PI	propidium iodide
pKa	acid-base dissociation constant
qRT-PCR	quantitative Real-Time Polymerase Chain Reaction
PKR	RNA-activated protein kinase
RSD	relative standard deviation
Sa/So	sphinganine / sphingosine
SAPK/JNK	stress-activated protein kinase/c-Jun N-terminal kinase
SD	standard deviation
SGLT-1	D-glucose/D-galactose sodium-dependent transporter
T-2	T-2 toxin
$t_{1/2el}$	elimination half-life
TCA	trichloroacetic acid
TEER	trans epithelial electrical resistance
TJ	tight junction
t_{max}	time to maximal plasma concentration
UPLC-MS/MS	ultra performance liquid chromatography tandem mass spectrometry
WST-1	water soluble tetrazolium salt
ZEA	zearalenone

General Introduction

1. *MYCOTOXINS*

1.1 General

The term mycotoxin was coined in 1960 with the outbreak of the mysterious turkey X disease in England, during which approximately 100 000 turkey poults died because of consumption of a groundnut meal contaminated with secondary metabolites from the fungus *Aspergillus flavus* (aflatoxins). Secondary metabolites are low-molecular weight organic compounds that are not essential for normal growth, development and reproduction but provide a number of fitness benefits to the producing fungus (Moss, 1991). To other organisms, these secondary metabolites can be beneficial (Vaishnav and Demain, 2011) or deleterious. Secondary metabolites which are deleterious are called mycotoxins, referring to their fungal origin and toxic nature (Bennet and Klich, 2003). It is estimated that there may be 20 000 to 300 000 unique mycotoxins, but only a relative few have been well characterized (Jacobson et al., 2006). The mycotoxins of most significance from a public health and agronomic perspective are those produced by molds present on feed and food. As mycotoxins are chemically very stable, they are not degraded during normal food processing (Eriksen, 2003) or autoclaving (Wannemacher et al., 2000) and thus end up in the feed and food chain. When consumed, they can cause a variety of adverse health effects (Hussein and Brasel, 2001; Jacobson and Coppock, 2006). The pathological states arising from the consumption of feeds contaminated with mycotoxins are referred to as ‘mycotoxicoses’ (Bouhet et al., 2004). The main toxic effects are carcinogenicity, genotoxicity, nephrotoxicity, hepatotoxicity, oestrogenicity, reproductive disorders, immunosuppression or dermal effects. The severity depends, however, on various factors including the type and concentration of mycotoxin, the route and duration

of exposure, mode of action of the toxin, the animal species as well as gender, age, body weight and health status of the animal (Hussein and Brasel, 2001; Avantaggiato et al., 2005). Of all animal species, pigs are the most sensitive to *Fusarium* mycotoxins and are economically important species in our regions. Furthermore, as their diet mainly consists of cereals, pigs are frequently exposed to these secondary metabolites making them an interesting subject to study the effect of *Fusarium* mycotoxins.

1.2 Mycotoxin characteristics

Mycotoxins present in animal feed and human food chains are mainly produced by fungi of the genera *Aspergillus, Penicillium* and *Fusarium* (Sweeney *et al.*, 1998). Their prevalence depends on their substrate preferences and their individual tolerance to water, pH and temperature (Abramson *et al.*, 1997; Magnoli *et al.*, 1998; Müller *et al.*, 1998; Sweeney et *al.*, 1998). *Aspergillus* species are typically found in tropical or subtropical areas, as average temperatures above 24 °C are required for optimal fungal growth and particular mycotoxin production. Mycotoxins produced by *Aspergillus* species are referred to as 'storage mycotoxins' because they generally develop after harvesting (Surai and Dvorska, 2005). The most prominent toxins produced by *Aspergillus* species are aflatoxins (Fink-Gremmels, 2005). In contrast to *Aspergillus*, *Penicillium* species prefer cool and moderate climates. They are also typical storage fungi, invading feed commodities at the post harvest stage. An exception, however, are the ochratoxin A (OTA) producing *Penicillium* strains found on oats and barley prior to harvest. Ochratoxin A is the most prominent *Penicillium* toxin found in feed commodities (Fink-Gremmels, 2005). *Fusarium* is probably economically the most important toxigenic genus of filamentous fungi in cereals worldwide (Bottalico and Perrone, 2002). They are known to invade grains during the growth of the plant. The mycotoxins they

produce are therefore called ‘field mycotoxins’ because they are present on developing grain. The most important *Fusarium* mycotoxins are the trichothecenes deoxynivalenol (DON) and T-2 toxin (T-2), next to zearalenone (ZEA) and fumonisin B1 (FB1).

1.2.1 Trichothecenes

Trichothecenes are a large group of structurally related mycotoxins mainly produced by fungi of the genus *Fusarium*. They have a tetracyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene ring in common, with the epoxy ring being responsible for the toxicological activity (Figure 1) (Desjardins et al., 1993; Sudakin, 2003).

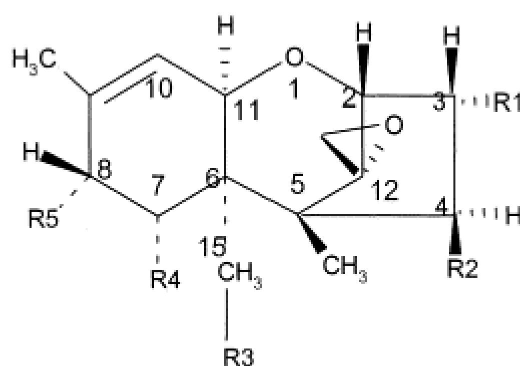

Figure 1. General chemical structure of trichothecenes (Cavret *et al.*, 2006). All trichothecenes have a tetracyclic sesquiterpenoid 12,13-epoxytrichothec-9-ene ring in common. The 12,13-epoxy ring is responsible for the toxicological activity.

On the basis of the absence or presence of functional groups, trichothecenes have been classified into four types being type A, B, C and D (Ueno, 1977; Ueno, 1985). Type A and B trichothecenes are produced by *Fusarium* species like *F. graminearum*, *F. sporotrichioides*, *F. poae* or *F. equiseti* while other species, such as *Myrothecium* and *Cephalosporium* are those producing type C and D trichothecenes (Wu et al., 2010).

Type A trichothecenes do not contain carbonyl on C-8 and are mainly represented by T-2 and its metabolite HT-2 toxin (HT-2). Type B trichothecenes, with DON being the most important

mycotoxin, differ from type A by the presence of a carbonyl group on C-8. Trichothecenes of type C, such as crotocin, have a second epoxy ring between the C-7 and C-8 or C-8 and C-9 positions, respectively. Type D trichothecenes, such as satratoxin G, contain a macrocyclic ring between C-4 and C-15. The different structural conformations are shown in Figure 2.

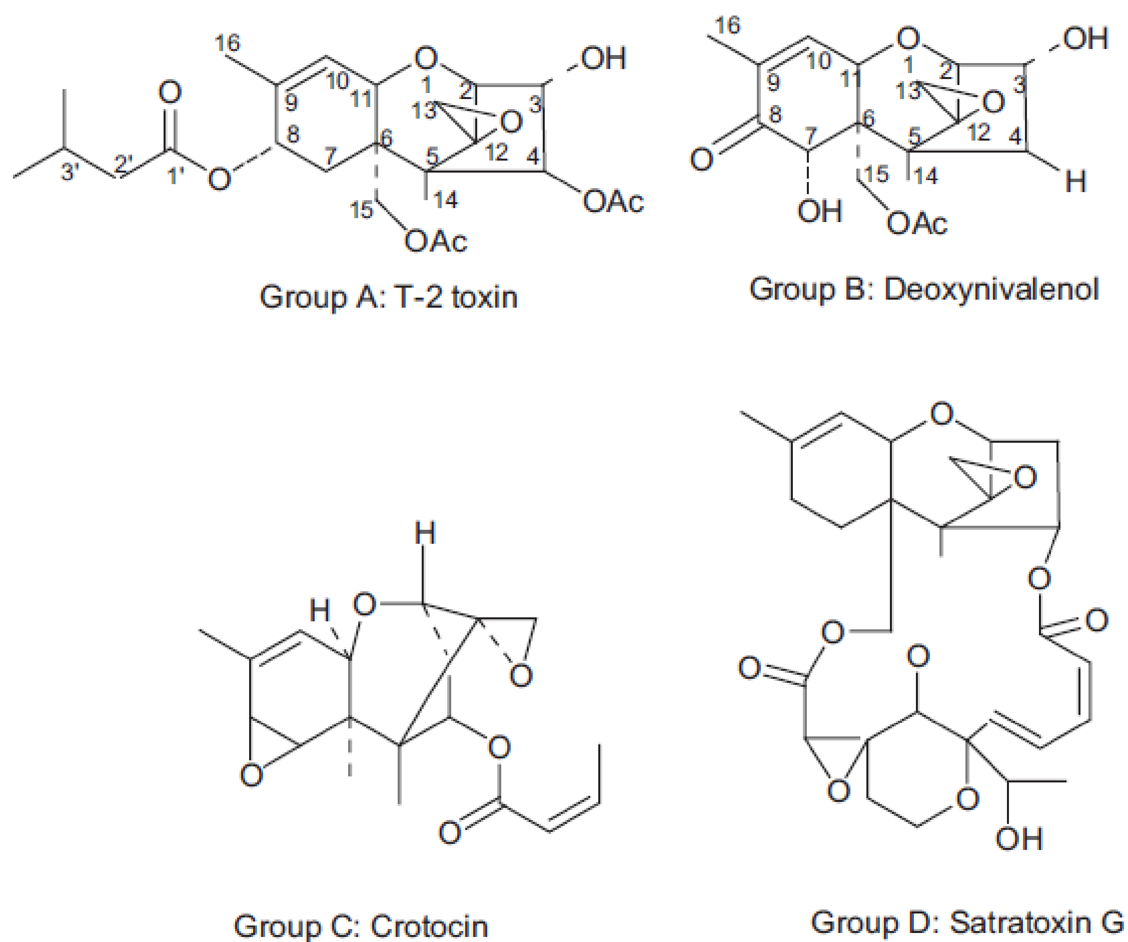

Figure 2. Chemical structure of trichothecenes with examples of groups A, B, C and D (Wu et al., 2010).

The trichothecenes causing most concern are T-2, which is the most acute toxic trichothecene (Calvert et al., 2005) and DON, which is the most frequently occurring one (Eriksen and Pettersson, 2004). They are both known to be inhibitors of protein synthesis by interfering with the active site of peptidyl transferase, an integral part of the 60S mammalian ribosomal subunit, resulting in impairment of initiation and elongation. Additionally, inhibitors of the

peptidyl transferase reaction can trigger a ‘ribotoxic stress response’. Alteration of 28s rRNA by trichothecenes was postulated to be an initiation signal for activation of stress-activated protein kinases/cJun N-terminal kinases (SAPK/JNK), which is a mitogen-activated protein kinase (MAPK). Trichothecene-induced MAPK activity drives activation of transcription factors that promote expression of pro-inflammatory genes as well as induce apoptosis (Yang et al., 2000; Moon and Pestka, 2002; Zhou et al., 2003) (Figure 3).

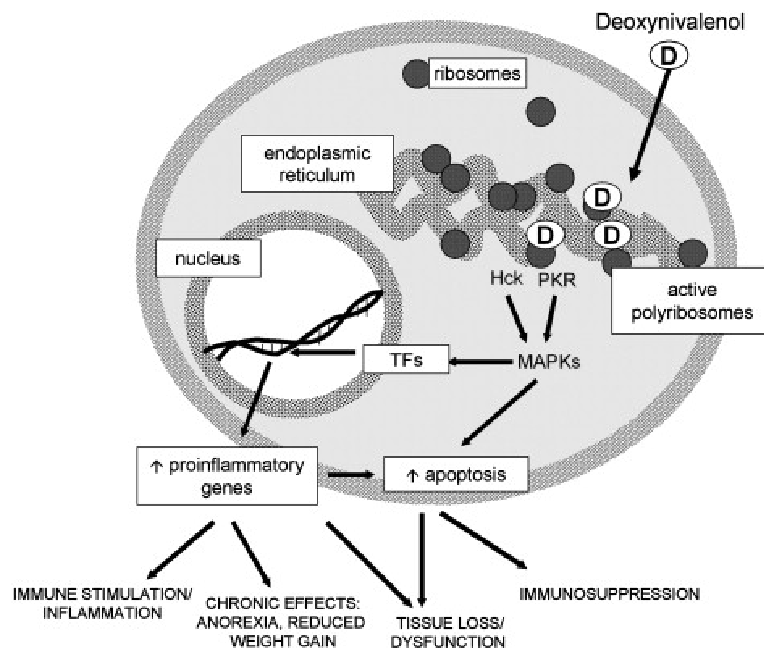

Figure 3. Molecular mode of action for deoxynivalenol and other trichothecenes. Deoxynivalenol (D) enters the cell via diffusion and binds to active ribosomes, which transduces signal to RNA-activated protein kinase (PKR) and hematopoietic cell kinase (HCK). Subsequent phosphorylation of mitogen-activated protein kinases (MAPKs) drives transcription factor activation and the resultant chronic and immunotoxic effects (Pestka et al., 2007).

- **T-2 toxin**

T-2 toxin is the most commonly occurring type A trichothecene, produced predominantly by *F. sporotrichioïdes*, *F. poae* and *F. langsethiae* (Williams, 1989; Meissonnier et al., 2008; Yanshen et al., 2011). This type A trichothecene received much attention because it has the highest toxicity of all the trichothecenes (D’Mello et al., 1999; Sudakin, 2003).

In pigs, T-2 is relatively poorly absorbed from the stomach (Ellison and Kotsonis, 1974; Beasely et al., 1986). Nevertheless, once T-2 toxin enters the small intestine, absorption occurs rapidly. Less than 30 min after its ingestion, T-2 is detected in pig blood (Eriksen and Petterson, 2004).

After absorption, T-2 is rapidly metabolized resulting in a half-life of elimination of less than 20 min. In pigs, more than 80% of T-2 is converted into HT-2 and metabolites thereof (SCF, 1999a; Bernhoft et al., 2000). HT-2 toxin may be further deacetylated, conjugated or hydroxylated by various metabolic pathways. In urine and bile, respectively 63% and 77% of the total T-2 metabolites in pigs are glucuronic acid conjugates (Corley et al., 1985). Hydroxylation of T-2 and HT-2 into their less toxic metabolites, 3'-hydroxy-T-2 and 3'-hydroxy-HT-2, respectively, is probably mediated by cytochrome P450 of intestinal and hepatic microsomes (CYP3A22) (Ge et al., 2010). Also Wu et al. (2011) showed that CYP3A29 could catalyze the hydroxylation of T-2, indicating that the CYP3A gene subfamily plays a role in the biotransformation of T-2 in pigs.

Elimination of T-2 and its metabolites occurs mainly via urine and faeces (Swanson and Corley, 1989). In pigs, after exposure to high doses of T-2, the toxin and its metabolites are also excreted in milk (Vanyi et al., 1991). Since T-2 is rapidly metabolized and secreted, no significant accumulation of T-2 is observed.

As already described, trichothecenes have a strong affinity for the 60S ribosomal subunit resulting in inhibition of the activity of the peptidyl transferase and consequently protein synthesis (Riley, 1998). T-2 inhibits initiation of protein synthesis, probably by inhibiting the binding of a second amino acid to the first in the amino acid sequence (Beasley et al., 1989). Furthermore, T-2 also inhibits the synthesis of DNA and RNA, interferes with the metabolism

of membrane phospholipids, and increases the level of liver lipid peroxides (Paterson and Lima, 2010).

The observed effects after acute oral intake of T-2 contaminated feed (ranging from 0.06 to 10 mg/kg body weight) include non-specific symptoms like weight loss, feed refusal, dermatitis, vomiting, diarrhea, haemorrhages and necrosis of bone marrow, spleen, testis, ovary and the epithelium of the stomach and intestine. Also disturbance of the circulatory system such as hypotension and arrhythmia due to an effect on blood pressure and catecholamine elevation, is described. The oral median lethal dose (LD50) is determined at 5 mg/kg body weight (b.w.) (Shuhmacher-Wolz, 2010).

Effects observed after repeated exposure to T-2 include signs such as poor weight gain, weight loss, dermal necrosis and immunological effects. Also alterations of various blood parameters are described. Seven week old pigs fed for 3 weeks 1 mg/kg T-2 contaminated feed resulted in lowered levels of glucose, inorganic phosphorous and magnesium (Rafai et al., 1995). Reduced haemoglobin and serum alkaline phosphatase values were seen in pigs receiving 8 mg T-2/kg feed during 30 days (Harvey et al., 1994). Necrotizing dermatitis on the snout, buccal commissures and prepuce resulting in increased serum triglyceride and decreased serum iron concentrations were seen after *ad libitum* treatment of pigs with 10 mg T-2/kg feed during 28 days (Harvey et al., 1990).

- **Deoxynivalenol**

Deoxynivalenol is chemically described as 12,13-epoxy-3,4,15-trihydroxytrichotec-9-en-8-one (SCF, 1999b). It is one of the primary trichothecene metabolites found in wheat and is commonly produced by *F. graminearum* and *F. culmorum*. It is the most commonly occurring trichothecene in nature. DON is also known as vomitoxin because of its potent emetic properties.

In animal studies with pigs fed DON contaminated feed, it was demonstrated that absorption of DON from the pig gastrointestinal tract is very rapid reaching maximal plasma concentrations within 15-30 min of dosing with an oral bioavailability around 80% (Prelusky et al., 1988; Dänicke et al., 2004; Eriksen and Pettersson, 2004). The rapid appearance indicates an important absorption from the proximal part of the digestive tract, involving stomach and small intestine. Compared to other species, the plasma elimination of DON is slower in pigs with a half-life of elimination of about 4 h (Prelusky et al., 1988).
The main metabolite of DON is the non-toxic DOM-1, which is formed by cleaving the 12,13-epoxygroup by faecal micro-organisms (Dänicke et al., 2004). Analysis of digesta from consecutive segments of the digestive tract revealed that DON is mostly metabolized in the caudal segments of the gut (Dänicke et al., 2004). However, in distinct contrast to other animal species, only a small part is metabolized as about 95% of intragastrically administered DON at a dose of 0.6 mg/kg of body weight was recovered as unchanged DON in blood, urine, bile and faeces of pigs (Prelusky et al., 1988). Accumulation of DON in tissues is very limited (Prelusky and Trenholm, 1991).

DON exerts its effects by various mechanisms as described for T-2. It inhibits protein synthesis as well as the synthesis of DNA and RNA (Ueno, 1983).

The most common signs of acute DON exposure in pigs include gastrointestinal problems, diarrhea, increased salivation, malaise and anorexia (Vesonder and Hesseltine, 1981; Pestka et al., 2007). A single oral dose of DON as little as 0.1-0.2 mg/kg body weight was described to cause vomiting in pigs (Forsyth et al., 1977)
Diminished feed consumption and lower weight gain are the principal clinical effects seen in pigs chronically exposed to feed naturally contaminated with low concentrations of DON (< 2

mg/kg) (Rotter et al., 1994; Trenholm et al., 1984). Complete feed refusal is only seen at 12 mg/kg and more than 20 mg/kg causes vomiting (Forsyth et al., 1977; Young et al., 1983; Abbas et al., 1986; Haschek et al., 2002). Although considered to be one of the least lethal trichothecenes, DON's emetic and anorectic potencies are equal to or greater than those reported for the more acutely toxic trichothecenes (Rotter et al., 1996), which demonstrates the sensitivity of pigs to DON. Pigs fed DON also exhibit altered blood parameters but these effects cannot be easily separated from nutritional status, i.e. weight loss as a result of significantly decreased feed intake (Young et al., 1983; Lun et al., 1985). Extensive lesions are not typically described as pigs regulate toxin ingestion by adjusting their feed intake (Friend et al., 1986; Harvey et al., 1989).

1.2.2 Fumonisin B1

Fumonisins are a group of mycotoxins produced by *Fusarium* species like *F. verticillioides* and *F. proliferatum*. Fumonisin B1 (Figure 4) can constitute up to 70% of all fumonisins in feed and is the most frequent cause of fumonisin toxicosis in animals. FB1 has been found as natural contaminant in maize (SCF, 1999c).

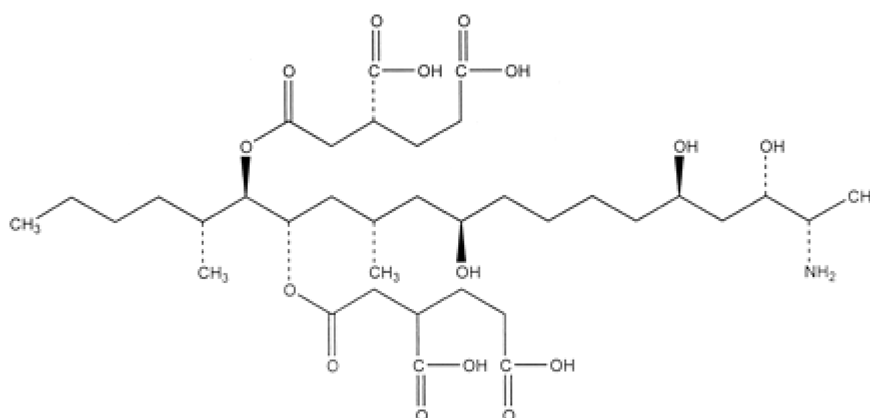

Figure 4. Chemical structure of fumonisin B1 (FB1) (Bennet and Klich, 2003).

In swine, a single oral administration of 5 mg FB1/kg of feed resulted in plasma concentrations within 30 min indicating rapid absorption of the toxin. Peak plasma concentrations were reached around 2 hours, followed by a reduction at 6 hours, until complete disappearance at 24 hours (Dilkin et al., 2010). Also Prelusky et al. (1994a) indicated detectable plasma levels 30-45 min after intravenous administration of the toxin. A limited excretion in urine (1.5% of the FB1 quantity taken up) and larger amounts of FB1 in faeces (76.5%) may indicate a low absorption rate and/or intense enterohepatic circulation of the toxin (Szabo-Fodor et al., 2008). Prelusky et al. (1996) stated that the oral bioavailability of FB1 in swine is limited to approximately 4%.

FB1 is metabolized by pig fecal microbiota into PHFB1, partially hydrolyzed FB1 (Gelderblom et al., 2001). A carry-over in edible tissues from swine was considered not to be of toxicological relevance.

Mechanisms of toxicity of fumonisins are complex. Fumonisins interfere with the biosynthesis of sphingolipids. The chemical structure of FB1 resembles the sphingolipids of mammalian cell membranes (Riley, 1996). Therefore FB1 is able to inhibit the enzyme ceramide synthetase, an N-acetyl-transferase catalyzing the synthesis of complex sphingolipids, thereby disrupting the *de novo* biosynthesis of ceramides and sphingolipids. Inhibition of ceramide synthase leads to reduced levels of ceramide and subsequently the intracellular concentration of free sphinganine (Sa) and sphingosine (So) increases to toxic levels (Figure 5). Furthermore, phosphorylation of the free sphingoid bases can also contribute to FB1 toxicity (Soriano et al., 2005; Voss et al., 2007). Increase in the Sa/So ratio in plasma can be used as a sensitive marker for fumonisin exposure. In general, the kidney appears to be the most sensitive organ to fumonisin-induced sphingolipid alterations since renal tubular cells are highly enriched in sphingolipids.

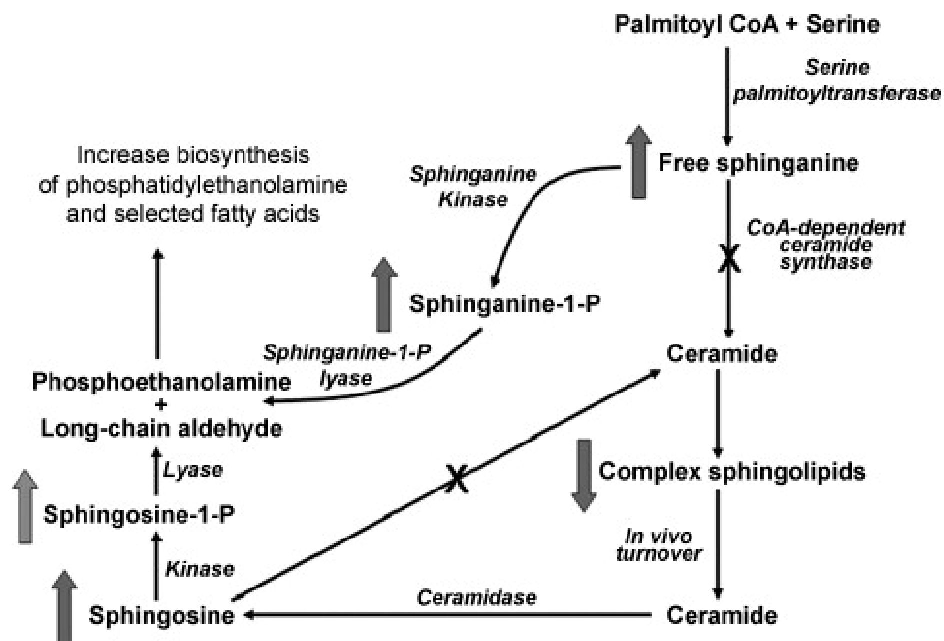

Figure 5. Biochemical mechanism of action of fumonisin B_1. Inhibition of ceramide synthase (x) by fumonisins results in increased tissue and serum concentrations (up arrows) of sphinganine, sphingosine and their 1-phosphate metabolites (Voss et al., 2007).

The main lesions in swine after chronic dietary exposure to FB1 are pulmonary edema, hydrothorax, hepatic nodular degeneration, hyperplasia and oesophageal lesions (Casteel et al., 1994). According to Hascheck et al. (2001), pigs develop lethal pulmonary edema in 4-7 days when fed FB1 containing feed at concentrations of ≤ 92 mg/kg of b.w./day. Dilkin et al. (2003) concluded that a dose of 10 mg of FB1/ kg of feed is safe for swine.

1.2.3 Zearalenone

Zearalenone is a resorcyclic acid lactone, chemically described as 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1,7(8*H*)-dione (SCF, 1999d) (Figure 6). It is produced by *Fusarium* species like *F. graminearum, F. sporotrichioides, F. oxysporum, F. moniliforme* and *F. crookwellense* (Conkova *et al.*, 2003). It is found particularly in corn (Fink-Gremmels, 2005).

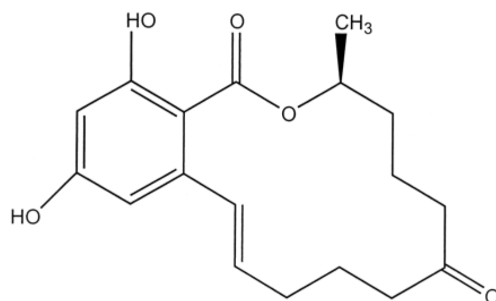

Figure 6. Chemical structure of zearalenone (ZEA) (Bennet and Klich, 2003).

In pigs, ZEA is rapidly absorbed after oral administration. The uptake of ZEA after a single oral dose of 10 mg/kg b.w. was estimated to be 80-85% (Biehl et al., 1993). Approximately 60% of ZEA is metabolized to α-zearalenol and its epimer β-zearalenol in a mean ratio of 3/1. Both intestinal mucosa and gut microflora metabolize ZEA to α-zearalenol and to the glucuronides of both compounds (Kollarczik et al., 1994). After oral administration of ZEA in pigs, 45% of the administered dose was recovered in the urine during the first 48 h, 22% was recovered in the faeces, and the total accumulated recovery in urine and faeces after 48 h was 67% (Biehl et al., 1993). Analysis of muscle tissue revealed high amounts of α-zearalenol together with traces of ZEA (Zöllner et al., 2002).

Zearalenone is not acutely toxic but is a problem because of its estrogenic effects in mammals. It competes with 17β-oestradiol for binding to the estrogen receptor. Binding of ZEA to the receptor results in a disordered protein synthesis of the estrogen dependent proteins (Figure 7). Even the metabolites of ZEA, α-zearalenol and β-zearalenol, can bind to the estrogen receptor with α-zearalenol having a greater affinity for the receptor compared to β-zearalenol.

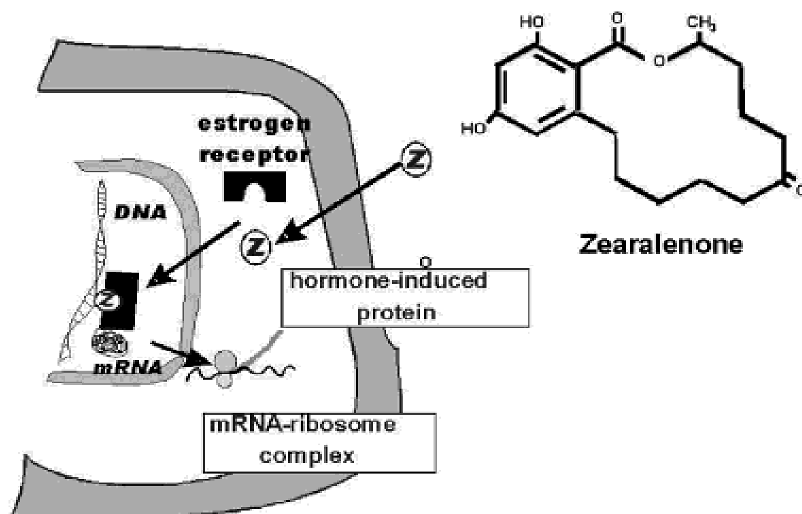

Figure 7. Mechanism of action of zearalenone. Interaction of zearalenone (Z) with the cytosolic estrogenic receptor illustrating hormonal mimicry. Zearalenone passively crosses the cell membrane and binds to the cytosolic estrogen receptor. The receptor-zearalenone complex is rapidly transferred into the nucleus where it binds to specific nuclear receptors and generates estrogenic responses via gene activation resulting in the production of mRNAs that code for proteins that are normally expressed by receptor-estrogen complex binding (Cousin et al., 2005).

Swine are the most commonly affected animals although cattle, poultry and laboratory rodents also are affected (Hagler et al., 2001). Hyperestrogenism is the typical syndrome exerted by ZEA. It is seen after intake of dietary concentrations of 1 mg/kg or more (Weaver et al., 1978a). In female swine it is manifested as swelling of the vulva and enlargement of the mammary glands, expecially in prepubescent gilts. ZEA has also been associated with feminization in young male swine, including testicular atrophy, swollen prepuce and mammary gland enlargement. In severe cases, this syndrome may progress to rectal and vaginal prolapse. High concentrations of ZEA (50 to 100 mg/kg) in swine diets have been reported to adversely affect cycling, conception, ovulation and implantation (Chang et al. 1979; Sundloff and Strickland, 1986). Other effects related to higher concentrations include anestrus, nymphomania and pseudopregnancy.

1.3 Sensitivity to mycotoxins

The impact of mycotoxins in the human food chain is an important issue worldwide. In human food, mycotoxins have gained increasing importance as they have been discussed in the etiology of various diseases. The first historically recorded case of human mycotoxicosis was ergotism, also known as St. Anthony's Fire, caused by the ingestion of grains contaminated with ergot mycotoxins produced by *Claviceps purpurea*. Ochratoxin A is suspected in the etiology of human nephropathies and tumors of urinary organs, fumonisin contamination has been linked with oesophageal cancer and aflatoxin B_1 causes liver cancer.

Among animal species, ruminants are generally be considered more resistant to the adverse effects of mycotoxins (Helferich et al., 1986; Prelusky et al., 1994b; Fink-Gremmels, 2008) because the rumen microbiota are able to degrade certain mycotoxins (Ribelin et al., 1978; Kiessling et al., 1984; Swanson et al., 1987). Kiessling et al. (1984) reported the ability of rumen microorganisms from sheep to degrade T-2, OTA, ZEA and diacetoxyscirpenol (DAS) *in vitro*. Also DON was reported to be metabolized by rumen microorganisms (King et al., 1984). On the other hand, a number of mycotoxins resist microbial degradation, and some are able to modify the rumen microflora resulting in a decreased capacity to degrade other mycotoxins. This may lead to unexpected high concentrations of mycotoxins in the systemic circulation and thus toxic effects in ruminants with high rumen stability.

In monogastrics, variable responses have been shown with all mycotoxins. Pigs have been shown to be very sensitive to T-2, DON (Friend et al., 1992) and ZEA (Biehl et al., 1993), while poultry seem to be very resistant to the estrogenic effects of ZEA. Chickens fed a diet containing up to 800 mg/kg ZEA did not express signs of toxicity or impairment of reproductive performance (Chi et al., 1980).

Initial studies reported that detrimental effects can be observed when purified mycotoxins are added to the feed (Trenholm et al., 1984); however, the situation with naturally contaminated diets is more complex because *Fusarium* species produce many metabolites and mycotoxicoses may be caused by multiple toxins. Unidentified/bound toxins, conjugated mycotoxins, or toxic agents of other origin might contribute substantially to the animal response (Prelusky et al., 1994b). At present, it is challenging to study the effect of masked mycotoxins and co-contamination of mycotoxins.

1.4 Occurrence of mycotoxins

Mycotoxins are omnipresent. Binder et al. (2007) reported a two-year survey programme undertaken to evaluate the incidence of mycotoxins in feed and feed raw materials and concluded that 52% of 1 507 samples taken from European and Mediterranean markets tested positive for aflatoxin B1, OTA, DON, T-2, ZEA and fumonisins. Monbaliu et al. (2010) analysed three different feed matrices (sow feed, wheat, and maize) for the evaluation of the toxin exposure in animal production and concluded that, in total, 67 samples out of 82 (82%) were contaminated with mycotoxins. Type B trichothecenes and fumonisins occurred most often and the majority of the infected feed samples (75%) were contaminated with more than one type of mycotoxin. Streit et al. (2012) summarized the results of all surveys evaluating mycotoxin contamination and co-occurrence in European feed and feed raw materials since 2004. The data clearly show that mycotoxins are ubiquitously present in feed material throughout Europe and that maximum contamination levels exceeding the EU maximum levels or guidance values are likely to occur. Multi-mycotoxin studies reported 75%-100% of the samples to contain more than one mycotoxin which could impact animal health at low doses. Co-occurrence of mycotoxins is thus very common. Contamination of feed with multiple mycotoxins can happen in many ways: by a single fungus that produces

more than one mycotoxin in a given feed ingredient; by two separate ingredients containing different mycotoxins being used in the manufacturing of feed; or by contamination of a single ingredient with two separate fungi that produce different mycotoxins. There is thus a distinct possibility that animals are exposed to a mixture of different toxins. It is important to consider the presence of multiple mycotoxins when estimating the safe levels of mycotoxins that can be fed. Additive, synergistic and antagonistic effects have been reported for mixtures of mycotoxins (Gutleb et al., 2002). An example of the interaction between mycotoxins is the interaction between DON and fusaric acid, a mycotoxin also produced by *Fusarium* species. Fusaric acid by itself is not toxic to animals, even at very high concentrations, but the combination of DON together with fusaric acid is highly toxic (Smith et al., 1997).

1.5 Regulation of mycotoxins in food and feed

National and international organizations are constantly evaluating the risk that mycotoxins pose to animals and humans. This has resulted in statutory maximum permissible limits. On a worldwide basis, at least 99 countries had mycotoxin regulations for food and/or feed in 2003. The guidance values in Europe on the presence of DON, ZEA, and FB1 in products intended for animal feeding are presented in Table 1. There are currently no European regulations for T-2 or HT-2 in animal feeding.

Table 1. The guidance values on the presence of deoxynivalenol, zearalenone, ochratoxin A and fumonisins in products intended for animal feeding, as determined in the Commission Recommendation of 17 August 2006 (2006/576/EC). These regulations apply to products intended for animal feed as soon as they enter the EU.

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg (ppm) relative to a feedingstuff with a moisture content of 12%
Deoxynivalenol	Feed materials (*)	
	- Cereals and cereal products (**) with the exception of maize by-products	8
	- Maize by-products	12
	Complementary and complete feedingstuffs with the exception of:	5
	- Complementary and complete feedingstuffs for pigs	0.9
	- Complementary and complete feedingstuffs for calves (< 4 months), lambs and kids	2
Zearalenone	Feed materials (*)	
	- Cereals and cereal products (**) with the exception of maize by-products	2
	- Maize by-products	3
	Complementary and complete feedingstuffs	
	- Complementary and complete feedingstuffs for piglets and gilts	0.1
	- Complementary and complete feedingstuffs for sows and fattening pigs	0.25
	- Complementary and complete feedingstuffs for calves, dairy cattle, sheep (including lamb) and goats (including kids)	0.5
Ochratoxin A	Feed materials (*)	
	- Cereals and cereal products (**)	0.25
	Complementary and complete feedingstuffs	
	- Complementary and complete feedingstuffs for pigs	0.05
	- Complementary and complete feedingstuffs for poultry	0.1
Fumonisin B1+B2	Feed materials (*)	
	- Maize and maize products	60
	Complementary and complete feedingstuffs for:	
	- pigs, horses (Equidae), rabbits and pet animals	5
	- fish	10
	- poultry, calves (< 4 months), lambs and kids	20
	- adults ruminants (> 4 months) and mink	30

(*) Particular attention has to be paid to cereals and cereals 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 dairy 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. (***) The term 'Maize and maize products' includes not only the feed materials derived from maize listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in the Annex, part B of Directive 96/25/EC but also other feed materials derived from maize in particular maize forages and roughages.

2.1 General

The pig gastrointestinal tract (GIT) consists of the stomach, small intestine (duodenum, jejunum and ileum) and large intestine (caecum, colon and rectum). The total length of the fully grown pig GIT is 23.5 m and has a capacity of 27.5 l (Moran, 1982). The small intestine has observable folds of mucosa (circular folds or plicae circulares) that increase the surface area threefold. From the circular folds, microscopic finger-like pieces of tissue, called villi, project. These villi increase the surface area by 10-fold. Furthermore, each villus is covered in microvilli, which increase the surface area by 20-fold (Figure 8). Unlike the small intestine, the large intestine surface area does not have villi. In addition, the large intestine enterocytes differ slightly from these of the small intestine and its microvilli are less packed (Kararli, 1995). Overall, this significantly contributes to the smaller surface area of the large intestine and is consistent with the fact that small intestine is the major site of absorption, also in comparison to the stomach.

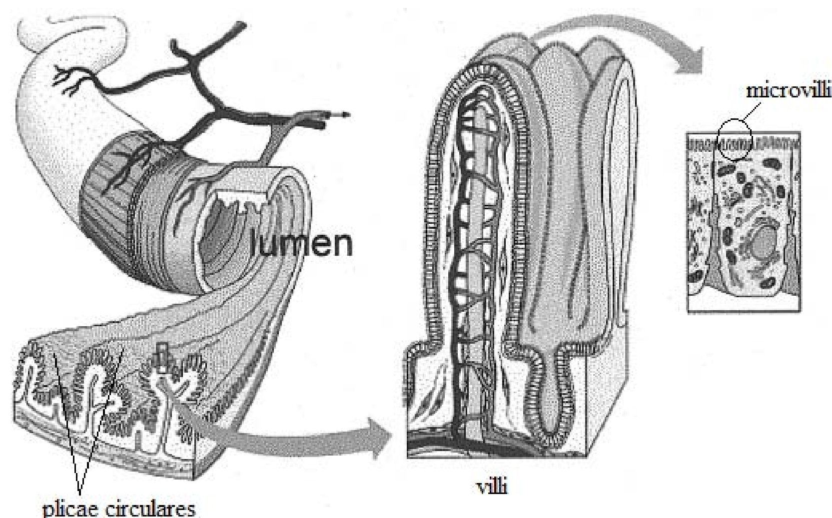

Figure 8. Representation of plicae circulares, villi and microvilli of the small intestine. Figure adapted from Strader and Woods (2005) with slight modifications.

2.2 Absorption of drugs across the gastrointestinal tract

The gastrointestinal tract represents the largest interface between the external environment and the internal host environment (Fasano, 2000). Its main function is to provide a selective barrier which allows the absorption of water, electrolytes, minerals and nutrients but also excludes harmful substances such as toxins, antigens and microorganisms (Oswald, 2006). Translocation of a molecule across the cellular barrier can occur by several routes (Figure 9). Passive diffusion is the most common mechanism of absorption across the intestinal membrane and is divided into two pathways: the paracellular pathway, in which a drug diffuses through the pores between the intestinal enterocytes; and the transcellular pathway, which requires drug diffusion across the lipid cell membrane of the intestinal enterocyte. The active transport pathway is mediated by transporters and is divided into active drug influx and efflux.

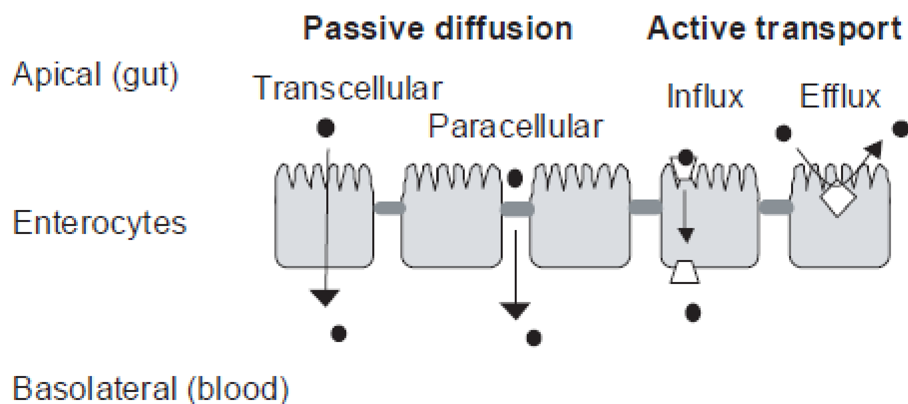

Figure 9. Multiple pathways for the intestinal absorption of a compound (Thomas et al., 2006).

1) Passive diffusion

Passive diffusion is the process by which molecules spontaneously diffuse from a region of higher concentration to a region of lower concentration. There are several factors that play a role in passive intestinal permeability. They are separable into two groups: extrinsic barriers within the lumen, and the barrier intrinsic to the epithelium. The extrinsic barriers stabilize the microenvironment at the apex of epithelial cells. The intrinsic barrier, which is the main physical barrier restricting passive molecular permeation, consists of two components: the epithelial cells (transcellular pathway) and the spaces around the epithelium (paracellular pathway) (Madara, 1990).

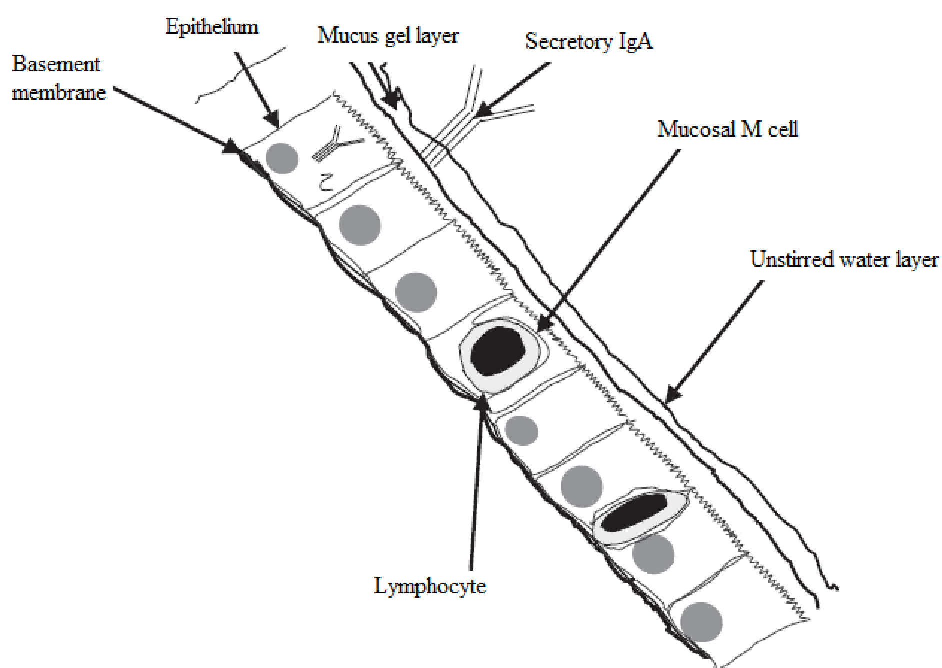

Figure 10. Components of the intestinal barrier system. Figure adapted from Farhadi et al. (2003) with slight modifications.

- *Extrinsic barrier*

The extrinsic barrier comprises of two components that may limit the rate of absorption of various drug molecules across the intestinal membrane. The outermost component of the intestinal pre-epithelial diffusion barrier is the unstirred layer of water molecules (Winne, 1976). The size of this layer has been estimated to be 100-800 µm in depth (Anderson et al., 1988). Next, there is the mucus layer, a viscous and elastic gel that covers the intestinal cell lining and is produced by the goblet cells of the villous epithelium. It is composed of various proteins and enzymes involved in digestion and absorption of nutrients. The mucus layer is resistant to mucolytic and proteolytic agents and protects the epithelial villi from physical friction, chemical digestion and adhesion of bacteria (Smithson et al., 1981). Its physicochemical properties can influence the rate of diffusion from the bulk to the site of absorption (Winne, 1979).

- *Intrinsic barrier*

Paracellular transport involves the passage of drugs through the tight junctions (TJ), well-organised protein structures, between the cells of the gastrointestinal tract (Oswald, 2006). Transport is dependent on the size of the junction and the size of the drug molecule and pH of the different compartments. The junctions between adjacent cells of the epithelium membrane vary from one tissue to another. The junctions between the cells in the gastrointestinal membrane are very tight. As a result, paracellular diffusion of drugs across the intestinal membrane is a very minor route of absorption.

Transcellular diffusion is determined by a drug's permeability across the lipophilic matrix of the membrane and depends on the lipophilicity, polarity and size of the drug molecule. A drug's lipophilicity is the most important determinant of permeability and is assessed by

measuring its distribution between the immiscible phases of n-octanol and water at a certain pH. The ratio of the drug's concentration in n-octanol and water at a certain pH is the drug's distribution coefficient (D). Drugs with large positive log D values are lipophilic, have a high permeability across the lipophilic core of the membrane and diffuse easily. As the log D value decreases among a series of drugs, lipophilicity and permeability both decrease and transcellular passage becomes increasingly difficult.

Polarity is another factor involved in transcellular transport of molecules. Due to the hydrophobic nature of the phospholipids, polar molecules cannot diffuse freely across the membrane. They are transported across membranes by proteins that form transmembrane channels. Only small non-polar molecules can diffuse easily across the membrane. A polar and ionized drug will be poorly absorbed. Most drugs are chemically weak acids or weak bases which become ionized when dissolved in water. When in solution, the ionized form of the drug will be in equilibrium with the un-ionised form. How much of a drug changes to the ionized form will depend on the acid-base dissociation constant (pKa) and the pH of the compartment.

The size of drug molecules also affects permeability as large molecules experience difficulty in diffusing. Drugs with a molecular mass greater than 500 Da are likely to have poor membrane permeability.

In summary, transcellular permeability is highest for small lipophilic, non-polar drugs (Rosenbaum, 2011).

2) Active transport

Although many drugs are passively transported through cells, for many others transport is facilitated, which means that transport across the membrane is faster than expected from their physicochemical properties. Active transport systems require energy and are capable of

moving compounds against an opposing concentration gradient. The direction of movement may be either into the cell, influx transport, or out of the cell, efflux transport. Transporters may reside on either the basolateral side or on the apical side of the membrane. The most extensively studied efflux transporter which functions as a barrier by extruding toxins and drugs into extracellular fluid, is P-glycoprotein (P-gp).

P-glycoprotein is one of the most clinically important transmembrane transporters. It is a member of the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily of transporters. Its normal function involves the excretion of drugs and their metabolites (Thiebaut et al., 1987; Dean, 2002). Due to the high concentrations of P-gp on the epithelial cells lining the intestine (Dean, 2002), P-gp can alter the absorption and bioavailability of orally administered drugs by pumping the drug out of the mucosal cell back into the intestinal lumen (Gottesman and Pastan, 1993; Mealey, 2004).

2.3 Biotransformation

Intestinal absorption and oral bioavailability can, however, also be linked to intestinal phase I metabolism. This metabolism is called the first pass effect and is mediated by different families of enzymes that result in the oxidation, reduction or hydrolysis (phase I reactions) and/or conjugation (phase II reactions) of drugs. Cytochrome P450 (CYP) enzymes are the major enzymes involved in phase I drug metabolism (Antonovic and Martinez, 2011). They are expressed both in the intestinal mucosa, and mainly in the liver. Inhibition of intestinal and/or hepatic CYP can thus result in increased bioavailability of drugs which are a substrate to these enzymes.

3. IMPACT OF MYCOTOXINS ON GASTROINTESTINAL TRACT AND BIOTRANSFORMATION MECHANISMS

3.1 Mycotoxins alter the intestinal barrier function

The GIT represents the first barrier against food and feed contaminants as well as the first target for these toxicants. After ingestion of mycotoxin contaminated food or feed, the GIT can be exposed to high concentrations of mycotoxins which are able to alter the intestinal barrier function. Epithelial integrity can be studied *in vitro* by using a Transwell insert system® and measuring the transepithelial electrical resistance (TEER) and the passage of tracers, such as FITC-dextran, across the epithelial monolayer. A drop in TEER value and an increased passage of tracer reflects a disturbance in epithelial tightness (Diesing et al., 2011a). Several *in vitro* studies indicated that DON is able to increase the intestinal permeability of human enterocytes (Kasuga et al., 1998; Maresca et al., 2002; Sergent et al., 2006). Also with intestinal epithelial cell lines from porcine origin (IPEC-1 cells), DON was shown to decrease TEER and increase paracellular permeability in a time- and dose-dependent manner (Pinton et al., 2009). The alterations in these two parameters are the result of a decrease in the expression of specific tight junction proteins (Pinton et al., 2009; Diesing et al., 2011a). The role of DON can be explained by the fact that the structure and function of these proteins can be regulated by signaling molecules involved in MAPK pathways (Matter and Balda, 2003), which are known to be activated by DON (Pestka et al., 2004). In this way, DON is able to decrease the expression of tight junctions, which in turn reduces the barrier function of the intestine. Also FB1 was shown to diminish the TEER of IPEC-1 cells (Bouhet et al., 2004) and Caco-2 cells (De Angelis et al., 2005). The mechanism of FB1 disruption of the TEER is still unclear but it is hypothesized that FB1 may result in the activation of signaling pathways

or alter the sealing function via the depletion in glycosphingolipids (Nusrat et al., 2000). The effect of ZEA and T-2 on barrier integrity is not reported in literature yet.

3.2 Mycotoxins affect cell viability, proliferation and intestinal morphology

Effects of mycotoxins on cellular viability were measured *in vitro* by various methods in different cell types. It is clear that there is a great heterogeneity in sensitivity between cell types and detection methods. Studies concerning the effect of *Fusarium* toxins on intestinal cell viability reveal that cytotoxicity is concentration- and time-dependent. At high concentrations, DON induces toxic effects compromising the intestinal barrier integrity whereas low concentrations of DON do not exhibit toxic properties, but has modulatory effects on cellular regulation (Diesing et al., 2011b). A cytotoxic effect of FB1 is also only seen at high concentrations and differs between proliferating and non-proliferating cells. This selective sensitivity of dividing cultures versus non-dividing ones, correlates with a higher rate of *de novo* sphingolipid biosynthesis in dividing cells, leading to a greater accumulation of free sphinganine upon FB1 treatment (Schmelz et al., 1998; Yoo et al., 1996). When comparing the toxicity of DON, T-2 and ZEA on the human intestinal cell line Caco-2, it was clear that T-2 exhibited the most cytotoxic response (Calvert et al., 2005).

When studying the effect of mycotoxins on cell proliferation it has been shown that a high concentration of DON affects cell growth by arresting the cell cycle in the G2/M phase (Yang et al., 2008). Also FB1 inhibits proliferation of IPEC-1 cells by blocking the cell cycle in the G0/G1 phase (Bouhet et al., 2004).

Examination of histopathological lesions revealed that T-2 causes necrosis of epithelial and crypt cells of the jejunum and ileum of pigs (Marrs et al., 1986; Pang et al., 1987a;1987b, 1987c; Weaver et al., 1978b).

3.3 Mycotoxins affect the production of intestinal mucus

Intestinal mucus, secreted by goblet cells, covers the gut epithelium and protects the epithelium against chemical, enzymatic, physical and bacterial aggressors that may be present in the gut lumen (Montagne et al., 2004). Obremski et al. (2008) demonstrated that the combination of low doses of T-2, DON and ZEA in the feed reduces the number of mucus-producing cells and the tightness of the intestinal glycocalix in pigs. The opposite effect, an increasing activity of the goblet cells, was however demonstrated when ZEA was fed alone to pigs at higher concentrations (Obremski et al., 2005). Also a high dose of FB1 leads to an increase in goblet cells in chickens (Brown et al., 1992). At present, the mechanisms responsible for the effect of these mycotoxins on mucus production have not been characterized.

3.4 Mycotoxins affect biotransformation mechanisms

Cytochrome P450 is a superfamily of enzymes which play a major role in drug metabolism. An effect of mycotoxins on enzymatic activity could result in a changed bioavailability of drugs that are a substrate of CYP enzymes. Reduced expression of hepatic CYP1A proteins was reported after intake of 2102 µg T-2/kg feed in pigs for 28 days (Meissonnier et al., 2008), after intragastric administration of 0.25 mg/kg b.w. T-2 in rabbits for 5 days (Guerre et al., 2000) and after intragastric administration of 1 mg/kg b.w. T-2 in rats for 8 days (Galtier et al., 1989). Osselaere et al. (2013) demonstrated a significant down-regulation in mRNA expression of hepatic CYP1A4, CYP1A5 and CYP3A37 when broilers were fed T-2 contaminated feed at a concentration of 752 µg T-2/kg feed. The same investigators showed that even a contamination level of 68 µg/kg T-2 caused a significant decrease in expression at mRNA level for CYP1A4. Effects of mycotoxins on intestinal CYP has not been described yet.

4. *PREVENTION OF MYCOTOXICOSIS*

Ever since the first report of a mycotoxicosis in the early 1960s, researchers have been meticulously researching ways to eliminate or minimize the effects of these contaminants. Due to the wide range of mycotoxins that can contaminate animal feed and their variable chemical compositions, prevention of mycotoxicosis is not easy. If unacceptably high levels of mycotoxins occur, removal or dilution of the contaminated feed is advised. Dilution of contaminated feed is not authorized within the European Union (Avantaggiato et al., 2005; Kolosova and Stroka, 2011) and since the incidence of mycotoxins in animal feed is quite high (Binder et al., 2007), it is not always possible to remove the contaminated feed. Therefore, many attempts have been made to find ways to prevent colonization of crops (pre-harvest strategies) and to detoxify contaminated feedstuffs (post-harvest strategies) in order to reduce the mycotoxin contamination level in feed (Döll and Dänicke, 2004).

4.1 Pre-harvest strategies

The steps of prevention that are the most effective are those carried out before the fungal infestation and before mycotoxin production occurs on plant material. Pre-harvest strategies primarily consist of tactics designed to reduce infection of crops by mycotoxin-producing fungi. It includes biological control, development of resistant varieties of crops through new strategies and good agricultural practices, which includes crop rotation, tillage practices, cropping pattern, reduction in plant stress through irrigation, timely planting and harvesting and protection of insect damage by the use of biopesticides (Choudhary and Koumari, 2010).

4.2 Post-harvest strategies

Prevention of mycotoxin contamination in the field is the main goal of agricultural food and feed industries, but even the best management of agricultural strategies cannot totally eradicate mycotoxin contamination (Jouany, 2007). Therefore several physical, chemical and biological methods have been developed in order to remove mycotoxins from contaminated feed (Bhatnager et al., 1991; Kabak and Dobson, 2009). Physical methods like cleaning, mechanical sorting and separation, washing, density segregation, thermal inactivation, irradiation, ultrasound and solvent extraction, and chemical procedures like treatment with acid/base solutions or other chemicals, ammoniation and ozonation have been tested (CAST, 2003). However, these physical and chemical detoxification methods often do not work, are expensive and often destroy or remove essential nutrients from the feedstuff and reduce palatability (Scott, 1991). Biological detoxification, which comprises binding of mycotoxins by adsorptive materials as well as microbial inactivation by specific microorganisms or enzymes (biotransforming agents), is nowadays therefore the most prominent approach to reduce the risk for mycotoxicoses in farm animals. Table 2 gives an overview of the current mycotoxin detoxifiers.

Table 2. Overview of the current mycotoxin detoxifiers (European Food Safety Authority, 2009).

Mycotoxin-detoxifying agents

Mycotoxin-adsorbing agents	**Mycotoxin-biotransforming agents**
* **Aluminosilicates** - Bentonites - Montmorillonites - Zeolite - HSCAS (Hydrated sodium calcium aluminosilicate)	* **Bacteria** - Gram-positive, anaerobic bacteria: *Eubacterium* - Gram-positive, aerobic bacteria: *Corynebacterium, Mycobacteria* - Gram-negative, aerobic bacteria: *Pseudomonas*
* **Activated carbons** * **Yeast cell walls** * **Micronized fibers** * **Bacteria**	* **Fungi** - *Aspergillus* - *Eurotium herbariorum* - *Rhizopus sp.* - *Penicillium raistricki* - *Rhinocladiella atrovirens*
* **Polymers** - Cholestyramine - Polyvinylpyrrolidone	* **Yeast** - *Trichosporon mycotoxinivorans* - *Phaffia rhodozyma* and *Xanthophyllomyces dendrorhous* isolates
	* **Enzymes** - Protease A - Pancreatin - Carboxypeptidase A - Epoxidase - Lactonohydrolase

Mycotoxin-adsorbing agents present in animal feed are compounds that should be able to bind mycotoxins during passage through the digestive tract under all pH-, temperature- and moisture-conditions of the digestive tract (Döll and Dänicke, 2004). The toxin-adsorbing agent complex passes through the GIT and is eliminated via the faeces whereby mycotoxin uptake by the animal is prevented. Mycotoxin-adsorbing agents can be divided into aluminosilicates, activated carbons, yeast cell walls, micronized fibers, bacteria and polymers. On the other hand, some microorganisms have enzymes that are able to degrade (biotransform) mycotoxins into a non-toxic or less toxic metabolite(s). Such enzymes have been described in bacteria, yeasts and fungi. Also purified enzymes can be used for this purpose. The concept is for each mycotoxin separately to select the appropriate microorganism that specifically degrades the toxin.

The addition of mycotoxin binders to contaminated diets has been considered the most promising dietary approach to reduce the negative impact of mycotoxins. Regarding the use of these feed additives, a primary concern is that the *in vivo* efficacy in sequestering mycotoxins and the safety towards livestock of most of these commercial products have not yet been thoroughly tested (Ledoux et al., 2001). Therefore, the European Commission (EC 386/2009) defined a new functional group of feed additives entitled “substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion or modify their mode of action” and stated that, before any feed additive is applied to prevent mycotoxin intoxication, both its efficacy and safety need to be tested. In association with the European Food Safety Authority (EFSA), it was defined that not only *in vitro* but also *in vivo* studies will be required for the assessment of mycotoxin binders with regard to efficacy testing. A major risk for mycotoxin binders, however, is the lack of selectivity with possible consequences for nutritional aspects through interactions with dietary compounds. Also the possible interactions with veterinary medicinal products such as

antibiotics, anthelmintics and coccidiostats need to be investigated (EFSA, 2010). Besides efficacy, also the safety of use of the additive for both the target animal species and the consumer needs to be investigated.

4.3 Mycotoxins and mycotoxin detoxifying agents: combined effect on intestinal absorption of drugs?

The effects of relative high concentrations of mycotoxins on pig health are well described. The consequences for animal health after intake of or exposure to mycotoxin concentrations below the guidance values, on the other hand, are less well studied. Furthermore, the effect of mycotoxins on the absorption of orally administered drugs is not yet investigated.

The efficacy and safety of most mycotoxin detoxifying agents is not fully understood. It was therefore stated by EFSA that the efficacy and safety of these feed additives needs to be investigated not only *in vitro*, but also *in vivo*. One of the parameters that needs to be studied is the possible interaction of the mycotoxin detoxifying agents with veterinary medicinal products.

At the time the research in this thesis was started, following scientific questions arised:
Do mycotoxins affect the bioavailability of orally administered drugs? Do mycotoxin detoxifying agents affect the bioavailability of orally administered drugs? What would be the effect of the combination of a mycotoxin and a mycotoxin detoxifying agent on the absorption of orally administered drugs?

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Scientific Aims

In our temperate regions *Fusarium* species often colonize fodder crops resulting in the contamination of feed and food commodities with potentially hazardous mycotoxins. This is especially of great concern for the pig industry, as pigs are the most sensitive species to mycotoxins and their diet mainly consists of cereals which give rise to a high exposure level of mycotoxins. The most important *Fusarium* mycotoxins are deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZEA) and fumonisin B1 (FB1). The visible clinical signs after exposure to high concentrations of these mycotoxins have been well described, however, the impact of low and in practice more relevant concentrations is until now poorly understood.

The gastrointestinal tract is the first barrier to come in contact with mycotoxins after ingestion of mycotoxin contaminated food or feed. Mycotoxin induced disruption of this barrier may result in a modified absorption of, among others, orally administered veterinary drugs. In this way, mycotoxins may also have indirect effects on animal and human health. Increased passage of drugs can, however, have important consequences with respect to toxicity for the animal, withdrawal time of veterinary drug formulations and consequently jeopardize public health with respect to tissue residues of the drug.

To minimize the negative impact of mycotoxins, yeast cell wall derived feed additives claiming to bind mycotoxins in the gastrointestinal tract, are often used in the feed industry. A great concern, however, is that the *in vivo* efficacy and safety of those feed additives have not been thoroughly tested yet (Ledoux et al., 2001).

The main goal of this thesis was to examine the hypothesis that even low concentrations of the most important *Fusarium* toxins alter the oral absorption and bioavailability of commonly used antibiotics in pigs. The antibiotics used in the experiments were doxycycline and chlortetracycline, both belonging to the group of tetracyclines; and paromomycin, an

aminoglycoside. Tetracyclines are frequently used in pig industry. Paromomycin, although somewhat less commonly used, was also selected as the drug is known to have a very low oral bioavailability due to its polar nature. The low degree of absorption of this antibiotic is mainly due to paracellular transport. On the other hand, tetracyclines are absorbed by transcellular passage due to passive non-ionic diffusion. Also a difference in oral bioavailability has been reported between tetracyclines in pigs. A percentage of absorption of about 25-30 % has been noticed for doxycycline in this animal species (Baert et al., 2000), while chlortetracycline shows a very low fraction of oral absorption (about 5 %, Nielsen and Gyrd-Hansen, 1996).

The specific objectives of this thesis were as follows:

- To examine the effects of the *Fusarium* mycotoxins DON, T-2, ZEA and FB1 on the intestinal barrier function in pigs using an *in vitro* model of porcine intestinal epithelial cells;
- To investigate the influence of the most potent of these four *Fusarium* mycotoxins (i.e. DON and T-2) on the oral absorption of the selected antibiotics in pigs, using *in vivo* pharmacokinetic studies;
- To study the effects of the *Fusarium* mycotoxins DON and T-2 on intestinal and hepatic biotransformation and transporter mechanisms, using *in vivo* studies in pigs;
- To evaluate the *in vivo* efficacy of a yeast-derived feed additive that claims to bind DON and T-2.

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Experimental Studies

Chapter 1

Porcine intestinal epithelial barrier disruption by the *Fusarium* mycotoxins deoxynivalenol and T-2 toxin promotes transepithelial passage of doxycycline and paromomycin

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Abstract

The gastrointestinal tract is the first target for the potentially harmful effects of mycotoxins after intake of mycotoxin contaminated food or feed. With deoxynivalenol (DON), T-2 toxin (T-2), fumonisin B1 (FB1) and zearalenone (ZEA) being important *Fusarium* toxins in the northern hemisphere, this study aimed to investigate *in vitro* the toxic effect of these mycotoxins on intestinal porcine epithelial cells derived from the jejunum (IPEC-J2 cells). Viability of IPEC-J2 cells as well as the proportion of apoptotic and necrotic IPEC-J2 cells was determined by flow cytometry after 72 h of exposure to the toxins. Correlatively, the integrity of the intestinal epithelial cell monolayer was studied using Transwell® inserts, in which the trans-epithelial electrical resistance (TEER) and passage of the antibiotics doxycycline and paromomycin were used as endpoints. We demonstrated that the percentage of Annexin-V-fluorescein isothiocyanate (Annexin-V-FITC) and propidium iodide (PI) negative (viable) cells, Annexin-V-FITC positive and PI negative (apoptotic) cells and Annexin-V-FITC and PI positive (necrotic) IPEC-J2 cells showed a mycotoxin concentration-dependent relationship with T-2 toxin being the most toxic. Moreover, the ratio between Annexin-V-FITC positive and PI negative cells and Annexin-V-FITC and PI positive cells varied depending on the type of toxin. More Annexin-V-FITC and PI positive cells could be found after treatment with T-2 toxin, while more Annexin-V-FITC positive and PI negative cells were found after exposure to DON. Consistent with the cytotoxicity results, both DON and T-2 decreased TEER and increased cellular permeability to doxycycline and paromomycin in a time- and concentration-dependent manner. It was concluded that *Fusarium* mycotoxins may severely disturb the intestinal epithelial barrier and promote passage of antibiotics.

Introduction

Mycotoxins are naturally occurring secondary metabolites produced by fungi. They can be formed in the field on crops as well as during storage and are chemically very stable, enabling them to survive processing, and thereby end up in the feed and food chain. In the northern temperate regions *Fusarium* species are the most prevalent toxigenic fungi (Nelson et al., 1994; CAST, 2003) with trichothecenes, fumonisins and zearalenone being the most important toxins produced (D'Mello et al., 1999). If ingested, these mycotoxins can cause a variety of adverse health effects, both on humans and animals (Gutleb et al., 2002). Since the gastrointestinal epithelium is the first barrier to come in contact with mycotoxins after ingestion, several studies investigated the effects of mycotoxins on intestinal cells. Most of these were conducted on human intestinal epithelial Caco-2 cells (Kasuga et al., 1998; Caloni et al., 2002, 2005; Calvert et al., 2005; De Angelis et al., 2005; Kouadio et al., 2005; Sergent et al., 2006; Maresca et al., 2008; Videmann et al., 2008; Pinton et al., 2009, 2010; Van de Walle et al., 2010), but also the porcine intestinal epithelial cell lines IPEC-1 (Bouhet et al., 2004) and IPEC-J2 (Awad et al., 2011; Diesing et al., 2011a,b) have been used to demonstrate that mycotoxins can alter the integrity of the gut barrier. Moreover, Pinton *et al.* (2009, 2010) showed that the trichothecene deoxynivalenol (DON) selectively affects the expression of tight junction proteins in porcine intestinal epithelial cell monolayers (IPEC-1) and Caco-2 cell monolayers resulting in increased paracellular passage of fluorescein isothiocyanate (FITC)-dextran and *Escherichia coli*. It was also demonstrated that fumonisin B_1 (FB1) affects the membrane composition of IPEC-1 cells resulting in an increased passage of this toxin (Loiseau et al., 2007). Intestinal epithelial cells are thus important targets for the toxic effects of mycotoxins and it is clear that an altered barrier could result in an altered passage of mycotoxin co-contaminants, xenobiotics and pathogens.

The aim of the present study was to evaluate the cytotoxic effect of the four most important *Fusarium* mycotoxins i.e. DON, T-2 toxin (T-2), zearalenone (ZEA) and FB1 in intestinal epithelial cells derived from the jejunum of pigs (IPEC-J2 cells). Therefore the percentage viable, apoptotic and necrotic IPEC-J2 cells was determined using flow cytometry. Correlatively, it was investigated whether differences in cell viability result in a changed transepithelial passage of the antibiotics doxycycline and paromomycin. This was studied using Transwell® devices which are commonly applied and widely accepted to perform permeability studies. The hypothesis tested in this study was that an altered integrity of the gut barrier provoked by mycotoxins, could result in an increased passage of antibiotics through the epithelium. Increased passage of these antibiotics can indeed have toxic consequences for the animal, withdrawal time, and consequently public health with respect to possible residues of drugs in edible tissues. To our knowledge, this is the first time that transport of antibiotics is used as a marker to study possible damage of mycotoxins to intestinal epithelial cells.

Materials and Methods

Cell line and culture conditions

The IPEC-J2 cell line is a continuous intestinal cell line originally derived from jejunal epithelia isolated from a neonatal, unsuckled piglet and maintained as a continuous culture (Schierack et al., 2006). Cells were grown in Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F12 (1:1) medium (Invitrogen ™ Life Technologies, Carlsbad, CA, USA), supplemented with 5% fetal calf serum (FCS, HyClone, Cramlington, England, UK), 1% (v/v) insulin/transferrin/Na-selenite (Gibco, Life Technologies, Paisley, Scotland), 1% (v/v) penicillin/streptomycin (Gibco, Life Technologies) and 1% (v/v) kanamycin (Gibco, Life Technologies), further referred to as culture medium. The cells were routinely seeded at a

density of 1.5×10^5 cells/ml with 18 ml medium in plastic tissue culture flasks (75 cm², Nunc, Denmark), maintained in a humidified incubator at 37 °C under 5% CO_2, and passaged every 2-3 days. All experiments were performed with cells within maximal 10 passages.

Chemicals

DON, T-2, FB1 and ZEA (Sigma-Aldrich, Bornem, Belgium) stock solutions of 2000 µg/ml were prepared in methanol for DON, ethanol for T-2 and acetonitrile for FB1 and ZEA (Merck, Darmstadt, Germany). Doxycycline and paromomycin stock solutions (Sigma-Aldrich, 2000 µg/ml) were prepared in high-performance liquid chromatography (HPLC) grade water. All stock solutions were stored at -20 °C. Serial dilutions were prepared in culture medium in order to obtain non-cytotoxic concentrations of organic solvents and antibiotics, and allowing the addition of a similar volume of vehicle in all experiments.

Determination of non-cytotoxic concentrations of the organic solvents

IPEC-J2 cells were seeded into a 96-well plate at a concentration of 1.5×10^5 cells/ml in a total volume of 200 µl. The cells were maintained in an atmosphere of 5% CO_2 at 37 °C. After overnight incubation, the cells were treated for 72 h with a final concentration of 1, 5, 10 or 20% of the organic solvents (methanol, ethanol or acetonitrile) in the total volume of culture medium. Next, to assess cytotoxicity, 10 µl of a water soluble tetrazolium salt (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) (WST-1) was added to each well and the plate was incubated at 37 °C for an additional 3 h. The absorbance was determined at 450 nm using a microplate ELISA reader (Multiscan MS, Thermo Labsystems, Helsinki, Finland). Each experiment was conducted in triplicate. The percentage of viable cells was calculated using the following formula: % viability = 100 x ((c-b) – (a-b))/(c-b).

In this formula a = OD_{450nm} derived from the wells incubated with solvent, b = OD_{450nm} derived from blank wells, c = OD_{450nm} derived from untreated control wells.

Assessment of cytotoxicity after exposure of IPEC-J2 cells to the investigated antibiotics doxycycline and paromomycin

A flow cytometric technique was used to assess the direct cytotoxic effect of the antibiotics doxycycline and paromomycin. IPEC-J2 cells were seeded at a concentration of 5 x 10^5 cells/ml in 24-well plates in 1 ml of culture medium and allowed to grow for 24 h. Next, cells were treated for 24 h with 0, 5, 10, 20 and 40 µg/ml of doxycycline or paromomycin. Each experiment was conducted in triplicate. After the incubation period, cells were trypsinized, culture medium and cells were collected, centrifuged for 5 min at 524 x *g* and 4 °C and resuspended in 0.5 ml Hank's Buffered Salt Solution (HBSS, Gibco). This procedure was repeated three times in order to remove cellular debris and antibiotics. Cell death was assessed using dual staining with Annexin-V-fluorescein isothiocyanate (Annexin-V-FITC, Roche Diagnostics, Belgium) and propidium iodide (PI, Sigma-Aldrich) which allows the discrimination of viable cells ($FITC^-/PI^-$), apoptotic ($FITC^+/PI^-$) and necrotic cells ($FITC^+/PI^+$). Cells were incubated for 20 min in the dark on ice with 100 µl of a solution containing 20 µl Annexin-V-FITC and 20 µl PI dissolved in 1000 µl incubation buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 140 mM NaCl and 5 mM $CaCl_2$. Cells were assessed by a FACSCanto flow cytometer (Becton, Dickinson and Company, Erembodegem, Belgium) and the percentage of viable, apoptotic and necrotic cells was obtained using the FACSDiva Software (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Assessment of cytotoxicity after exposure of IPEC-J2 cells to *Fusarium* toxins

The flow cytometric technique as described above was also used to assess the direct cytotoxic effect of mycotoxins. IPEC-J2 cells were seeded at a concentration of 5 x 10^5 cells/ml in 24-well plates in 1 ml of culture medium and allowed to grow for 24 h. Next, cells were treated for 72 h with increasing concentrations of T-2 (0-10 ng/ml), DON (0-10 µg/ml), ZEA (0-20 µg/ml) and FB1 (0-15 µg/ml). The molar concentration corresponding with 1 µg/ml DON, FB1 and ZEA and 1 ng/ml T-2 is 3.37 µM, 1.39 µM, 3.14 µM and 2.14 nM, respectively. Each experiment was conducted in triplicate. After the incubation period, cells were processed as described above before analysis on the FACSCanto flow cytometer. The concentration corresponding to 50% reduction in viable cells (IC50) was calculated with linear regression curves. A positive control was provided by cells single stained with Annexin-V-FITC and cells single stained with PI, also used to determine spectral overlap, after treatment for 72 h with 10 µg/ml DON. Furthermore, unstained cells (no Annexin-V-FITC and no PI) were also included to determine autofluorescence of IPEC-J2 cells.

Assessment of TEER after exposure of IPEC-J2 cells to DON and T-2

The effect of DON and T-2 on barrier integrity was determined by measuring the transepithelial electrical resistance (TEER) using a Millicell Electrical Resistance System (Millipore, World Precision Instruments, Sarasota, FL, USA). Cells were grown and differentiated on Transwell® collagen-coated polytetrafluoroethylene (PFTE) membrane inserts (0.4 µm pore diameter, 6.5 mm diameter, Corning Inc., NY, USA) at a density of 1.5 x 10^5 cells/ml. Culture medium was replaced three times a week and IPEC-J2 cells were used for TEER experiments at day 21 post-seeding. Next, cells were incubated for 72 h with different concentrations of T-2 (0 - 100 ng/ml) or DON (0-10 µg/ml). TEER was measured

every 24 h from the start of the toxin exposure until 3 days. TEER values were expressed as kΩ x cm^2. Each single experiment was conducted in threefold.

Passage of doxycycline and paromomycin after exposure of IPEC-J2 cells to DON and T-2

Cells were seeded on Transwell® collagen-coated PFTE membrane inserts as described above for the TEER experiment. Only cells which are cultured for 21 days show stable TEER values indicating cells have reached confluence and form a tight monolayer (data not shown). Next, cells were incubated for 72 h with different concentrations of T-2 (0 - 100 ng/ml) or DON (0-10 µg/ml), during which the last 24 h a non-cytotoxic concentration of both paromomycin (30 µg/ml) and doxycycline (10 µg/ml) was added to the apical (AP) compartment of the transwell. To evaluate the permeability of the monolayers, 50 µl of transport medium was withdrawn from the basolateral (BL) compartment at time 0 (before) and at 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after addition of the drugs and replaced immediately by 50 µl of fresh culture medium. Each experiment was conducted in triplicate.

Acceptor media were analyzed for the paromomycin content by high-performance liquid chromatography tandem mass spectrometry (LC-MS/MS). The LC-MS/MS system consisted of a Surveyor Autosampler Plus and MS Pump Plus, coupled to a TSQ Quantum Ultra® triple quadrupole mass spectrometer (all from ThermoFisher Scientific, Zellik, Belgium). Chromatographic separation was performed on a PLRP-S column (150 mm x 2.1 mm i.d., 100 Å, dp: 5 µm, Varian, Middelburg, The Netherlands) using a gradient programme (flow-rate: 200 µl/min). The mobile phase consisted of (A) 20 mM pentafluoropropionic acid (PFPA) (Sigma-Aldrich) in water and (B) 20 mM PFPA in water/acetonitrile (50/50, v/v) (VWR International, Leuven, Belgium). The MS instrument was operated in the selected reaction monitoring (SRM) mode, using two precursor ion > product ion transitions. Tobramycin

(Sigma-Aldrich) was used as internal standard (IS). Samples were prepared by pipetting 50 µl culture medium into an Eppendorf cup (Novolab, Geraardsbergen, Belgium). Each sample was spiked with 10 µl of the IS working solution of 1 µg/ml in HPLC grade water (VWR International) and diluted with 65 µl methanol, 100 µl HPLC grade water and 25 µl 200 mM PFPA. After vortexing, samples were centrifuged at 7 800 x *g* for 10 min to remove possible solid particles. The sample was transferred into screw-capped conical vials and 5 µl were injected onto the LC-MS/MS system.

Doxycycline concentrations were determined using the same LC-MS/MS configuration and HPLC column as for paromomycin analysis. The mobile phase consisted of (A) 0.5 % acetic acid in water and (B) acetonitrile. A gradient programme was applied and the flow-rate was 200 µl/min. The MS instrument was operated in the SRM mode, using two precursor ion > product ion transitions. Demethylchlortetracycline (DMCTC, Sigma-Aldrich) was used as IS. Samples were prepared by pipetting 25 µl culture medium into an Eppendorf cup. Each sample was spiked with 12.5 µl of the IS working solution of 10 µg/ml in HPLC grade water, followed by the addition of 75 µl 0.5 % formic acid in HPLC grade water. After vortexing briefly and centrifugation, 5 µl were injected onto the LC-MS/MS system.

The limit of quantification (LOQ) was 10 ng/ml for both doxycycline and paromomycin. A detailed description of both methods can be found in Goossens *et al*. (2012).

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) to address the significance of difference between mean values with significance set at $P < 0.05$.

Results

A concentration of 1% ethanol, 5% methanol or 5% acetonitrile of the total volume of culture medium was determined to be non-cytotoxic for IPEC-J2 cells

Figure 1 represents the percentage of cytotoxicity after exposure for 72 h to 1, 5, 10 or 20% ethanol, methanol or acetonitrile in the total volume of culture medium.

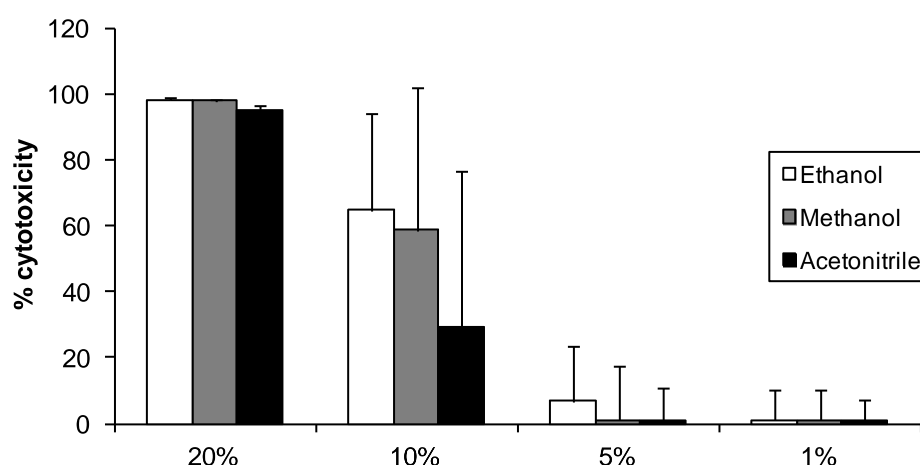

Figure 1. Percentage of cytotoxicity after exposure of IPEC-J2 cells for 72 h to 1, 5, 10 or 20 % ethanol, methanol or acetonitrile in the total volume of culture medium (n=3, mean + SD).

A final concentration of 5% acetonitrile, 5% methanol and 1% ethanol was accepted to use in the experiment as the viability of IPEC-J2 cells was on average > 95 %. Non-cytotoxic concentrations of the solvents were ensured by appropriate dilution of the toxins stock solutions in culture medium.

A concentration of 10 µg/ml doxycycline and 30 µg/ml paromomycin was determined to be non-cytotoxic for IPEC-J2 cells

Figure 2 represents the percentage of viable cells after exposure for 24 h to 5, 10, 20 or 40 µg/ml doxycycline (a) or paromomycin (b), respectively.

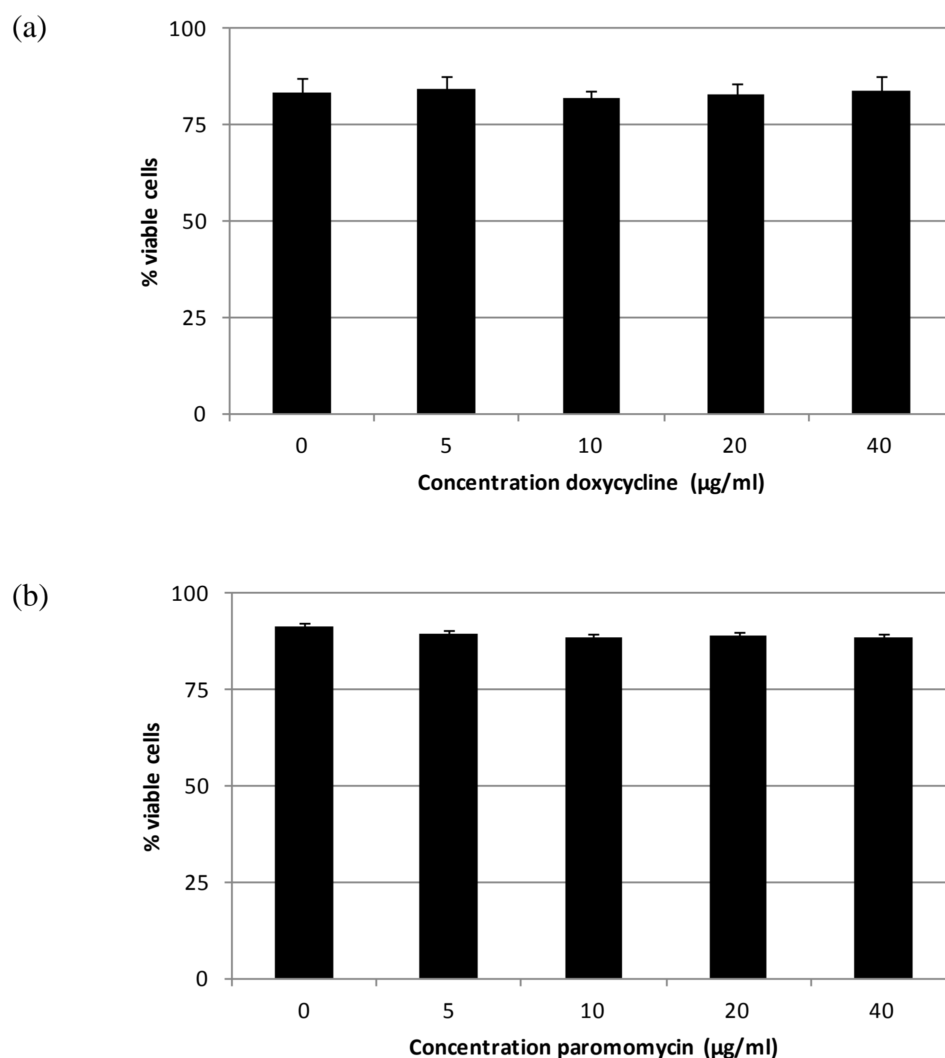

Figure 2. Percentage of viable cells after exposure of IPEC-J2 cells for 24 h to 0, 5, 10, 20 or 40 µg/ml doxycycline (a) or paromomycin (b) (n=3, mean + SD).

A concentration up to 40 µg/ml doxycycline or paromomycin caused no significant reduction in cell viability of IPEC-J2 cells. It was concluded that a concentration of 10 µg/ml doxycycline and 30 µg/ml paromomycin could be used in the experiments. The concentration

of the antibiotics was based on, on the one hand, the dose of the antibiotic that is usually administered to pigs, and on the other hand, the capacity of the intestine of an adult pig. When 10 mg/kg body weight doxycycline and 40 mg/kg body weight paromomycin is administered to a pig of 20 kg, and this dose ends up in an intestinal volume of 27.5 l, this corresponds with a concentration of about 8 and 29 µg/ml respectively.

Induction of necrosis and apoptosis in IPEC-J2 cells is *Fusarium* toxin dependent

Figure 3 represents the percentages of Annexin-V-FITC and PI negative, Annexin-V-FITC positive and PI negative and Annexin-V-FITC and PI positive IPEC-J2 cells, exposed for 72 h to different concentrations of DON, T-2, FB1 or ZEA, respectively.

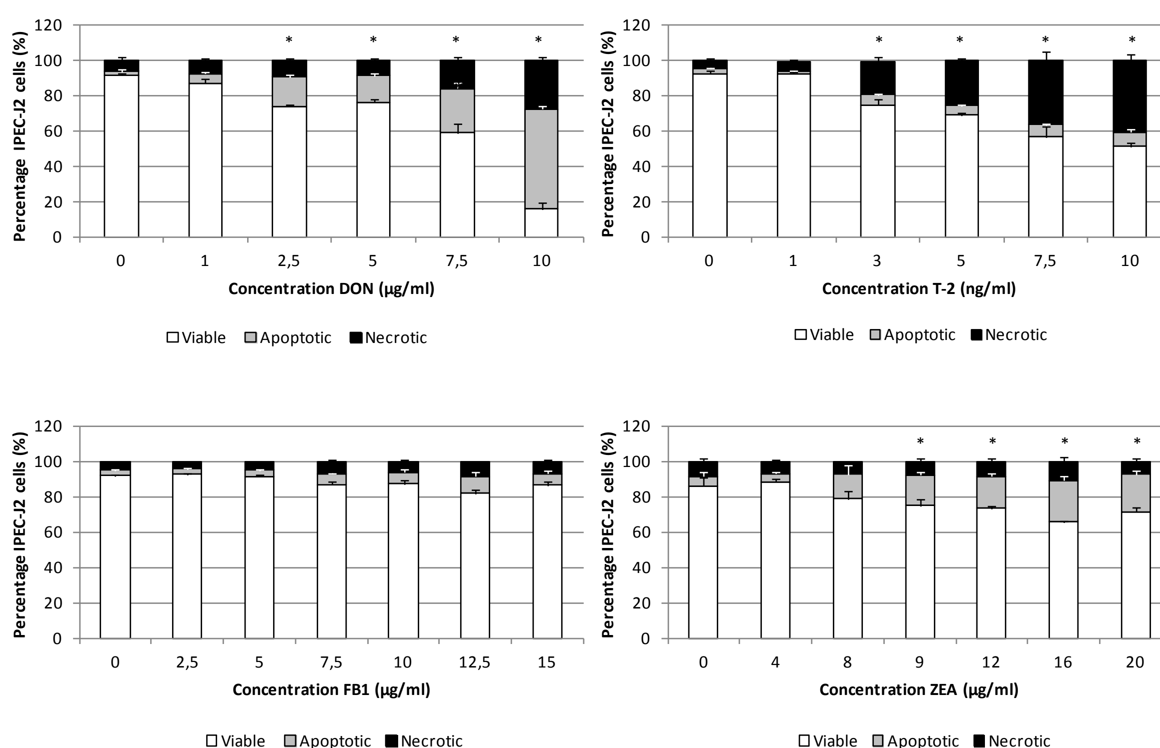

Figure 3. Percentage viable, apoptotic and necrotic cells after incubation of undifferentiated IPEC-J2 cells for 72 h with different concentrations of DON, T-2, FB1 or ZEA (n=3, mean + SD). *Significant difference in viable cells compared to control ($P < 0.05$). The molar concentration corresponding with 1 µg/ml DON, FB1 and ZEA and 1 ng/ml T-2 is 3.37 µM, 1.39 µM, 3.14 µM and 2.14 nM, respectively.

Exposure of IPEC-J2 cells for 72 h to different mycotoxin concentrations showed a concentration-dependent toxicity. T-2 was clearly the most toxic one with a significantly lower percentage of Annexin-V-FITC and PI negative cells in the ng/ml range compared to DON and ZEA which were toxic in the µg/ml range. FB1 was not toxic at the tested concentrations. For all mycotoxins, except T-2 toxin, a higher percentage of Annexin-V-FITC positive and PI negative cells rather than Annexin-V-FITC and PI positive cells can be found after exposure of undifferentiated IPEC-J2 cells for 72 h to different toxin concentrations. For T-2 toxin, the majority of dead cells were Annexin-V-FITC and PI positive. The IC50 for DON and T-2 was determined to be 6.98 µg/ml and 9.55 ng/ml, respectively.

Cytotoxic concentrations of DON and T-2 induce a decrease in transepithelial electrical resistance (TEER) across the IPEC-J2 monolayer

Figure 4 and 5 represent the effect of DON and T-2 on TEER of differentiated IPEC-J2 cells, respectively.

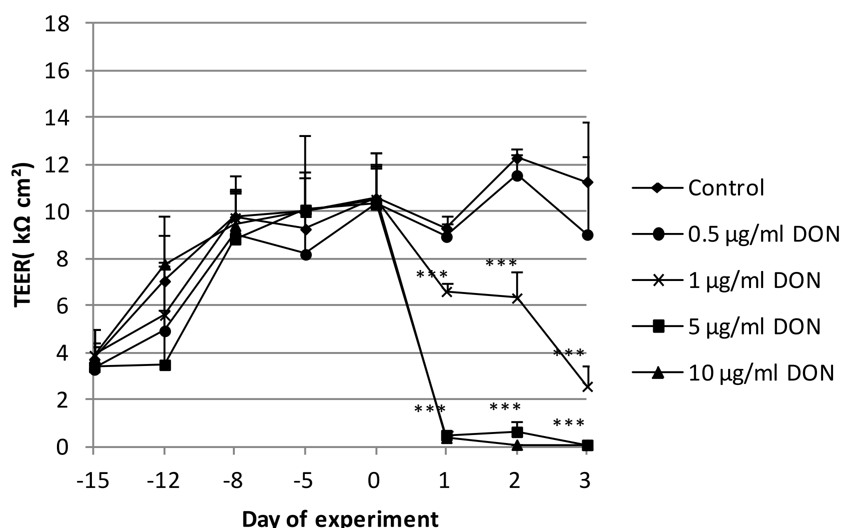

Figure 4. Effect of DON on trans-epithelial electrical resistance (TEER) of differentiated IPEC-J2 cells. Cells were grown for 21 days on Transwell® collagen-coated PTFE filters (0.33 cm², 0.4 µm). Next, at day 0 various concentrations of DON were added to the apical compartment for 72 h: untreated inserts, inserts treated with 0.5, 1, 5 or 10 µg/ml of DON. TEER values are expressed as mean + SD (n = 3). Significant differences in TEER value have been marked with *** ($P < 0.001$).

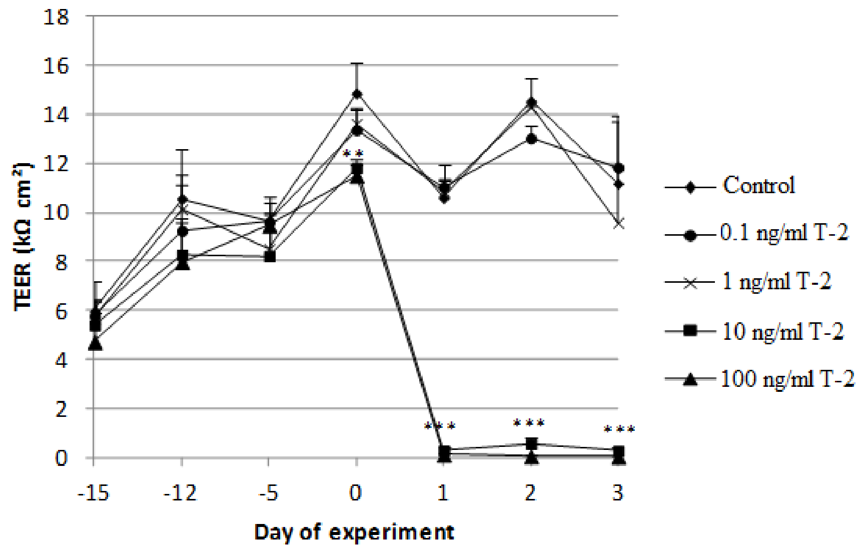

Figure 5. Effect of T-2 on trans-epithelial electrical resistance (TEER) of differentiated IPEC-J2 cells. Cells were grown for 21 days on Transwell® collagen-coated PTFE filters (0.33 cm², 0.4 µm). Next, at day 0 various concentrations of T-2 were added to the apical compartment for 72 h: untreated inserts, inserts treated with 0.1, 1, 10 or 100 ng/ml of T-2. TEER values are expressed as mean + SD (n = 3)**.** Significant differences in TEER value have been marked with ** ($P < 0.01$) or *** ($P < 0.001$) according to the significance level.

Both for T-2 and DON, a drop in TEER can be seen 24 h after incubation of IPEC-J2 cells with cytotoxic concentrations of the mycotoxins. Treatment with non-cytotoxic concentrations results in TEER values comparable to those of the control cells. Treatment of differentiated IPEC-J2 cells with 1 µg/ml DON shows a time-dependent decline in TEER.

Disruption of epithelial integrity of the IPEC-J2 cell monolayer results in increased passage of doxycycline and paromomycin

Figure 6 and 7 represent the passage of doxycycline and paromomycin across the IPEC-J2 cell monolayer treated for 72 h with several concentrations of DON and T-2, respectively.

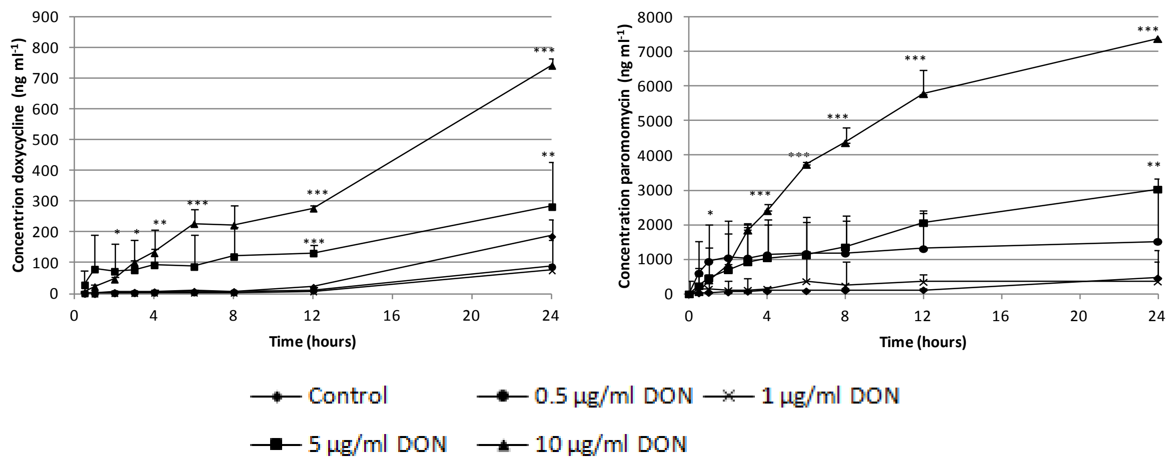

Figure 6. Passage of doxycycline (a) and paromomycin (b) across a differentiated IPEC-J2 cell monolayer, measured as the concentration in the basolateral compartment. Cells were grown for 21 days on Transwell® collagen-coated PTFE filters (0.33 cm², 0.4 µm). Next, at day 0, cells were incubated for 72 h with different concentrations of DON (untreated inserts, inserts treated with 0.5, 1, 5 or 10 µg/ml), during which the last 24 h a non-cytotoxic concentration of doxycycline and paromomycin was added to the apical compartment (n = 3, mean + SD). Significant differences in passage have been marked with * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$) according to the significance level.

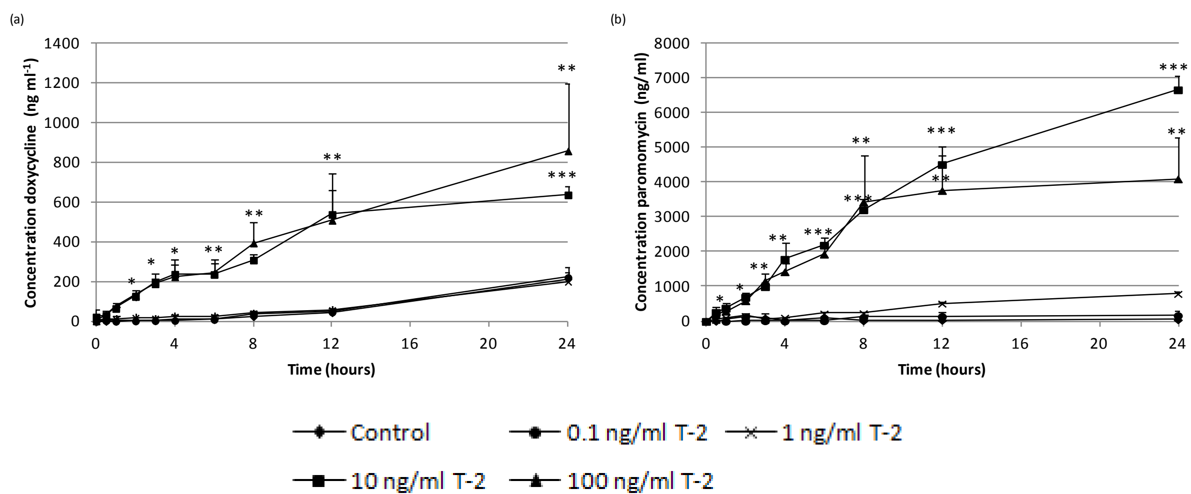

Figure 7. Passage of doxycycline (a) and paromomycin (b) across a differentiated IPEC-J2 cell monolayer, measured as the concentration in the basolateral compartment. Cells were grown for 21 days on Transwell® collagen-coated PTFE filters (0.33 cm², 0.4 µm). Next, at day 0, cells were incubated for 72 h with different concentrations of T-2 (untreated inserts, inserts treated with 0.1, 1, 10 or 100 ng/ml of T-2), during which the last 24 h a non-cytotoxic concentration of doxycycline and paromomycin was added to the apical compartment (n = 3, mean + SD). Significant differences in passage have been marked with * ($P < 0.05$), ** ($P < 0.01$) or *** ($P < 0.001$) according to the significance level.

In both the T-2 and DON experiment, increased passage of doxycycline and paromomycin was seen after incubation of IPEC-J2 cells with cytotoxic concentrations of the tested mycotoxins.

Discussion

The gastrointestinal tract can be exposed to high concentrations of mycotoxins after ingestion of mycotoxin contaminated food or feed. Since swine are the most sensitive domestic animal species to mycotoxins (Hussein et al., 2001), this study aimed to investigate the toxic effect of four common *Fusarium* toxins, T-2, DON, ZEA and FB1 on IPEC-J2 cells. Studies concerning the effects of mycotoxins on IPEC-J2 cells are limited. The concentrations of the mycotoxins tested in this study are the result of several optimization experiments performed to refine the concentration range to be used for these *in vitro* experiments. According to Sergent et al. (2006) DON concentrations in human intestine are estimated between 0.16 µg/ml and 2 µg/ml depending on the contamination level of the feed, including thus our tested range. Mycotoxin concentrations in the test solutions are not based on levels known to cause problems in pigs, but are based on the cytotoxicity results of the flow cytometer experiments, the solubility of the mycotoxin in aqueous solvents, and cost of the mycotoxin.

In vitro cell viability assays have a central role in predictive toxicology. The capability of commonly used colorimetric assays, such as the WST-1, MTT (3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide) and MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) or the lactate dehydrogenase (LDH) bioassay to detect cytotoxic effects is, however, limited. Cells that are apoptotic remain undetected with these assays. Our study used a flow cytometric technique, which allows to distinguish Annexin-V-FITC and PI negative (viable) cells from Annexin-V-FITC positive and PI negative (apoptotic) cells and Annexin-V-FITC and PI positive (necrotic) cells.

After 72 h of exposure with T-2, DON, FB1 or ZEA, T-2 clearly was the most cytotoxic with a toxicity in the ng/ml range, while the other mycotoxins are only toxic at concentrations in the µg/ml range. This finding is in accordance with the study of Calvert et al. (2005), who demonstrated that T-2 was most cytotoxic against three human cell lines tested as determined by the MTT assay.

When comparing the percentage Annexin-V-FITC positive and PI negative cells and Annexin-V-FITC and PI positive cells, it is striking that more Annexin-V-FITC and PI positive cells than Annexin-V-FITC positive and PI negative cells were found after treatment of the cells with T-2. This corresponds, however, with findings in practice where T-2, present in feed, causes skin necrosis (Pang et al., 1987; Harvey et al, 1990). Also necrosis of the lymphoid cells of the intestinal mucosa was seen after intake of T-2 contaminated feed (Pang et al., 1987; Fekete et al., 1989). DON, on the other hand, causes more Annexin-V-FITC positive and PI negative cells than Annexin-V-FITC and PI positive cells. The discrepancy between type A (T-2) and type B (DON) trichothecenes was also demonstrated by Nasri et al. (2006) who showed that DON causes rather apoptosis and T-2 rather necrosis in Jurkat T-lymphocytes. As it is known that type B trichothecenes activate the MAPK signaling pathway resulting in apoptosis, while type A trichothecenes are not able to activate the MAPK families (Shifrin et al., 1999; Moon et al., 2003; Zhou et al;, 2005) the authors suggested this as an explanation for the results. For ZEA and FB1, although FB1 was not cytotoxic at the tested concentrations, an approximately equal percentage of apoptotic and necrotic cells were seen after treatment of IPEC-J2 cells for 72 h with those toxins. Our results suggest that different toxins may induce different types of cell death in IPEC-J2 cells. To our knowledge, this is the first report of DON and T-2 induced different mechanisms of cell death in IPEC-J2 cells. Since it was not the principal aim of this study to elaborate further the differences in the mode of cell death induced by different mycotoxins, no further experiments were conducted.

Nevertheless, it could be interesting to investigate these differences in more detail, including the investigation of associated pathway molecules, the analysis of potential protective effects of caspase inhibitors and/or necrose inhibitors, etc. Also other types of cell death can be taken into account since there are more than two ways for a cell to die. Cell death can be classified according to its morphological appearance (apoptotic, necrotic, autophagic or associated with mitosis), enzymological criteria (with and without the involvement of nucleases or distinct classes of proteases, such as caspases, calpains, cathepsins and transglutaminases), functional aspects (programmed or accidental, physiological or pathological) or immunological characteristics (immunogenic or non-immunogenic) (Melino, 2001). It could be interesting exploring these differences in relation with mycotoxins.

In correlation with direct cytotoxicity, the consequences on barrier integrity (TEER and passage of drugs) were also assessed. The effect on TEER and passage was only studied for DON and T-2 because, of the four mycotoxins tested in this study, these are the most toxic ones and their ratio of Annexin-V-FITC positive and PI negative cells and Annexin-V-FITC and PI positive cells is antithetic. Notwithstanding the assertion of Geens and Niewold (2011) that IPEC-J2 cells cultured on Transwell®-collagen coated inserts with a surface area of 0.33 cm² are not appropriate to measure TEER values, our IPEC-J2 monolayer showed TEER values above 10 kΩ cm² after 3 weeks of culturing. According to Fromter and Diamond (1972), IPEC-J2 cell lines with TEER values of approximately 2 kΩ cm² can be considered to be tight epithelia. When IPEC-J2 cells were incubated with cytotoxic concentrations of DON (5 and 10 µg/ml) and T-2 (10 and 100 ng/ml) TEER values dropped to nearly 0 kΩ cm² after 24 h suggesting a complete disruption of the epithelial monolayer within 24 h. In contrast, TEER values of IPEC-J2 cells incubated with non-toxic concentrations (0.5 µg/ml DON and 0.1 and 1 ng/ml T-2) were comparable to the TEER of control cells. Treatment with 1 µg/ml

DON on the other hand shows a time-dependent reduction of the TEER. This was also reported by Pinton *et al.* (2009) who showed a time- and dose-dependent reduction of the TEER after exposure of IPEC-1 and Caco-2 cells to different concentrations (0, 3, 6, 15, 30 µg/ml) of DON. A decreased barrier function *in vitro* is reflected in both a decreased TEER and an increased tracer flux (Mahfoud et al., 2002; Bouhet et al., 2004). In our study, the decrease in TEER is related to the passage of the used tracers, doxycycline (molecular weight (m.w.) 513 Da) and paromomycin (m.w. 714 Da), two commonly used antibiotics in pig industry. The higher the toxin concentration, the higher the marker passage. Most studies use tracers such as FITC-dextran, while in this study and for the first time the antibiotics doxycycline and paromomycin were used as markers. We were, however, not capable of demonstrating that induction of more apoptosis or more necrosis results in differences in transepithelial passage of antibiotics. Both doxycycline and paromomycin pass the epithelial monolayer, regardless the induction of a different cell death mechanism.

It is very interesting to study the passage of these therapeutic compounds during *in vivo* trials, as veterinary drugs are often used in pig mass medication and frequently administered by the oral route, i.e. mixed in the feed or drinking water. When discussing the relevance of the *in vitro* results for the *in vivo* situation it needs to be taken into account that enterocytes are not continuously exposed to mycotoxins. Rather a time-dependent fluctuation in mycotoxin concentrations exists *in vivo*, whereas *in vitro* cells are exposed continuously at constant concentrations. Nevertheless, the current *in vitro* findings warrant further investigation *in vivo* because an increased passage of antibiotics may have important implications for both animal and human health. Increased absorption of antibiotics may lead to higher plasma levels, including the risk of exceeding the toxic threshold. Moreover, increased antibiotic plasma levels may also lead to a tissue residue problem in slaughter animals and inherent food safety

concerns. It could thus be interesting to investigate *in vivo* the effect of mycotoxins on the intestinal absorption of antibiotics.

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Chapter 2

Influence of mycotoxins and a mycotoxin adsorbing agent on the oral bioavailability of commonly used antibiotics in pigs

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Abstract

It is recognized that mycotoxins can cause a variety of adverse health effects in animals, including altered gastrointestinal barrier function. It is the aim of the present study to determine whether mycotoxin-contaminated diets can alter the oral bioavailability of the antibiotics doxycycline and paromomycin in pigs, and whether a mycotoxin adsorbing agent included into diets interacts with those antibiotics. Experiments were conducted with pigs utilizing diets that contained blank feed, mycotoxin-contaminated feed (T-2 toxin or deoxynivalenol), mycotoxin-contaminated feed supplemented with a glucomannan mycotoxin binder, or blank feed supplemented with mycotoxin binder. Diets with T-2 toxin and binder or deoxynivalenol and binder induced increased plasma concentrations of doxycycline administered as single bolus in pigs compared to diets containing blank feed. These results suggest that complex interactions may occur between mycotoxins, mycotoxin binders, and antibiotics which could alter antibiotic bioavailability. This could have consequences for animal toxicity, withdrawal time for oral antibiotics, or public health.

Introduction

Toxigenic fungi may often colonize fodder crops and feed components. Under varied environmental conditions they can produce toxic secondary metabolites, called mycotoxins. A recent study investigated the occurrence of mycotoxins in European feed samples and concluded that 82% of the samples were contaminated with mycotoxins (Monbaliu et al., 2010), indicating that mycotoxins are omnipresent. All farm animals can experience a negative impact from a dietary intake of mycotoxins but pigs are one of the species which are highly sensitive. The economic impact of mycotoxins includes increased mortality, increased veterinary care costs, reduced livestock production, disposal of contaminated foods and feeds and investment in research and applications to reduce the mycotoxin problem (Hussein et al., 2001).

Since the gastrointestinal epithelium is the first barrier exposed to mycotoxins after ingestion of contaminated feed, research has focused on the effects on barrier integrity. *In vitro* research revealed that mycotoxins are able to increase the permeability of intestinal epithelial cell monolayers. Indeed, deoxynivalenol (DON), ochratoxin A (OTA) and patulin (PAT) compromise the intestinal barrier function by altering the tight junction complex (Maresca et al., 2001, 2002; Mahfoud et al., 2002; McLaughlin et al., 2004; Sergent et al., 2006; Lambert et al., 2007). This reduced expression of tight junction proteins leads to an increased passage of tracers such as fluorescein isothiocyanate (FITC)-dextran and bacteria such as *Escherichia coli* (Pinton et al., 2009). In addition, *in vivo* experiments provide compelling evidence that mycotoxins can alter intestinal functions and lead to malabsorption of nutrients like glucose (Hunder et al., 1991; Subramanian et al., 1991).

To minimize exposure to mycotoxins, a variety of physical, chemical and biological methods has been developed in order to eliminate fungi and their mycotoxins from foods and feeds

(Jouany et al., 2007; Kabak et al., 2009). One of the most prominent post-harvest strategies is the use of mycotoxin adsorbents as feed additives. By including various mycotoxin adsorbing agents in the compound feed, the bioavailability of mycotoxins can decrease by reducing their uptake (Avantaggiato et al., 2005).

The extensive use of adsorbents in the livestock industry has led to the introduction of a wide range of new products, most of them offering high *in vitro* mycotoxin adsorption capacity. Regarding the use of these products as feed additives, a primary concern is that the *in vivo* efficacy in sequestering mycotoxins and the safety towards livestock of most of these commercial products have not yet been thoroughly tested (Ledoux et al., 2001).

The European Commission (EC, 2009) recently defined a new functional group of feed additives as "substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion or modify their mode of action" and requested technical advice from the European Food Safety Authority (EFSA) on the guidelines to be followed for authorization of these additives. EFSA stated that not only *in vitro* but also *in vivo* studies are required for the assessment of mycotoxin binders with regard to efficacy testing. A major risk for mycotoxin binders, however, is the lack of selectivity with possible consequences for nutritional aspects through interactions with dietary compounds. EFSA also reported that one of the parameters to be studied is the possible interactions of mycotoxin binders with veterinary medicinal products (EFSA, 2010). Additives that exert their activity mainly by binding, may affect the oral bioavailability of drugs.

To our knowledge, only a few studies deal with this topic. Amer et al. (2005) described a significantly lower maximal plasma concentration for lincomycin in broilers after pretreatment with a mycotoxin binder (C_{max} = 3.27 ± 0.15 µg/ml), compared to broilers receiving no binder in the feed (C_{max} = 10.65 ± 0.17 µg/ml). The Bureau of Veterinary Drugs

of Canada (1992) reported a lack of efficacy of tylosin in cattle after intake of a clay containing bentonite which binds tylosin and makes it unavailable to the animal. Shryock *et al.* (1994) demonstrated that bentonite (2%), mixed in the feed, renders tilmicosin completely ineffective in broiler chickens. This resulted in the prohibition of the use of a bentonite feed additive in combination with antibiotics, growth promoters, coccidiostats and other medical substances (EFSA, 2011).

Since there are no other scientific papers that investigate these possible interactions, the general aim of this study was to examine whether mycotoxins, a commercially available mycotoxin binder, or the combination of both in the feed, affect the oral bioavailability of frequently used antibiotics in pigs. It is generally accepted that, in the northern hemisphere, *Fusarium* moulds are among the most important toxigenic fungi involved in the animal feed chain (Nelson et al., 1994). Therefore the mycotoxins studied were T-2 toxin (T-2) and deoxynivalenol (DON). T-2 is the most potent and cytotoxic trichothecene (Calvert et al., 2005). Moreover , there are no recommended maximum levels in animal feed for this *Fusarium* mycotoxin available yet (EC, 2006). DON on the other hand, is one of the most frequent contaminants of maize and small grain cereals (EC, 2006). Both mycotoxins have already been proven in our laboratory to influence the passage of the antibiotics doxycycline and paromomycin across an intestinal epithelial monolayer of porcine origin (Goossens et al., 2012). These antibiotics were also used in this *in vivo* experiment as it is a common practice in current pig husbandry to administer these veterinary drugs by ‘mass’ medication in feed and/or drinking water.

Materials and Methods

Animals

Twenty-four clinically healthy 9-week-old pigs (Piétrain × Landrace, local commercial pig farm), with a mean (± SD) body weight of 22.6 ± 1.1 kg, were used in the T-2 experiment (experiment 1). The animals were randomized into a control group (*n* = 6) and three experimental groups (each *n* = 6).

A second experiment with DON was performed with another twenty-four pigs (Piétrain × Landrace), with a mean (± SD) body weight of 23.1 ± 1.4 kg. The animals were also randomized into a control group (*n* = 6) and three experimental groups (each *n* = 6).

All the animals were weighed daily and fed, once a day, 1.5 kg of the assigned feed during the first week and 2 kg of the assigned feed during the last two weeks of the experiment. Water was available *ad libitum*. The animals were housed in groups under natural light conditions.

Both animal experiments have been approved by the ethics committee of the Faculty of Veterinary Medicine, Ghent University (EC 2009/094 + expansion EC 2010/012 and expansion EC 2010/038 + EC2010/120).

Feed

Experiment 1: Influence of T-2 and Mycotoxin Binder on the Oral Absorption of Doxycycline and Paromomycin

Conventional pig feed was purchased (ILVO, Melle, Belgium) and analysed (n=1) for the presence of mycotoxins. Analysis with liquid chromatography tandem mass spectrometry (LC-MS/MS) (Monbaliu et al., 2010) revealed that the feed contained 479 ± 140 µg/kg DON and 44 ± 13 µg/kg zearalenone. All other mycotoxins (*n* = 21) tested were below the limit of

detection (LOD). Since the feed did not contain T-2 toxin, it was accepted for use in experiment 1. This feed is further referred to as blank feed and was used to feed the animals during the acclimatization period as well as to prepare the T-2 contaminated feed needed for the experiment.

To produce feed contaminated with 100 µg T-2/kg, a stock solution of 250 µg/ml T-2 toxin was prepared by dissolving 50 mg T-2 (Sigma-Aldrich, Bornem, Belgium) in 200.0 ml ethanol (Merck, Darmstadt, Germany). The contaminated feed was produced by adding 120 ml of the stock solution to 500 g of blank feed. This premix was then mixed with 5 kg of blank feed to assure a homogeneous distribution of the toxin. The final premix was then mixed for 20 min in the total amount of feed (300 kg) needed for the experiment. To test T-2 toxin 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 toxin. A mean concentration of 99 ± 13 µg/kg T-2 was found in this T-2 contaminated feed.

The binder used in the experiment was a commercially available glucomannan mycotoxin binder which claimed to bind T-2 and DON, added at a concentration of 2 kg per metric ton. To produce the binder supplemented feed (150 kg), binder was added to both blank feed and feed contaminated with 100 µg T-2/kg feed and mixed for 20 min. A mean concentration of 111 ± 4 µg/kg T-2 was found in the T-2 contaminated feed supplemented with binder.

Experiment 2: Influence of DON and Mycotoxin Binder on the Oral Absorption of Doxycycline and Paromomycin

Conventional feed was purchased (DANIS, Koolskamp, Belgium) and analysed (n=1) with LC-MS/MS for the presence of mycotoxins. As the feed only contained 11 ± 4 µg/kg T-2 and no other mycotoxins such as DON, the feed was used to feed the animals during the acclimatization period as well as to prepare the DON contaminated feed needed for the

experiment. The reference strain *Fusarium graminearum* MUCL 42841 (Mycothèque de l'Université catholique de Louvain) was used to produce DON, for inclusion in feed at a theoretical concentration of 1 mg/kg. The strain was grown in liquid mineral medium supplemented with L-arginin as a selective nitrogen source (Gardiner et al., 2009). After 14 days of cultivation, the culture was filtered and centrifuged. The supernatant was freeze-dried and mixed into the blank feed (300 kg) until a final DON concentration of 1 mg/kg was obtained. To test DON 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 DON. A mean concentration of 802 ± 23 µg/kg DON was found and the feed was accepted to be used in the experiment. Blank feed supplemented with binder (150 kg) was produced as described in experiment 1. A mean concentration of 813 ± 24 µg/kg DON was found in the DON contaminated feed supplemented with binder.

Study Design

Experiment 1

After an acclimatization period of one week, during which all animals received blank feed, the four groups of six animals received blank feed (control), feed contaminated with 99 ± 13 µg T-2/kg feed, feed contaminated with 111 ± 4 µg T-2/kg feed and supplemented with the mycotoxin binder or blank feed supplemented with the mycotoxin binder, respectively.

After the intake of the experimental diet for seven days, the animals received a single oral intragastric bolus of doxycycline (Soludox 50%®, Eurovet, Bladel, The Netherlands) and paromomycin (Gabbrovet 70®, CEVA Santé Animale, Brussels, Belgium). The oral solution was prepared by dissolving the powders in tap water. Doxycycline was given at a dose of 10 mg/kg body weight and paromomycin at 100 mg/kg body weight. Blood samples were collected in heparinised tubes (Venoject®, Terumo Corp., Tokyo, Japan) by puncturing the

external jugular vein with a 20 G needle (20 G, 0.9 × 38 mm, Terumo Corp., Tokyo, Japan) before and at 0.5, 1, 2, 3, 4, 6, 8, 12 and 24 h after administration. The samples were centrifuged at 2,851 *g* at 4 °C for 10 min and plasma was stored at −15 °C until assayed for paromomycin and doxycycline.

Experiment 2

To evaluate whether another trichothecene mycotoxin results in findings comparable to the first experiment, a second experiment was performed using the mycotoxin DON. After an acclimatization period of one week, four groups of six animals received for 13 days respectively blank feed (control), feed contaminated with 802 ± 23 µg/kg DON, feed contaminated with 813 ± 24 µg/kg DON and supplemented with the mycotoxin binder and blank feed supplemented with the mycotoxin binder. Subsequently the animals received a single oral bolus of doxycycline (10 mg/kg b.w.) and paromomycin (100 mg/kg b.w.), blood was drawn and plasma samples were collected as described in experiment 1.

Doxycycline Determination in Plasma

Plasma doxycycline concentration was determined by high-performance liquid chromatography (HPLC), using ultraviolet detection, based on the procedure described by Baert *et al.* (2000). The method was in-house validated by a set of parameters that were in compliance with the recommendations as defined in several EU documents (Knecht et al., 1974; Heitzman et al., 1994; Anonymous, 2002; Anonymous, 2011). The following parameters were evaluated: linearity, within-run and between-run accuracy and precision, limit of quantification (LOQ), limit of detection (LOD), specificity. Quantification was performed using matrix-matched calibration curves (concentration range: 200-1000 ng/ml and 2000-10 000 ng/ml) and the correlation coefficients (r = 0.9963 ± 0.0030, n = 6 and r =

0.9962 ± 0.0030) and goodness-of-fit coefficients (g = 5.65 ± 2.49% and 5.37 ± 2.44%, n = 6) fell within the accepted ranges, *i.e.*, $r \geq 0.99$ and $g \leq 10\%$, respectively. The internal standard (IS, demethylchlortetracycline, chemical reference substance, European Pharmacopoeia, Strasbourg, France) was added to all samples prior to the start of the sample preparation procedure. Since the principle of internal standardization was used for quantification, no correction factor had to be applied for analyte loss during sample preparation (extraction recovery). Within-run and between-run precision and accuracy were evaluated by analyzing 6 independently spiked samples at 2 concentration levels, *i.e.*, 1000 and 5000 ng/ml, respectively. The following mean results were obtained: within-run accuracy (n = 6): 908.0 ± 40.4 ng/ml (4.4%) and 4955 ± 223.0 ng/ml (4.5%); between-run accuracy (n = 6): 912.0 ± 43.3 ng/ml (4.7%) and 4810 ± 153.9 ng/ml (3.2%).

The results fell within the accepted ranges for accuracy (-20% to +10% of the theoretical concentration) and precision (within-run precision: relative standard deviation (RSD) ≤ RSD_{max} with $RSD_{max} = 2^{(1-0.5\log \text{Conc})} \times 2/3$, *i.e.*, 10.7% and 8.4% at 1000 ng/ml and 5000 ng/ml, respectively; between-run precision: RSD ≤ RSD_{max} with $RSD_{max} = 2^{(1-0.5\log \text{Conc})}$, *i.e.*, 16.0% and 12.6% at 1000 ng/ml and 5000 ng/ml, respectively). The LOQ was defined as the lowest concentration for which the method was validated with a within-run accuracy and precision that fell within the specified ranges. The LOQ was also the lowest point of the calibration curve and was set at 200 ng/ml (n = 6, mean result: 190.2 ± 3.44 ng/ml). The LOD was defined as the concentration corresponding with a signal-to-noise ratio of 3 and was found to be 82.6 ng/ml. The specificity of the method was shown, since no peaks of endogenous interferences could be determined in blank samples.

Paromomycin Determination in Plasma

The concentration of paromomycin in plasma was determined by LC-MS/MS. Samples were prepared by pipetting 500 µl plasma into an Eppendorf cup (Novolab, Geraardsbergen, Belgium). Each sample was spiked with 50 µl of the IS (tobramycin, Sigma) working solution of 5 µg/ml in HPLC grade water (VWR International, Leuven, Belgium). After vortexing briefly, 100 µl of a 20% trichloroacetic acid (TCA) solution in water were added to deproteinize. After vortexing for 15 sec, the samples were centrifuged at 7800 *g* for 10 min. The upper layer was transferred into screw-capped polypropylene vials for HPLC and 100 µl were injected onto the LC-MS/MS system. The HPLC system consisted of a quaternary gradient pump type P4000 and an AS3000 autosampler coupled to a LCQ® mass spectrometer with an electrospray ionization source operating in the positive ionization mode (ThermoFisher Scientific, Zellik, Belgium). Chromatographic separation was achieved using a Nucleosil column (100 mm × 3 mm i.d., dp: 5 µm, Varian) protected with a guard column of the same type (10 mm × 2 mm i.d., Varian). The mobile phase consisted of 20 mmol/l pentafluoropropionic acid (PFPA) (Sigma-Aldrich) in water (A) and 20 mmol/l PFPA in water/acetonitrile (50/50, v/v) (VWR International) (B). A gradient elution was performed at a flow-rate of 0.2 ml/min, *i.e.*, 0–4 min, 40% B; 4–6 min: linear gradient to 90% B, 6–6.1 min, linear gradient to 40% B, 6.1–10.5 min, 40% B. Paromomycin and the IS eluted at 5.45 and 5.68 min, respectively. A divert valve was used to direct the HPLC column effluent to the MS system from 4.5 to 7.5 min only, which prevented the MS system from quick contamination. The method was in-house validated by the same parameters as for the doxycycline analysis (Knecht et al., 1974; Heitzman et al., 1994; Anonymous, 2002; Anonymous, 2011). Quantification was performed using matrix-matched calibration curves (concentration range: 50-5000 ng/ml) and the correlation coefficients ($r = 0.9981 \pm 0.0005$, $n = 6$) and goodness-of-fit coefficients ($g = 8.29 \pm 1.71\%$, $n = 6$) fell within the accepted ranges,

i.e., $r \geq 0.99$ and $g \leq 10\%$, respectively. Within-run and between-run precision and accuracy were evaluated at 2 concentration levels, *i.e.*, 250 and 2500 ng/ml, respectively. The following mean results were obtained: within-run accuracy and precision (n = 6): 228.1 ± 11.5 ng/ml (5.0%) and 2603 ± 191.8 ng/ml (7.4%); between-run accuracy and precision ($n = 6$): 225.2 ± 15.7 ng/ml (7.0%) and 2410 ± 165.8 ng/ml (6.9%). The results fell within the accepted ranges for accuracy (-20% to +10% of the theoretical concentration) and precision ($RSD \leq RSD_{max}$ with RSD_{max} for within-run precision: 13.1% and 9.3% and RSD_{max} for between-run precision: 19.7% and 13.9% at a concentration level of 250 ng/ml and 2500 ng/ml, respectively). The LOQ was set at 50 ng/ml ($n = 6$, mean result: 46.9 ± 5.26 ng/ml), while the LOD was found to be 0.09 ng/ml. The specificity of the method was shown, since no peaks of endogenous interferences could be determined in blank samples.

Pharmacokinetic and Statistical Analysis

Plasma concentration *versus* time data were analyzed by means of WinNonlin®, Version 6.2.0 (Pharsight Corporation, Mountain View, CA, USA) software program using noncompartmental analysis. The area under the plasma concentration-time curve from dosing to the last measured concentration ($AUC_{0–24h}$) was calculated via the trapezoidal method. Data were statistically analyzed using SPSS 17.0 software for Windows (SPSS Inc., Chicago, IL, USA). Normally distributed data were analyzed using one-way analysis of variance (ANOVA) to address the significance of difference between mean values with significance set at $P \leq 0.05$. Bonferroni as post hoc test was used when equal variances were assessed. Not normally distributed data were analyzed using the non-parametric Kruskal-Wallis analysis, followed by a Dunn's Multiple Comparison test.

Results

Experiment 1

Intake of T-2 Contaminated Feed Supplemented with Mycotoxin Binder Results in Significant Higher Plasma Concentrations of Doxycycline

For the different groups, the plasma concentration-time curve of doxycycline after oral (p.o.) administration is presented in Figure 1.

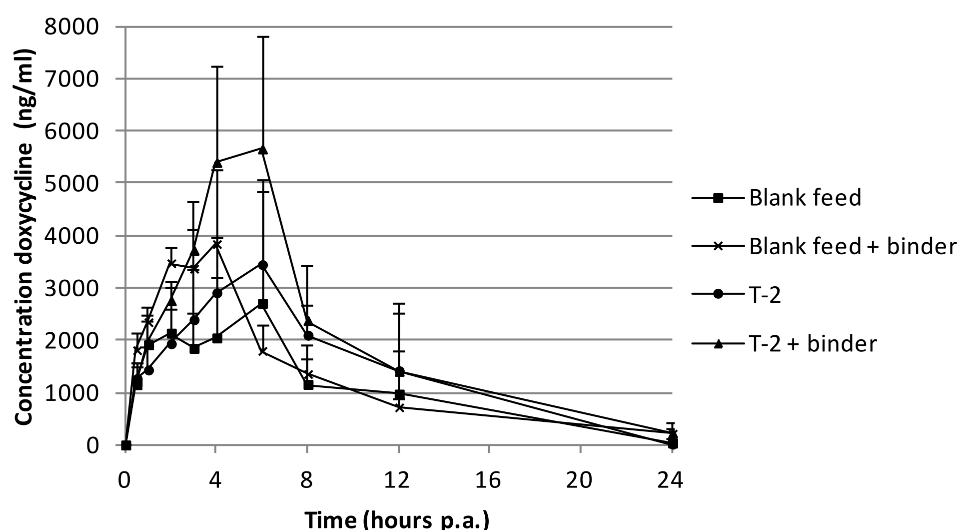

Figure 1. Mean plasma concentrations (+SD) in pigs after a single peroral administration of doxycycline at 10 mg/kg body weight (n = 6). Prior to the bolus, pigs received during 7 days, blank feed (control group), blank feed supplemented with mycotoxin binder, feed contaminated with 100 µg T-2/kg feed and feed contaminated with 100 µg T-2/kg feed supplemented with mycotoxin binder, respectively.

The group which received feed supplemented with 100 µg T-2/kg feed and binder showed significantly higher plasma concentrations compared to the control group (P = 0.033), but not to the other experimental groups. The group which received 100 µg T-2/kg feed and the one that received blank feed supplemented with the mycotoxin binder had plasma concentrations that were not different with the control group. The mean area under the plasma concentration-time curve ($AUC_{0–24h}$) is summarized in Table 1.

Table 1. Mean (± SD) area under the plasma concentration-time curve (AUC_{0-24h}) for doxycycline in the different groups. Prior to the bolus, pigs received during 7 days, blank feed (control group), blank feed supplemented with mycotoxin binder, feed contaminated with 100 µg T-2/kg feed and feed contaminated with 100 µg T-2/kg feed supplemented with mycotoxin binder, respectively. Superscript (*) refers to a significant difference compared to the control group ($P < 0.05$).

Group	AUC_{0-24h} (ng/ml*h)
Blank feed (control)	22 653 (± 16 275)
Blank feed + binder	29 343 (± 7 681)
100 µg/kg T-2	29 320 (± 13 334)
100 µg/kg T-2 + binder	43 961 (± 7 982) *

Intake of Binder Supplemented Feed Results in Higher Plasma Concentrations of Paromomycin

The plasma concentration-time curve of paromomycin after p.o. administration for the different groups is presented in Figure 2.

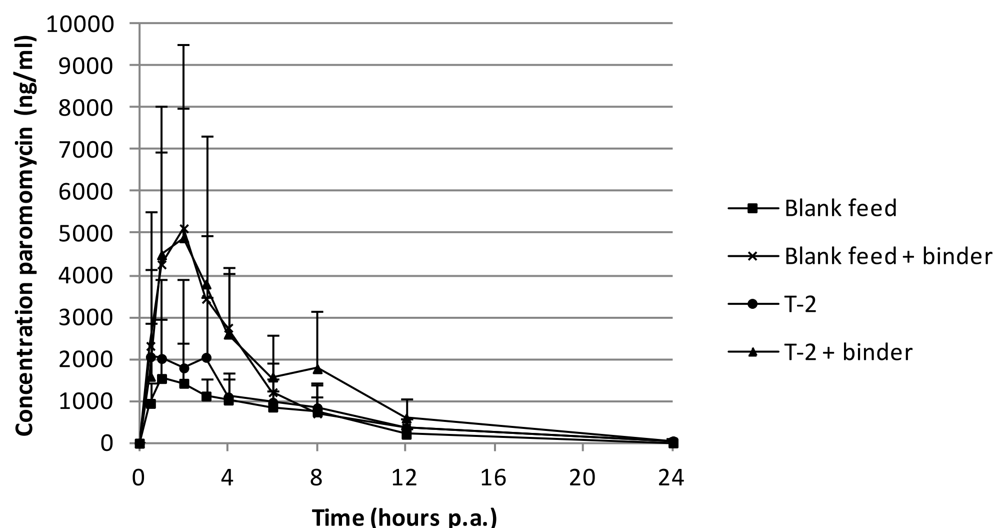

Figure 2. Mean plasma concentrations (+SD) in pigs after single peroral administration of paromomycin at 100 mg/kg body weight ($n = 6$). Prior to the bolus, pigs received during 7 days, blank feed (control group), blank feed supplemented with mycotoxin binder, feed contaminated with 100 µg T-2 toxin/kg feed and feed contaminated with 100 µg T-2 toxin/kg feed supplemented with mycotoxin binder, respectively.

Although a trend for higher plasma concentrations after intake of binder supplemented feed was seen, there were no significant differences in plasma concentrations or $AUC_{0–24h}$ of paromomycin on the basis of treatment.

Experiment 2

Intake of DON Contaminated Feed Supplemented with Mycotoxin Binder Results in Higher Plasma Concentrations of Doxycycline

The plasma concentration-time curve for doxycycline after p.o. administration is presented in Figure 3 for the different groups.

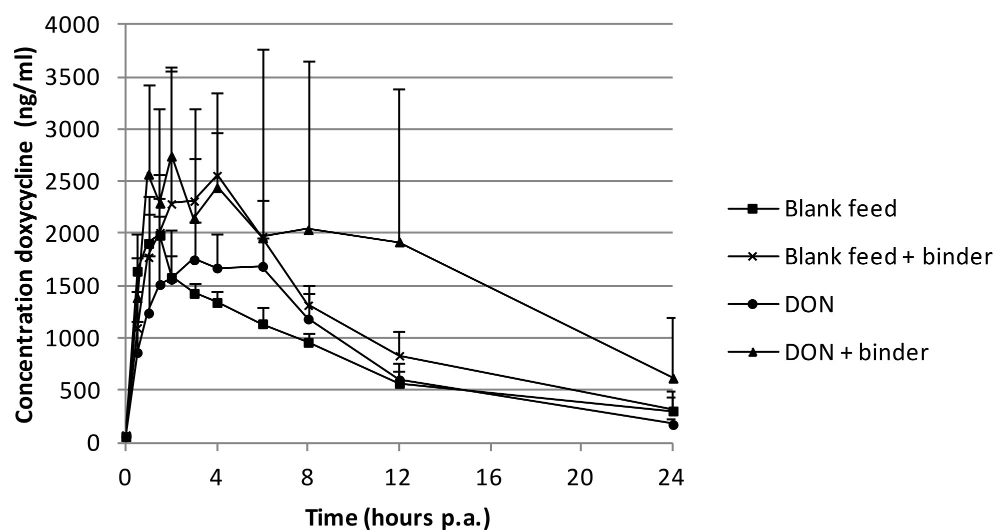

Figure 3. Mean plasma (+SD) concentrations in pigs after single peroral administration of doxycycline at 10 mg/kg body weight (n = 6). Prior to the bolus, pigs received during 13 days, blank feed (control group), blank feed supplemented with mycotoxin binder, feed contaminated with 1 mg DON/kg feed and feed contaminated with 1 mg DON/kg feed supplemented with mycotoxin binder, respectively.

The plasma concentration of doxycycline was significantly (P = 0.045) higher in the group which received DON contaminated feed supplemented with the mycotoxin binder, but not to the other experimental groups.

Mean area under the plasma concentration-time curve ($AUC_{0–24h}$) is summarized in Table 2.

Table 2. Mean (±SD) area under the plasma concentration-time curve ($AUC_{0–24h}$) for doxycycline in the different groups. Prior to the bolus, pigs received during 13 days, blank feed (control group), feed contaminated with 1 mg DON/kg feed, feed contaminated with 1 mg DON/kg feed supplemented with mycotoxin binder and blank feed supplemented with mycotoxin binder, respectively. Superscript (*) refers to a significant difference compared to the control group ($P < 0.05$).

Group	$AUC_{0–24h}$ (ng/ml*h)
Blank feed (control)	19 011 (± 2 805)
Blank feed + binder	26 783 (± 4 752)
1 mg/kg DON	20 107 (± 3 304)
1 mg/kg DON + binder	40 029 (± 22 775) *

No Statistical Different Plasma Concentrations of Paromomycin after Intake of DON Contaminated Feed

Plasma concentrations of paromomycin after intake of DON contaminated feed were not significantly different compared to the control group (Figure 4).

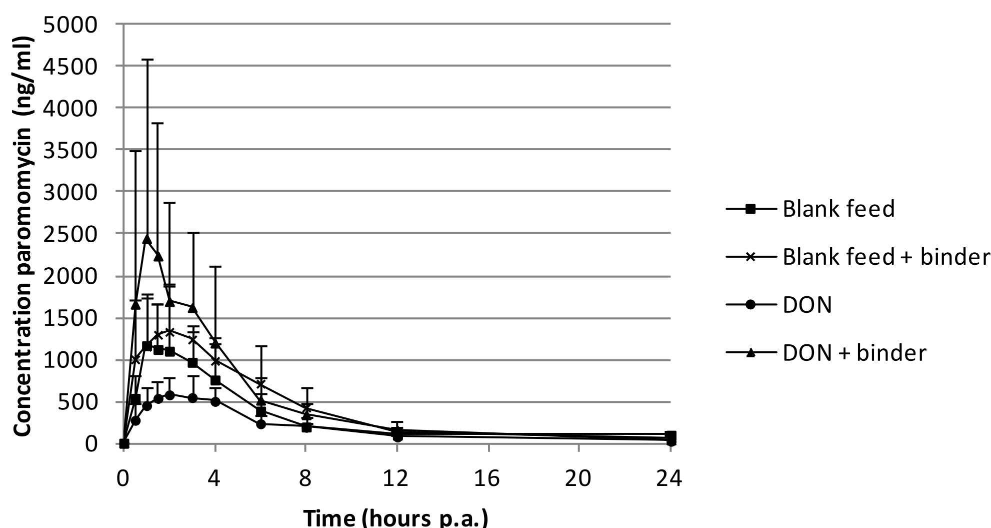

Figure 4. Mean (+SD) plasma concentrations in pigs after single peroral administration of paromomycin at 100 mg/kg body weight (n = 6). Prior to the bolus, pigs received during 13 days, blank feed (control group), blank feed supplemented with mycotoxin binder, feed contaminated with 1 mg DON/kg feed and feed contaminated with 1 mg DON/kg feed supplemented with mycotoxin binder, respectively.

Discussion

The results obtained indicate that there is a need for extensive research into the field of safety testing of mycotoxin detoxifying agents. Indeed, on request of the European Commission, the Panel on Additives and Products or Substances used in Animal Feed derived a proposal for the modification of Annex III of Commission Regulation (EC) No 429/2008 (EC, 2008). For the authorization of additives belonging to the functional group of substances for reduction of the contamination of feed by mycotoxins, one of the parameters that needs to be taken into account is the presence and characterization of possible interactions of the binder with, among others, veterinary medicinal products.

In the present study, the effect of mycotoxins and a commonly used mycotoxin binder on the absorption of two frequently used antibiotics in pigs was investigated *in vivo*. The study was conducted in pigs because, among farm animals, pigs react most sensitively to exposure to trichothecene mycotoxins (Eriksen et al., 2004). The antibiotics tested were doxycycline and paromomycin. These veterinary drugs are often used in pigs in 'mass' medication and are frequently administered via the oral route, *i.e.*, mixed in the feed or drinking water. The mycotoxins used were the trichothecenes DON and T-2. T-2 toxin is the most potent and toxic trichothecene for which there are no recommended maximum levels in animal feed (EC, 2006). The contamination level used in the experiment was based on Monbaliu *et al.* (2010) who analyzed 82 feed samples from different European countries for the presence of mycotoxins.

As T-2 was found at a concentration ranging from 10-122 µg/kg, it was decided to produce feed contaminated with 100 µg/kg. DON on the other hand is one of the most frequent contaminants of maize and small grain cereals with a recommended maximum concentration level of 900 µg/kg of DON in pig feed (EC, 2006). It was decided to respect this limit and

work with a concentration of approximately 1000 µg/kg. The mycotoxin adsorbing agent used was an esterified yeast cell wall that claims to bind DON and T-2.

Regarding the plasma concentration of doxycycline, the concentration-time curve of the control group shows a profile as described previously (Baert et al., 2000). At 6 h post administration two out of six pigs showed an extra peak in the plasma concentration-time profile. This may be due to the enterohepatic recirculation of doxycycline (Riond et al., 1990). Compared to this control group the plasma concentrations were significantly higher in the group which received feed contaminated with 100 µg T-2/kg supplemented with the mycotoxin binder. Remarkably, the AUC was almost double compared to the AUC of the control group. Significant higher plasma concentrations of doxycycline were also found when the mycotoxin DON was added together with the mycotoxin binder in the feed.

In an attempt to clarify the mechanism behind the increased plasma concentrations, a possible interaction of the binder with divalent ions such as Ca^{2+} and Mg^{2+}, which normally partially bind doxycycline (Berthon et al., 1983), was presented as potential explanation. With less bound doxycycline present in the intestinal lumen, more doxycycline could then enter the systemic circulation. To check this assumption an extra experiment was performed with oxytetracycline as antibiotic. Oxytetracycline also belongs to the class of tetracyclines like doxycycline and binds even stronger to divalent ions (Arias et al., 2007). If our assumption was correct, the effects seen with doxycycline should be even more pronounced with oxytetracycline. However, no statistical difference in plasma oxytetracycline concentration could be demonstrated between the groups receiving either blank feed or blank feed supplemented with binder. In the oxytetracycline experiment however, no T-2 toxin was added to the feed. Therefore, the increased plasma concentrations seen for doxycycline are probably the result of a more complex interaction between mycotoxin, binder and drug.

For paromomycin, there was no statistical difference in plasma concentrations between the groups. This is possibly due to the great variation in plasma concentration between the individual pigs. Although not significant, there is a tendency that the intake of binder supplemented feed also leads to an increased plasma concentration.

Recent *in vivo* experiments at our department with broiler chickens also confirm the possible role of binders in the absorption of antibiotics. In chickens, increased plasma concentrations of oxytetracycline were seen after intake of feed supplemented with a mycotoxin detoxifying agent (Osselaere et al., 2012). We also showed that, in pigs, administration of a single bolus of DON combined with a glucomannan binder leads to increased plasma concentrations of DON compared to the control group which received a bolus with only DON (Devreese et al., 2012).

The mechanisms for these phenomena still remain unknown but it demonstrates that effects can be binder, species and drug specific. Another possible explanation that we put forward is that the mycotoxin binder affects the intestinal epithelium in which the duration of exposure can play a role, for example by loosening of the tight junctions, stimulating the production of cytokines, influencing the length of villi and depth of crypts, influencing the production of mucus *etc*., which could result in increased passage of antibiotics across the intestinal wall.

In conclusion, our results demonstrate that the studied mycotoxin binder may significantly affect the oral bioavailability of doxycycline. This may be of importance for the animal, the withdrawal time of veterinary drug formulations containing doxycycline, and consequently for public health with respect to tissue residues of the antibiotic. On the other hand, if the mycotoxin binder leads to increased plasma concentrations, this can be compensated by reducing the dosage of the antibiotic. However, since the effects depend on the type of detoxifying agent, type of drug and the animal species, further research is needed to elucidate possible interaction mechanisms.

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Chapter 3

Chronic exposure to the mycotoxin T-2 promotes oral absorption of chlortetracycline in pigs

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Abstract

The aim of the present study was to investigate whether T-2 toxin, a potent *Fusarium* mycotoxin, affects the oral absorption of the antibiotic chlortetracycline in pigs. Animals were allocated to blank feed without T-2 toxin (controls), feed containing 111 µg T-2/kg feed, T-2 contaminated feed supplemented with a yeast-derived feed additive, or blank feed supplemented solely with the feed additive, respectively. After 21 days, an intragastric bolus of chlortetracycline was given to assess potential alterations of the pharmacokinetics of this commonly used antibiotic. A significantly higher area under the plasma concentration-time curve and maximal plasma concentration of chlortetracycline were observed after intake of T-2 contaminated feed compared to control. Thus exposure to T-2 contaminated feed can influence the oral bioavailability of chlortetracycline. This effect could have consequences for the withdrawal time of the drug and the occurrence of undesirable residues in edible tissues.

Introduction

T-2 toxin (T-2) is a potent mycotoxin in Europe, contaminating food and feed and consequently causing adverse health effects to humans and animals. Other mycotoxins such as deoxynivalenol (DON), ochratoxin A and patulin were shown to compromise the intestinal barrier function in human epithelial cell lines by altering the tight junction complex (Maresca et al., 2001, 2002; Mahfoud et al., 2002; McLaughlin et al., 2004; Lambert et al., 2007). The reduced expression of tight junction proteins leads to an increased passage of tracers such as fluorescein isothiocyanate (FITC)-dextran and bacteria such as *Escherichia coli* (Pinton et al., 2009). An increased absorption of antibiotics was also seen *in vitro* after exposure of intestinal porcine epithelial cells (IPEC-J2 cells) to DON or T-2 (Goossens et al., 2012a). To minimize the negative effects of mycotoxins, cell wall components derived from the *Saccharomyces cerevisiae* yeast, often claiming to bind mycotoxins, are frequently added to contaminated diets (EFSA, 2009). Recently, it has been demonstrated that feeding T-2 at 100 µg/kg feed for 7 days, in combination with such a feed additive, leads to an increased passage and enhanced oral absorption of the antibiotic doxycycline in pigs (Goossens et al., 2012b). Doxycycline is a second generation tetracycline derivative with a mean oral bioavailability of approximately 20% in pigs (Baert et al., 2000). It could be interesting to investigate the effect with a first generation tetracycline which has a much lower oral bioavailability than doxycycline. Chlortetracycline (CTC), for example, is a first generation tetracycline with a mean oral bioavailability of only 5% in pigs (Nielsen and Gyrd-Hansen, 1996). If the same effect is seen with CTC, i.e. increased oral bioavailability of CTC after intake of T-2 contaminated feed, this would have important implications for the treatment schedule. Furthermore, increased plasma concentrations of chlortetracycline could result in increased tissue residues, and consequently lead to an adjustment of the withdrawal time of the antibiotic. Next, it was decided to feed the animals for 21 days instead of 7 days, as was the

case in the T-2 experiment described in Goossens et al. (2012b). During the latter *in vivo* experiment, significant increased plasma concentrations of doxycycline were only seen after intake of T-2 toxin contaminated feed supplemented with the mycotoxin binder, and not with T-2 solely. We demonstrated, however, that *in vitro* exposure of IPEC-J2 cells to T-2 results in a time-dependent effect of T-2 on the passage of doxycycline across the IPEC-J2 monolayer (Goossens et al., 2012a).

It was therefore the aim of the present study to investigate if a prolonged dietary exposure to T-2, whether or not combined with a yeast-derived feed additive, influences the oral absorption of CTC in pigs. Oral bioavailability was studied using selected pharmacokinetic variables, i.e. area under the plasma concentration-time curve from time 0 to 24 p.a. (AUC_{0-24h}) and maximum plasma concentration (C_{max}); and the pharmacokinetic parameter time to reach C_{max} (t_{max}).

Materials and Methods

Twenty-four male 9-week-old pigs (Landrace) with a mean (± SD) body weight (b.w.) of 20.1 ± 2.1 kg were randomized into a control group (n = 6) and three experimental groups (each n = 6). After an acclimatization period of one week, the groups were given blank feed (control group), blank feed supplemented with a yeast-derived feed additive at a concentration of 0.5 g/kg (Alphamune®, Alpharma, Belgium), feed experimentally contaminated with 111 µg T-2/kg feed and T-2 contaminated feed (111 µg T-2/kg feed) supplemented with the same additive (0.5 g/kg), respectively. The contamination level used in the experiment was based on the results obtained by Monbaliu et al. (2010) who analyzed 82 feed samples from different European countries for the presence of mycotoxins. As T-2 was found at a concentration ranging from 10-122 µg/kg it was decided to produce feed experimentally contaminated with approximately 100 µg T-2/kg feed.

Twenty-one days after the start of the feeding trial, a CTC feed-grade premix was administered by gavage using an intragastric tube at a concentration of 20 mg CTC/kg b.w. (Aurofac®, Alpharma). CTC feed-grade premix was chosen as it is the only CTC-containing registered veterinary medicinal product available on the Belgian market. The dose of 20 mg/kg b.w. was chosen as this is the recommended daily dose according to the leaflet of the product. The premix was mixed with a small amount of feed and the tube was rinsed sufficiently with water to assure complete delivery of CTC in the stomach. The CTC feed-grade premix was administered by gavage to assure that all animals received the same dose of the antibiotic, based on their body weight. Blood samples were taken by puncturing the external jugular vein with a 20 G needle (20 G, 0.9 × 38 mm, Terumo Corp., Tokyo, Japan) before and at 0.5, 1, 1.30, 2, 3, 4, 6, 8, 12 and 24 h post administration (p.a.) of CTC. Thereafter, the animals were sedated with a combination of xylazine (XylM® 2%, VMD, Arendonk, Belgium), zolazepam and tiletamine (Zoletil 100®, Virbac, Wavre, Belgium), followed by killing by exsanguination. Next, intestinal sections of duodenum, jejunum and ileum for light-microscopic evaluation of villus height and crypt depth were collected, fixed for 24 h in formalin, embedded in paraffin wax, sectioned (5 µm) and stained with haematoxylin and eosin. Villus height and crypt depth were measured using MOTIC Images Advanced 3.2 digital microscopy software. Villus height was measured from the top of the villus to the villus-crypt junction. The total mucosal thickness was measured from the top of the villus to the border over the *muscularis mucosae*. Crypth depths were determined by calculating the difference between the total mucosal thickness and the villus height. The animal experiment has been approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2011/186).

Plasma concentrations of CTC were determined using an in-house validated HPLC-UV method (high-performance liquid chromatography-ultraviolet detection) with a limit of

quantification of 0.2 µg/ml (Baert et al., 2000). The main pharmacokinetic variables $AUC_{0\text{-}24h}$ and C_{max}; and the main pharmacokinetic parameters t_{max}, terminal half-life ($t_{1/2}\lambda_z$) and terminal decay slope (λ_z) were determined by means of the WinNonlin® software program using non-compartmental analysis, version 6.2.0 (Pharsight Corporation, Mountain View, CA, USA). Data were statistically analyzed using SPSS 19.0 software for Windows (SPSS Inc., Chicago, IL, USA). Normally distributed data were analyzed using two-way analysis of variance (ANOVA) to address the significance of difference between mean values with significance set at $P \leq 0.05$. Not normally distributed data were analyzed using the non-parametric Kruskal-Wallis analysis, followed by a Dunn's Multiple Comparison test.

Results

Significantly higher plasma concentrations of CTC, as reflected by the $AUC_{0\text{-}24h}$ and C_{max}, compared to the control group were seen after intake of T-2 contaminated feed (Figure 1, Table 1).

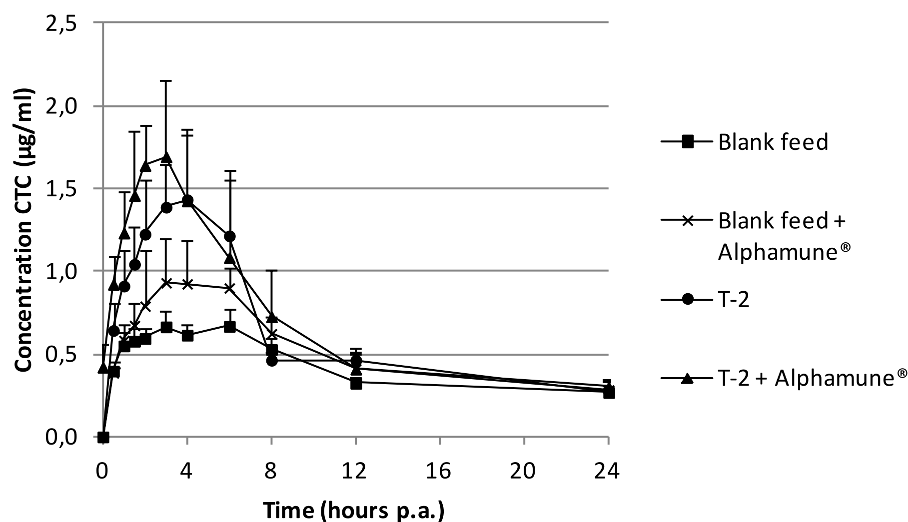

Figure 1. Plasma concentration-time curves for intragastric administered chlortetracycline (dose: 20 mg/kg b.w.) in pigs, determined 21 days after the start of the feeding trial during which the animals received blank feed (control group), blank feed supplemented with a yeast-derived feed additive (Alphamune®, 0.5 g/kg feed), feed experimentally contaminated with 111 µg T-2 toxin/kg feed or feed contaminated with 111 µg T-2 toxin/kg feed supplemented with the yeast-derived additive (0.5 g/kg feed). Results are given as mean + SD (*n* = 6 pigs).

No significant differences could be demonstrated for the other pharmacokinetic parameters t_{max}, $t_{1/2}\lambda_z$ and λ_z. (Table 1).

Table 1. Selected pharmacokinetic variables and parameters of intragastric administered chlortetracycline in pigs, determined 21 days after the start of the feeding trial during which the animals received blank feed (control group), blank feed supplemented with a yeast-derived additive (Alphamune®, 0.5 g/kg feed), feed experimentally contaminated with 111 µg T-2 toxin per kg feed or feed contaminated with 111 µg T-2 toxin per kg feed supplemented with the yeast-derived additive, respectively. Results are given as mean ± SD (*n* = 6 pigs). Numbers marked with different letters indicate a significant difference ($P < 0.05$).

	Blank feed	**Blank feed + Alphamune®**	**T-2**	**T-2 + Alphamune®**
AUC_{0-24h} (µg h/ml)	6.04 ± 0.71[a]	8.23 ± 1.66[a,b]	11.18 ± 1.93[b,c]	13.34 ± 2.66[c]
C_{max} (µg/ml)	0.74 ± 0.06[a]	1.00 ± 0.24[a,b]	1.52 ± 0.39[b,c]	1.80 ± 0.42[c]
t_{max} (h)	4.17 ± 1.47	4.17 ± 1.60	3.50 ± 0.55	2.83 ± 0.75
$t_{1/2}\lambda_z$ (h)	6.76 ± 1.68	5.55 ± 1.40	5.53 ± 2.24	5.44 ± 2.09
λ_z (1/h)	0.11 ± 0.03	0.13 ± 0.03	0.14 ± 0.06	0.14 ± 0.05

Note: AUC_{0-24h} = area under the plasma concentration-time curve from time 0 to 24 h; C_{max} = maximal plasma concentration; t_{max} = time to maximal plasma concentration; terminal half-life ($t_{1/2}\lambda_z$); terminal decay slope (λ_z)

Addition of a yeast-derived feed additive to T-2 contaminated feed did not change these observations. No significant differences could be demonstrated for the other pharmacokinetic parameters t_{max}, $t_{1/2}\lambda_z$ and λ_z.

Discussion

In vitro experiments already described that DON is able to decrease the expression of tight junctions in IPEC-1, IPEC-J2 and Caco-2 cell lines (Pinton et al., 2009; Diesing et al., 2011). Since T-2 is structurally related to DON, it is likely that T-2 also results in a decreased expression of the tight junctions making it partly responsible for the increased paracellular

passage of CTC and the increase of the bioavailability after intake of T-2 contaminated feed administered for 21 days. Although not significant compared to the control group, there is a tendency to higher plasma concentrations of CTC in the group which received blank feed supplemented solely with Alphamune®. The effect of the toxin itself combined with the effect of Alphamune® on the plasma concentrations of CTC could explain that the highest plasma concentrations are seen in the group which received both T-2 and Alphamune® into the feed simultaneously. Therefore, it was investigated whether the toxin and/or the feed additive affect the intestinal absorption surface area. It is indeed described that a yeast-derived feed additive may enhance villus height, surface area, lamina propria thickness, crypt depth and goblet cell density in the ileum, and to some degree in the jejunum and duodenum of turkey poults after 21 days of intake (Solis de los Santos et al., 2007). A changed absorption area could result in enhanced absorption of the antibiotic. However, in our study, no significant differences in villus length or crypth depth could be demonstrated between the different groups after 21 days of feeding (Table 2).

Table 2. Villus length and crypth depth of duodenum, jejunum and ileum fragments of pigs, determined 21 days after the start of the feeding trial during which the animals received blank feed (control group), blank feed supplemented with a yeast-derived additive (Alphamune®, 0.5 g/kg feed), feed experimentally contaminated with 111 µg T-2 toxin per kg feed or feed contaminated with 111 µg T-2 toxin per kg feed supplemented with the yeast-derived additive (0.5 g/kg feed), respectively. Results are given as mean ± SD (*n* = 6 pigs).

	Duodenum (µm)		**Jejunum (µm)**		**Ileum (µm)**	
	Villus	Crypth	Villus	Crypth	Villus	Crypth
Blank feed	490.6 ± 70.0	440.0 ± 68.9	502.5 ± 131.9	250.4 ± 60.9	357.72 ± 79.6	251.51 ± 64.1
Blank feed + Alphamune®	531.9 ± 128.3	461.2 ± 89.0	462.7 ± 100.6	247.1 ± 75.9	376.74 ± 68.9	277.66 ± 76.8
T-2	450.2 ± 114.2	489.7 ± 122.5	561.0 ± 72.0	235.6 ± 41.5	392.7 ± 68.3	282.2 ± 90.1
T-2 + Alphamune®	522.8 ± 123.5	486.3 ± 90.9	494.1 ± 94.6	256.9 ± 65.2	394.0 ± 60.6	267.6 ± 52.0

Our findings are important to the field since an enhanced oral absorption of CTC could have consequences for the withdrawal time of the antimicrobial and the occurrence of undesirable residues in edible tissues. On the other hand, given the low bioavailability of CTC, an

increased intestinal absorption of CTC in combination with T-2 could also be beneficial from a theoretical point of view. However, we do not recommend to feed the animals T-2 contaminated feed in order to achieve higher plasma concentrations of CTC, as T-2 itself may be detrimental for animal health.

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Chapter 4

The mycotoxin T-2 inhibits hepatic cytochrome P4503A activity in pigs

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Abstract

Mycotoxins are toxic metabolites produced by fungi that readily colonize crops. After ingestion, these mycotoxins can compromise intestinal health, and once entering the blood stream, even affect the liver and its metabolizing enzymes. It was therefore the aim of the present study to investigate the effect of T-2 toxin, an emerging and potent *Fusarium* mycotoxin, on the enzymatic activity of cytochrome P4503A (CYP3A) metabolizing enzymes in the liver of pigs. In addition, a yeast-derived feed additive that claims to bind T-2 toxin was included in the study to evaluate its efficacy. Our results demonstrated that a 14-days intake of T-2 toxin contaminated feed at a dose of 903 µg/kg feed, whether or not combined with the mycotoxin binder, results in a substantial inhibition of the CYP3A activity in the liver of pigs. This result may be of importance for animal health, the pharmacokinetics and the withdrawal time of drugs that are substrate of CYP3A enzymes, and consequently can be a threat for public health with respect to tissue residues of these drugs.

Introduction

Mycotoxins are secondary metabolites produced by fungi that can cause several adverse health effects in humans and animals. The mycotoxins of most significance, from a health and agronomic perspective, are those produced by molds present on food and feed. One of the important mycotoxins in temperate regions is T-2 toxin (T-2), an acute toxic trichothecene produced by *Fusarium* species present on oats, wheat and barley. To minimize the negative effects of mycotoxins biological detoxification, which comprises binding of mycotoxins by adsorptive feed additives in the gastro-intestinal tract, is the most prominent approach to reduce the risk for mycotoxicoses in farm animals (Döll and Dänicke, 2004). As pigs are the most sensitive species to several *Fusarium* mycotoxins, including T-2, several different types of such feed additives are frequently added to pig feed in order to restrict the negative impact of mycotoxins.

After ingestion, the gastro-intestinal tract is the first barrier mycotoxins come into contact with. Consequently, this intestinal barrier can be compromised by mycotoxins resulting in an altered passage of xenobiotics, mycotoxins and pathogens. Moreover, when entering the blood stream after oral absorption, mycotoxins firstly reach the liver, and can interfere with the expression and activity of drug metabolizing enzymes belonging to the cytochrome P450 superfamily (CYP). A reduced expression of CYP1A proteins after intake of T-2 contaminated feed has already been described in pigs, rats and rabbits by Meissonnier et al. (2008), Galtier et al. (1989) and Guerre et al. (2000), respectively. In these studies, mainly the CYP protein expression was evaluated, and few further post-translational enzymatic activities were determined. Osselaere et al. (2013a) demonstrated a significant down-regulation in mRNA expression of CYP1A4, CYP1A5 and CYP3A37 when broilers were fed a diet contaminated with T-2. In humans (Hardman, 2001) and in pigs (Thorn et al., 2011), most drugs undergo deactivation by the CYP3A subfamily. It was therefore the aim of the present

study to evaluate whether T-2 toxin affects CYP3A enzymatic activity in the liver of pigs. In addition, a yeast-derived feed additive claiming to bind T-2 was included in this study to evaluate its binding efficacy.

Materials and Methods

Experimental diets

Conventional pig feed consisting of 30% wheat, 26% barley, 4% wheat gluten, 11% soybean, 5% danex, 6% corn and 3% melasse was purchased from DANIS nv (Ardooie, Belgium) and analysed for the presence of mycotoxins. Danex is a soy product obtained through a series of hydro-thermo-mechanical treatments. The soy beans are put through this process to obtain a product to give maximum digestibility of proteins and fats. The 'toasting' process gives a double guarantee, namely a maximum PDI (Protein Dispersibility Index) and minimum urease activity. Following vitamins and trace minerals were also included: vitamin A: 12000 IE/kg, vitamin D3: 1800 IE/kg, vitamin E: 50 mg/kg, manganese: 45 mg/kg, copper: 160 mg/kg, sodium selenite: 0.40 mg/kg, iron(II)sulphate monohydrate (E1) – iron: 100 mg/kg, potassium iodide – iodine (E2): 1 mg/kg, zinc oxide – zinc (E6): 88 mg/kg.

Analysis with liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Monbaliu et al., 2010) revealed that the feed contained 69 ± 20 µg/kg deoxynivalenol and 5.8 ± 2.2 µg/kg 3-acetyldeoxynivalenol. All other mycotoxins (n = 22) tested were below the limit of detection. Since the feed did not contain T-2, it was accepted for use in the experiment. It was used on the one hand, as blank feed and, on the other hand, to contaminate and supplement it with T-2 and/or the feed additive, respectively. To produce feed contaminated with 1 mg/kg T-2, a stock solution of 5 mg/ml T-2 toxin was prepared by dissolving 500 mg T-2 (Sigma-Aldrich, Bornem, Belgium) in 100.0 ml ethanol (Merck, Darmstadt, Germany). The contaminated feed was produced by adding 80 ml of the stock solution to 500 g of blank feed.

This premix was then mixed with 5 kg of blank feed to assure a homogeneous distribution of the toxin. The final premix was then mixed for 20 min in the total amount of feed (400 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 903 ± 85 µg/kg T-2 was found in this T-2 contaminated feed.

The binder used in the experiment was a commercially available glucomannan mycotoxin binder which claimed to bind T-2, added at a concentration of 2 g/kg. To produce the binder supplemented feed (200 kg), binder was added to both blank feed and feed contaminated with 1 mg T-2/kg feed and mixed for 20 min.

Animal experiment

In our animal experiment, twenty-four male 9-week-old pigs (Landrace) with a mean (± SD) body weight of 22.1 (± 1.8) kg were randomized into four dietary treatment groups (each n = 6). During fourteen days one group was fed a diet not contaminated with T-2, i.e. the control group. A second group received feed artificially contaminated with 903 ± 85 µg/kg T-2. The third group received blank feed supplemented with a yeast-derived mycotoxin binder at a concentration of 2 g/kg, and the fourth group received feed artificially contaminated with 903 ± 85 µg/kg T-2 and supplemented with the binder (2 g/kg). At the end of the feeding trial, the animals were sedated with a combination of xylazine (XylM® 2%, VMD, Arendonk, Belgium), zolazepam and tiletamine (Zoletil 100®, Virbac, Wavre, Belgium), followed by killing by exsanguination. Liver fragments of 1 cm^3 were collected from each animal, rinsed in phosphate buffered saline (PBS, Gibco, Life Technologies, Paisley, Scotland), immediately frozen in liquid nitrogen and preserved at -80 °C till further analysis. The animal experiment was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC2012/046).

Determination of CYP3A activity

The method to evaluate CYP3A activity is already described by Osselaere et al. (2013b). Only minor changes have been applied to the existing protocol. In short, CYP3A enzymatic activity was determined in hepatic microsomes, prepared using a process of differential ultracentrifugation (Wilson et al., 2003). All processing of tissue was performed on a bed of ice. After thawing on ice, liver tissue was minced and homogenized in 16 ml of homogenization buffer (pH 7.25, 0.25 M phosphate buffer, 1.15% KCl). After centrifugation at 10 000 x *g* for 25 min at 4 °C, the resulting supernatant was centrifuged at 100 000 x *g* for 80 min (4 °C). The resulting microsomal pellet was washed by resuspending it in homogenization buffer (16 ml) and centrifuging again following the latter conditions. The final microsomal pellet was resuspended in resuspension buffer (1.5 ml/gram tissue, pH 7.25, 0.25 M phosphate buffer, 1.15% KCl, 30% glycerol). The obtained microsomes were quickly frozen in liquid nitrogen and stored at -80 °C until activity measurements. The total protein concentration in all microsomes was measured using the Bradford assay (Bradford, 1976). CYP3A activity was determined by monitoring the formation rate of 1-hydroxy-midazolam, the major metabolite of midazolam which is a substrate for CYP3A enzymes (Hosagrahara et al., 1999). First, a mixture of 50 µl of 1.15% KCl in water, 50 µl of 0.05 M aqueous phosphate buffer (pH 7.4) and 50 µl of midazolam in water (25 µM) was pre-heated at 37 °C, mixed with 50 µl NADPH (final concentration in incubation medium of 1 mM) and pre-incubated for 3 minutes. Next, the enzymatic reaction was initiated by the addition of 50 µl of the hepatic microsomal dilution. Each microsomal sample was diluted in a 1.15% KCl solution in such a way that a final concentration of 0.275 mg total protein/ml incubation mixture was obtained. After exactly 20 minutes incubation at 37°C in a shaking heating block, the enzymatic reaction was terminated by adding 25 µl of a water/acetonitrile/formic acid mixture (42/55/3, (v/v/v)), supplemented with the analytical internal standard chlorpropamide

at a final concentration in the incubation mixture of 0.072 µM. Finally, the samples were vortexed and cooled on ice, after which they were centrifuged at 20 000 x *g* for 10 min (4 °C). The supernatants were collected and frozen at -20 °C until analysis. All incubations of the microsomes were performed in triplicate for each pig. The amounts of 1-hydroxy-midazolam that were formed during the incubations were quantified using a validated UPLC-MS/MS method (De Bock et al., 2012). Each sample was measured in duplicate. Results of the enzymatic activities, expressed as pmol 1-hydroxy-midazolam/mg protein/min, were analyzed using two-way ANOVA to address the significance of difference between mean values, with significance set at $P \leq 0.05$.

Results

A statistically significant inhibition in hepatic CYP3A activity was seen in the groups which received T-2 contaminated feed, whether or not combined with the feed additive (Figure 1).

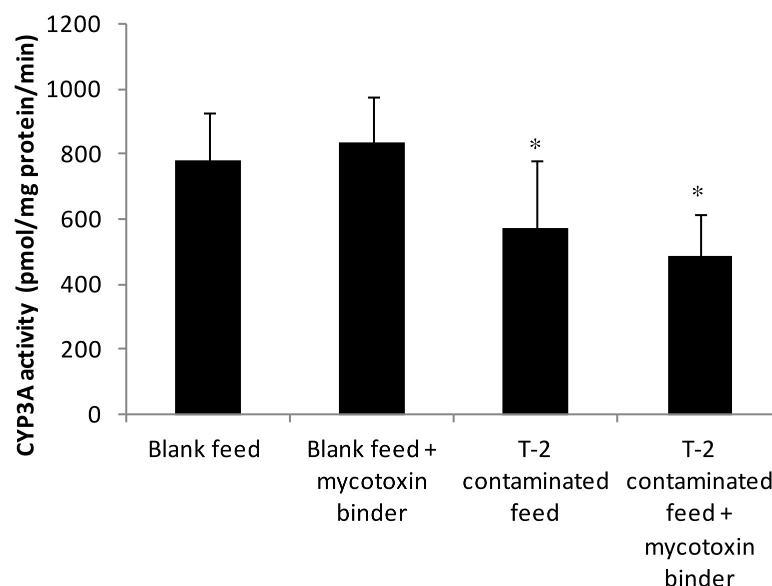

Figure 1. CYP3A enzymatic activity in the liver of pigs which received during 14 days blank feed, blank feed supplemented with a yeast-derived feed additive (mycotoxin binder), T-2 toxin contaminated feed containing 903 ± 85 µg/kg T-2 and T-2 toxin contaminated feed supplemented with the mycotoxin binder, respectively. Results are expressed as means (+ SD) of 3 replicate incubations per pig and six pigs per group. Superscript (*) refers to a significant difference compared to non-contaminated feed ($P < 0.05$).

The results demonstrate that intake of feed contaminated with 903 ± 85 µg/kg T-2 during 14 days leads to an inhibition in hepatic CYP3A enzymatic activity in pigs. Moreover, addition of a yeast-derived mycotoxin binder did not prevent this inhibition in enzymatic activity. Intake of solely binder supplemented feed did not exert an effect. Results of the two-way ANOVA revealed a significant P-value for the factor “toxin”. The interaction term and the factor “additive” were not found to be statistically significant.

Discussion

In this study, results of the CYP3A enzymatic activity were expressed as pmol of 1-hydroxy midazolam formation per mg protein/min, and not as pmol/nmol P450/min since it has not been demonstrated that T-2 has no effect on CYP450 protein content at the dose and duration of T-2 exposure tested in pigs of 9-week-old. If a normalization and correction for the global P450 content would be done, it could be that the possible effect on CYP3A we want to study is masked. Moreover, Meissonnier et al. (2008) found there was no observable variation in the total protein content of microsomal or cytosolic fractions extracted from liver tissue in piglets receiving different doses of T-2 toxin, justifying our approach.

Reduced expression of hepatic CYP1A proteins was already reported after intake of 2102 µg T-2/kg feed in pigs for 28 days (Meissonnier et al., 2008), after intragastric administration of 0.25 mg/kg body weight (b.w.) T-2 in rabbits for 5 days (Guerre et al., 2000) and after intragastric administration of 1 mg/kg b.w. T-2 in rats for 8 days (Galtier et al., 1989). Next, Osselaere et al. (2013a) demonstrated a significant down-regulation in mRNA expression of CYP1A4, CYP1A5 and CYP3A37 when broilers were fed T-2 contaminated feed at a concentration of 752 µg T-2/kg feed. The same investigators showed that even a

contamination level of 68 µg/kg T-2 caused a significant decrease in expression at mRNA level for CYP1A4.

Cytochrome 3A enzymes are known to play an important role in the metabolism of several therapeutic agents. A decreased expression and/or activity of CYP3A enzymes could result in increased plasma concentrations of drugs that are metabolized by these enzymes. Furthermore, the impaired gastro-intestinal barrier function caused by trichothecene mycotoxins such as T-2 and deoxynivalenol can also result in an increased intestinal passage of drugs (Goossens et al., 2012).

Meissonnier et al. (2008) reported no effect of T-2 at concentrations up to 2102 µg/kg feed on the expression of CYP3A in pigs. Important to remark, however, is that it is demonstrated that CYP expression and CYP activity are not always correlated. In other words, a normal expression level of CYP enzymes does not necessarily corresponds with a normal CYP activity. This was also reported by Meissonnier et al. (2008) who demonstrated a reduced activity of CYP1A enzymes in pigs after intake of 540, 1324, 2102 µg T-2/kg feed, while the expression level of CYP1A was only decreased in pigs fed 2102 µg T-2/kg feed. Also Osselaere et al. (2013a) indicated that CYP activity and CYP expression level after intake of T-2 contaminated feed in broiler chickens are not correlated with each other. Our study is the first to describe a significant effect of T-2 on CYP3A activity in pigs using midazolam as substrate drug.

Due to the design of the pig's facility, i.e. housing in group on a grid floor and using one feed trough, it was not possible to determine individual daily feed intake. However, during the 14-day experimental period, each group of 6 pigs was offered every day about 9 kg of feed, and it was recorded that the next day, the pig trough was completely empty for each group. Based on the initial body weight of the pigs (i.e. mean b.w. of 22.1 kg at the start of the experiment),

and based on the daily intake of about 9 kg of contaminated feed at 903 µg T-2/kg feed for the group of 6 pigs, we can roughly estimate that the mean daily toxin exposure was 61 µg T-2/kg b.w.

In this study, daily weight gain was not determined since Meissonnier et al. (2008) examined three T-2 toxin doses that were fed for 28 days (540, 1324 or 2102 µg T-2/kg feed) and found that only a concentration of 2102 µg T-2/kg feed affected the body weight gain of pigs, when compared to control pigs. Beside weight gain, Meissonnier et al. (2008) also evaluated the effect of T-2 toxin on liver weight. Following 28 days exposure to T-2 toxin (540, 1324 or 2102 µg T-2/kg feed) no effect on liver weight (expressed as % b.w.) could be demonstrated. As a consequence, we did not expect our feed artificially contaminated with 903 ± 85 µg/kg T-2 to affect liver weight or total body weight gain of the animals.

In conclusion, a reduced hepatic CYP3A enzymatic activity could result in substantially increased plasma concentrations of drugs that are a substrate of CYP3A enzymes.

This may be of importance for the animal, the pharmacokinetics and withdrawal time of drugs that are substrate of CYP3A, and consequently for public health with respect to tissue residues of these drugs.

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Chapter 5

Reduced expression of intestinal P-glycoprotein following ingestion of deoxynivalenol (DON) contaminated feed in pigs

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Abstract

Mycotoxins, which are secondary metabolites produced by fungi, are known to have effects at the level of the intestine. They can influence intestinal mucus production, compromise the intestinal barrier function and affect epithelial transport systems. Permeability-glycoprotein (P-gp) is a 170-180 kDa membrane glycoprotein which mediates the active transmembrane transport of a variety of substrates. Expression is particularly noted in epithelia such as those of the gastrointestinal tract. Bioavailability of certain drugs after peroral administration may be limited by an intestinal secretion process that is mediated by P-gp. Up- or downregulation of P-gp could thus influence the oral bioavailability of drugs that are a substrate for P-gp. In contrast to the effect of food components on the expression of intestinal P-gp, the effect of mycotoxins on intestinal P-gp is yet unknown. It was therefore the aim of the present study to investigate whether the *Fusarium* mycotoxin deoxynivalenol (DON) alters the expression of P-gp at the level of the intestines of pigs. Our results demonstrated a reduced expression of P-gp in the ileum of pigs fed for 10 days feed experimentally contaminated with 1 mg/kg DON. Furthermore, intake of feed experimentally contaminated with 3 mg/kg DON for 10 days resulted in a complete absence of P-gp.

Introduction

Permeability-glycoprotein (P-gp) was the first drug transporter described (Juliano and Ling, 1976). This adenosine triphosphate (ATP)-dependent drug transporter is highly conserved and is abundantly expressed at the brush border membrane of columnar epithelial cells of small and large intestine. The physiological role of intestinal P-gp might be to prevent entry of xenobiotics, bacterial toxins and food-borne pathogens into the gut wall (Neudeck et al., 2004). These efflux transporters excrete their substrates back into the gastrointestinal lumen thereby reducing the net absorption into the systemic circulation. Various classes of antibiotics have been shown to be a substrate to P-gp (Saitoh et al., 1995; Zhou, 2008). Variability in P-gp expression can thus influence the pharmacokinetic characteristics of P-gp substrates. The literature has provided extensive data supporting the modulation of drug bioavailability through P-gp regulation by components in foods such as fruit juices, spices, herbs and green tea (Zhang et al., 2009). Only scarce information is available on the effect of mycotoxins on transporters.

Mycotoxins are toxic secondary metabolites produced by fungi. It is known they can affect epithelial transport systems after oral ingestion of mycotoxin contaminated feed. Deoxynivalenol (DON) selectively affected the activities of the intestinal transporters D-glucose/D-galactose sodium-dependent transporter (SGLT1), the passive D-fructose transporter GLUT5 and the active and passive L-serine transporter (Maresca et al., 2002). Koraichi et al. (2012) demonstrated that daily exposure of pregnant rats to 1 mg/kg b.w. zearalenone (ZEA) during 14 days results in modulation of mRNA levels of ABC transporters, in particular P-gp, in fetal liver and maternal tissues. Duca et al. (2012) showed that intraperitoneal treatment of rats with 25 mg/kg b.w. ZEA during 3 days resulted in an upregulation of mRNA expression of P-gp. Osselaere et al. (2013) demonstrated that broilers fed T-2 toxin at 752 µg/kg feed had a significantly lower hepatic mRNA expression of MRP2

(multidrug resistance-associated protein 2), compared to the control group. To what extent mycotoxins can modulate intestinal P-gp expression in pigs is not yet examined. It was therefore the aim of the present study to investigate whether the *Fusarium* mycotoxin deoxynivalenol (DON) alters the expression of P-gp at the level of the intestines of pigs.

Materials and Methods

Nine male pigs with a mean body weight of 5.90 ± 0.169 kg were randomized into three experimental groups each consisting of 3 pigs. During 10 days, the first group received blank feed *ad libitum* and was assigned the control group. The second group received *ad lib* feed experimentally contaminated with 1 mg/kg DON and the third group received feed experimentally contaminated with 3 mg/kg DON. At the end of the feeding trial, the animals were sedated with a combination of xylazine (XylM® 2%, VMD, Arendonk, Belgium), zolazepam and tiletamine (Zoletil 100®, Virbac, Wavre, Belgium), followed by killing by exsanguination. Samples of small intestine and colonic wall were fixed in formalin, embedded in paraffin wax, sectioned (5 µm) and immunohistochemically stained for P-gp using the monoclonal antibody C219 (Covance, Princeton, New Jersey, USA). A description of the method can be found in detail in Van der Heyden et al. (2011).

P-gp immunolabelling was scored using a semiquantitative scoring system: 0, no epithelial P-gp expression; 1, P-gp expression by epithelial cells at the level of the villus tips in the small intestine or multifocal loss of P-gp expression at the plasma membrane of colonic enterocytes; and 2, continuous P-gp labelling at the brush border of the surface epithelium. The results of the different groups are presented in Figure 1.

Results

Figure 1 represents sections of the ileum immunohistochemically stained with a P-gp specific antibody (C219) after feeding pigs for 10 days respectively blank feed, feed experimentally contaminated with 1 mg/kg DON and feed experimentally contaminated with 3 mg/kg DON.

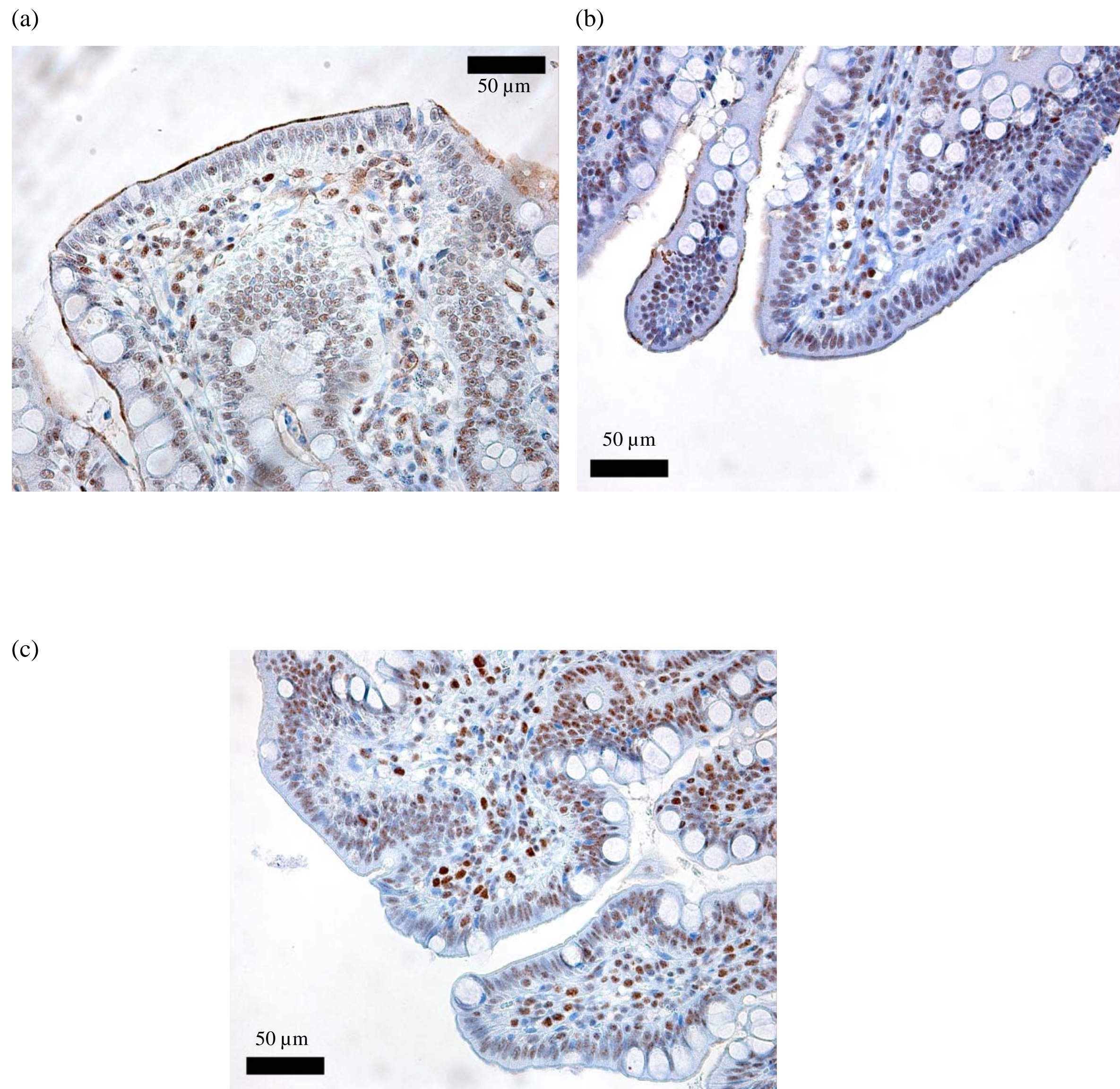

Figure 1. Staining of ileum sections with a P-gp specific antibody (C219) after feeding pigs for 10 days respectively blank feed (a), feed experimentally contaminated with 1 mg/kg DON (b) and feed experimentally contaminated with 3 mg/kg DON (c).

The use of antibody C219 results in a brown-coloured staining of P-gp where present. In the pigs receiving blank feed (a), a continous brown line indicating P-gp expression at the apical membrane of all enterocytes was present. A reduced expression of P-gp (intermittent staining) was seen when pigs were fed 1 mg/kg contaminated feed (b). A dose of 3 mg/kg DON resulted in a complete absence of P-gp, i.e. no staining at the apical side of the enterocytes (c).

Discussion

There are extensive data concerning the modulation of drug bioavailability through P-gp regulation by components in foods such as fruit juices, spices, herbs, cruciferous vegetables and green tea (Zhang et al., 2009). Only a few studies describe an effect of mycotoxins on P-gp expression, and only in rats (Koraichi et al., 2012; Duca et al., 2012). However, to our knowledge, the effect of mycotoxins on intestinal P-gp in swine has not been investigated yet. Our results indicate that intake of DON contaminated feed may result in a decreased expression of P-gp. This might have consequences for the bioavailability of compounds which are a substrate for P-gp. The reduced expression and/or complete disappearance of P-gp may result in a higher uptake of noxious substances present in the intestinal lumen such as xenobiotics, explaining partly the reduced zootechnical performance of pigs exposed to DON.

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General Discussion

General

As indicated by several surveys is the prevalence of mycotoxins in animal feedstuffs quite high in Europe (Monbaliu et al., 2010; Griessler et al., 2010; Rodrigues and Naehrer, 2012; Streit et al., 2012). Next to aflatoxin and ochratoxin A , the *Fusarium* toxins deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZEA) and fumonisin B1 (FB1) are most frequently found. These mycotoxins are small and stable molecules which are extremely difficult to remove or eradicate and furthermore keep their toxic properties when entering the feed chain (Eriksen, 2003). As a consequence, consumption of mycotoxin contaminated feed may have a negative impact on animal health.

Among animal species pigs are considered to be the most sensitive to mycotoxins and are economically important species in our regions. Moreover, since pigs have a cereal rich diet they are frequently exposed to mycotoxins. Although the clinical effects of acute exposure to high doses of mycotoxins are well documented, only a few papers described the effects of mycotoxins present at low and permitted feed concentrations (Drochner et al., 2004; Chen et al., 2008).

After intake of mycotoxin contaminated feed, the gastrointestinal tract is the first organ to be encountered. When this barrier is compromised by mycotoxins, not only harmful substances such as toxins, enzymes, antigens and microorganisms, but also nutrients, drugs, etc. are more likely to be absorbed at higher levels in the systemic circulation.

It was therefore the aim of the present study to evaluate the effect of low and in practice relevant mycotoxin concentrations in feed on the absorption of commonly used antibiotics in pigs.

Effect of mycotoxins on drug absorption

After oral administration, drugs must be absorbed through the gastrointestinal tract to achieve the systemic circulation and exert their pharmacological effects. Intestinal permeability is regulated either by the tight junctions or by the transcellular pathway, with or without active carrier mediation. Furthermore, intestinal absorption and bioavailability can also be influenced by intestinal phase I and phase II metabolism and to multidrug efflux pumps. Mycotoxins have been shown to influence all of these important factors.

To have a first indication concerning the toxicity of the most important *Fusarium* mycotoxins, *in vitro* experiments were performed to determine the viability of IPEC-J2 cells. In contrast to most studies, which apply commonly used colorimetric tests such as WST-1, MTT or LDH assays, a flow cytometric technique was used allowing discrimination between viable, apoptotic and necrotic cells. In general, it could be concluded there is a dose- and time-dependent effect of all tested mycotoxins on the viability of IPEC-J2 cells. Furthermore, of the four mycotoxins tested, T-2 was clearly the most toxic one, with a cytotoxic effect even in the ng/ml range. DON, FB1 and ZEA were only toxic to IPEC-J2 cells in the µg/ml range. This is a very important remark since there are no maximum permissible guidance levels for T-2 in animal feed available yet. Recently the European Food Safety Authority (EFSA) published a scientific opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed (EFSA, 2011a). However, because of the limited knowledge on the effects of T-2 and HT-2 toxins on farm and companion animals, and the absence of a comprehensive database on feed contamination levels of T-2 and HT-2 in the EU, it was not possible to assess the risks of these toxins for animal health. Nevertheless, based on dose-response data, a benchmark dose of 10 µg/kg b.w. per day was advised for pigs.

In correlation with cell viability, the integrity of the intestinal IPEC-J2 cell monolayer after exposure to mycotoxins was evaluated. Our results indicated a dose- and time-dependent effect of all tested mycotoxins on the trans-epithelial electrical resistance (TEER) of the porcine intestinal epithelial cell monolayer. These findings are in concordance with those of several other studies indicating that mycotoxins can alter the gastrointestinal barrier properties (Kasuga et al., 1998; Sergent et al., 2006; Pinton et al., 2009; Van De Walle et al., 2010; Diesing et al., 2011). To our knowledge, our study was the first to use drugs as a marker for the effect of mycotoxins on barrier integrity.

Results indicated, both *in vitro* and *in vivo*, that exposure of intestinal cells to mycotoxins results in increased passage of certain antibiotics. This is an important finding as increased plasma concentrations could have consequences for the withdrawal time of the antibiotics, toxicity in treated pigs and consequently for public health.

In order to investigate how mycotoxins can influence the absorption of antibiotics, it is important to understand how antibiotics are absorbed. The rate and extent of passive non-ionic diffusion of a drug through the different layers of the intestinal wall depends on several factors, i.e. the diffusion coefficient, the surface area, the diffusion distance and the concentration gradient over this diffusion distance. Changes in one or more of these parameters can lead to changes in bioavailability of drugs which are absorbed by passive diffusion. Therefore, the effect of T-2 toxin and/or a modified glucomannan mycotoxin binder, which is commonly used in feed industry, on several of these parameters were studied.

As passive diffusion only takes place for non-ionized drugs and as the diffusion coefficient of a drug varies with varying pH, it was tested whether mycotoxin and/or mycotoxin binder and/or antibiotic cause changes in acidity of the environment. This could modify the charge of the antibiotic resulting in a changed absorption. It was demonstrated that addition of the tested

modified glucomannan mycotoxin binder to water causes a drop in pH with two units. This pH shift provoked by the binder does, however, not explain our results as the increased plasma concentrations were not seen in the group which received blank feed supplemented with the mycotoxin binder. Chlortetracycline and T-2 toxin were not shown to influence the pH.

Another parameter involved in passive diffusion of drugs is the diffusion distance. Intestinal mucus, secreted by goblet cells, covers the gut epithelium and protects the epithelium against chemical, enzymatic, physical and bacterial aggressors that may be present in the gut lumen (Montagne et al., 2004). It is a part of the intestinal barrier and changes in thickness of the mucus layer results in a changed diffusion distance. Obremski et al. (2008) demonstrated that the combination of low doses of T-2, DON and ZEA in the feed reduces the number of mucus-producing cells and the tightness of the intestinal glycocalix in pigs. The opposite effect, an increasing activity of the goblet cells, was however demonstrated when ZEA was fed alone to pigs (Obremski et al., 2005). Increases in the number of goblet cells were also seen in chickens fed a high dose of FB1 (Brown et al., 1992). Furthermore, it was suggested by Braybrooks et al. (1975) that tetracyclines are bound to the mucin macromolecules. These authors investigated the bioavailability of tetracycline in the presence and absence of a porcine gastric mucin layer by the rat everted gut sac technique and revealed an approximate 50% reduction in tetracycline movement across the membrane in the presence of the mucin layer. Considering these findings, it was hypothesized that a mycotoxin induced reduction in thickness of the pig mucosal layer could result in an increased passage of doxycycline. A quantitative Real-Time PCR (qRT-PCR) method was applied to determine the effect of intake during 21 days of 100 µg/kg T-2 contaminated feed on the transcription levels of MUC1, MUC2 and MUC5 in swine. However, no significant differences in transcription level of these mucins in the duodenum of pigs could be demonstrated.

In addition to passive absorption, intestinal absorption and bioavailability of a drug may also be influenced by intestinal phase I and/or phase II metabolism and to multidrug efflux pumps. Therefore, the effect of T-2 or DON on CYP activity and P-gp expression, respectively, was also studied. A significant reduction in CYP3A activity, as well as in P-gp expression was noted after intake of mycotoxin contaminated feed. These findings can have important consequences for drugs which are a substrate for these proteins regarding the dosage scheme and treatment efficacy.

Of the antibiotics used in our experiments, namely doxycycline and chlortetracycline, it is not known whether they are substrates for CYP and/or P-gp in pigs. Only doxycycline is found to be a substrate for P-gp in humans (Zhou, 2008). More research concerning the role of P-gp and CYP in the bioavailability of these drugs in pigs and other animal species is necessary.

Role of mycotoxin detoxifying agents

A changed bioavailability of drugs after intake of mycotoxin contaminated feed supplemented with a mycotoxin detoxifying agent was only limited described in literature. Amer (2005) found significant lower maximal plasma concentrations for lincomycin in broilers fed a mycotoxin detoxifying agent, compared to broilers receiving no detoxifying agent in the feed. Unfortunately, the type of binder was not specified by the authors. The Bureau of Veterinary Drugs of Canada (Anonymous, 1992) reported a lack of efficacy of tylosin in cattle after intake of a clay containing bentonite. Also Shryock et al. (1994) demonstrated that bentonite (2%), mixed in the feed, renders tilmicosin completely ineffective in broiler chickens. As a consequence, it was recommended by EFSA that the simultaneous oral use of of certain medicinal substances and bentonite, should be avoided (EFSA, 2010). Recently the European Commission requested the Panel on Additives and Products or Substances used in Animal

Feed (FEEDAP) to modify Commission Regulation (EC) No 429/2008. It was suggested, for the authorization of additives belonging to the functional group of substances for reduction of the contamination of feed by mycotoxins, that one of the parameters that needs to be taken into account is the presence and characterization of possible interactions of the additive with, among others, veterinary medicinal products. It was in this context that our research group included mycotoxin detoxifying agents in the experiments performed to determine the effect of mycotoxins on the absorption of commonly used antibiotics in pigs. In this thesis it was decided to use feed additives belonging to the class of the yeast cell walls. These additives are derived from the yeast *Saccharomyces cerevisiae* and consist mainly of α-D-mannans and β-D-glucans, the two main components of a yeast cell wall. All experiments were conducted with the same commercial binder, except for the experiments described in chapter 3. For the latter, another commercial feed additive was used also derived from *S. cerevisiae*. It could be concluded that intake of T-2 or DON contaminated feed supplemented with the mycotoxin binder results in increased plasma concentrations of doxycycline and chlortetracycline. Remarkably, Osselaere et al. (2012) also demonstrated increased plasma concentrations of oxytetracycline in chickens after intake of feed supplemented with a mycotoxin detoxifying agent, more specifically a mycotoxin biotransforming agent which is supposed to degrade the mycotoxin into non-toxic compounds. In pigs it was demonstrated that administration of a single oral bolus of DON combined with a glucomannan mycotoxin binder enhances the intestinal absorption of DON compared to the control group receiving only DON (Devreese et al., 2012). How exactly intake of mycotoxin detoxifying agents lead to changed plasma concentrations of mycotoxins or antibiotics in animals remains still unknown.

Several attempts were made in order to elucidate the mechanism underpinning the increased plasma concentrations of doxycycline in pigs receiving T-2 contaminated feed supplemented with a yeast-derived glucomannan mycotoxin binder.

A first hypothesis stemmed from the research of Shryock et al. (1994) who demonstrated, as already mentioned, that bentonite, a mycotoxin adsorbing aluminosilicate, mixed in the feed, renders tilmicosin completely ineffective in broiler chickens. The investigators postulated that the exchangeable mineral cations of bentonite are susceptible to being replaced by organic cations (such as tilmicosin) which likely become tightly bound and thus not available to the chicken. Considering our experimental set-up, it was hypothesized that the mycotoxin binder could interact with divalent ions such as Ca^{2+} and Mg^{2+}. As a consequence, doxycycline, which is normally partially bound by divalent ions (Berthon et al., 1983), would be less bound by those divalent ions in the intestinal lumen and more doxycycline would be available to enter the systemic circulation. An extra experiment was performed in which plasma levels of oxytetracyline, a tetracycline which is more likely to be bound by divalent ions compared to doxycycline, were measured in pigs one week after intake of either blank feed or blank feed supplemented with the feed additive. However, no changed plasma concentrations of oxytetracycline between the two groups could be demonstrated (Goossens et al., 2012a).

Next, the effect of toxin and/or mycotoxin binder on surface area was studied. Solis de los Santos (2007) demonstrated that a mannan-oligosaccharide (MOS) and β-glucan additive enhances villus height, surface area, lamina propria thickness, crypt depth and goblet cell density in the ileum, and to some degree also in the jejunum and duodenum of turkey poults. A changed surface area is directly related to digestion and nutrient absorption, because villi play an important role in these processes (Uni et al., 1999; Sklan, 2001). The mycotoxin binder used in our experiments consists mainly of both mannan-oligosaccharides and β-glucans, two major components of the yeast cell wall. It was thus hypothesized that the mycotoxin binder could influence the parameter surface area, i.e. villus length and crypt depth, resulting in a changed absorption area and therefore a modified absorption capacity.

Significant changes in villus length and crypt depth by histology of the intestinal tissues could, however, not be demonstrated in our experiment (Goossens et al., 2012b).

Since the mycotoxin binder consists mainly of β-glucans, it was tested whether addition of pure β-glucans to the feed also induces increased plasma concentrations of doxycycline. In our study, no statistical difference could be demonstrated between the different experimental groups excluding the role of pure β-glucans in the enhanced absorption of doxycycline. Other components of the yeast cell wall, i.e. mannoproteins and chitin (Schreuder et al., 1996), could thus be involved in the increased plasma concentrations of doxycycline.

Finally, another hypothesis could be that addition of a binder may lead to a longer residence time of the mycotoxins in the intestinal tract. This prolonged presence could eventually result in a more pronouced impairment of the intestinal barrier function, leading to an enhanced absorption of drugs.

Although we were not able to reveal the exact mechanism behind the increased plasma concentrations of doxycycline after intake of T-2 or DON contaminated feed supplemented with a modified glucomannan mycotoxin binder, these are several hypotheses that can be excluded. The major problem with commercially available mycotoxin binders, however, is the fact that it is often not shown that these additives do work *in vivo*. Furthermore, there is also a lack of studies investigating the safety of the use of mycotoxin binders. Most studies nowadays performed with feed additives are mainly carried out *in vitro* but these results are difficult to be extrapolated to the *in vivo* situation. The main problem hereby is that several different factors need to be taken into account. In general, our findings underline clearly the need for scientifically based information concerning the safety and efficacy of these products.

Other factors influencing bioavailability of drugs

It has to be noticed that not only mycotoxins and mycotoxin detoxifying agents can influence the oral bioavailability of drugs. Bioavailability of orally administered drugs may also be indirectly affected by the diets' composition.

For example, in broilers, Teirlinck et al. (2009) showed that intake of a maize-based diet results in less villus fusion, a thicker tunica muscularis, and less apoptosis of epithelial cells in the mucosa than those given a wheat/rye-based diet.

In early-weaned pigs, it has been shown that soybean meal has a detrimental effect on villus length. However, this undesirable effect could be minimized when soybean meal was fed as part of a corn-based diet (Dunsford et al., 1989). The type and composition of dietary fiber has also been shown to influence the gut morphology as the length of the colon was significantly greater in pigs consuming a high-fibre diet than in those on a low-fibre diet (Jørgensen et al., 1996).

The effects described above may contribute to the extent to which a drug is passively absorbed since a changed gut morphology may result in a changed absorption area. However, this does not explain the results described in chapter 2 and 3, since the experimental diets had all the same basic composition. The study design was as such that both the control group and the experimentally contaminated diet groups all received the same basal diet.

Consequences of increased plasma concentrations of antibiotics

The antibiotics used in the experiments were doxycycline and chlortetracycline, both belonging to the group of tetracyclines; and paromomycin, an aminoglycoside. Tetracyclines are frequently used in pig industry. Paromomycin, although somewhat less commonly used, was also selected as the drug is known to have a very low oral bioavailability due to its polar nature. The low degree of absorption of this antibiotic is mainly due to paracellular transport.

On the other hand, tetracyclines are absorbed by transcellular passage due to passive non-ionic diffusion. Also a difference in oral bioavailability has been reported between tetracyclines in pigs. A percentage of absorption of about 25-30 % has been noticed for doxycycline in this animal species (Baert et al., 2000), while chlortetracycline shows a very low fraction of oral absorption (about 5 %, Nielsen and Gyrd-Hansen, 1996).

Increased plasma concentrations of tetracycline antibiotics in farm animals are not considered to have consequences for the animal itself. Tetracycline antibiotics are known to have a wide therapeutic-toxic window and increased plasma concentrations of these antibiotics are not expected to be toxic for the animal. However, caution is needed for antibiotics with a narrow therapeutic-toxic window, such as aminoglycosides who require strict control of the plasma levels. Although our results indicated no significantly increased plasma concentrations of paromomycin compared to the control group, there was a trend for higher plasma concentrations after intake of binder supplemented feed (solely or in combination with T-2). It is likely that inclusion of more animals in the study could result in smaller standard deviations and thus significant differences in the oral bioavailability of paromomycin compared to the control group.

The increased plasma concentrations of antibiotics after intake of mycotoxin contaminated feed could, however, also be seen as an advantage for antibiotics with a low oral bioavailability such as chlortetracycline. However, we would not recommend to feed the animals mycotoxin contaminated feed in order to achieve higher plasma concentrations, as the mycotoxin itself is detrimental for the animal.

Next, it has to be taken into account that increased plasma concentrations of antibiotics may give rise to increased tissue residues. This has no implications for the animal itself, but could possibly be a threat for consumers of animal meat and/or for the farmer with respect to violation of the maximum residue limit (MRL) legislation. Therefore, it has to be studied whether the MRL is not exceeded by means of residue depletion studies. In the case the MRL is exceeded, the withdrawal time of the antibiotic needs to be adapted.

Future perspectives

It is clear that mycotoxins have the potential to affect intestinal integrity and permeability in pigs resulting in increased passage of antibiotics. In summary, the following conclusions and suggestions for further research can be made:

1. As already stated by EFSA, very few studies investigated the effect of mycotoxins at levels lower than or at the maximum permissible guidance levels. Our study is one of the very few taking into account such low contamination levels which are commonly seen in practice.
   Among the mycotoxins tested in this project, T-2 was the most toxic one. However, no maximum permissible limits for T-2 in animal feed are available yet. Because of the limited knowledge on the effects of T-2 and HT-2 toxins on farm and companion animals, and the absence of a comprehensive database on feed contamination levels of T-2 and HT-2 in the EU, EFSA was not able to assess the risks of these toxins for animal health. More studies investigating the effect of T-2 and HT-2 on animal health are needed. However, also the effects of mycotoxins which are already regulated still need to be investigated as identification of mycotoxin hazards is based on mostly incomplete toxicological data and the maximum acceptable levels have not been based on pivotal studies.
2. Our *in vivo* results revealed a time-dependent effect of T-2 on the bioavailability of certain tetracycline antibiotics. Intake of T-2 contaminated feed for 7 days, or DON contaminated feed for 13 days did not result in increased plasma concentrations of doxycycline but intake of T-2 contaminated feed during 21 days, resulted in increased plasma concentrations of chlortetracycline. Increased plasma concentrations can have important implications concerning treatment efficacy, toxicity and withdrawal time of

the antibiotic. Therefore, it needs to be tested whether the current withdrawal time is still acceptable when antibiotics are administered in combination with mycotoxin contaminated feed.

3. It can be concluded that DON and T-2 can affect P-gp expression and hepatic CYP activity in pigs, respectively. Since it is not known whether the antibiotics used in our experiments are a substrate for these proteins, the effect of mycotoxins on P-gp and CYP cannot be used to explain the increased plasma concentrations. To study whether antibiotics are a substrate for P-gp, Madin-Darby Canine Kidney (MDCKII) confluent cell monolayers overexpressing human P-gp can be used. Transport of antibiotics across these monolayers, with or without the presence of inducers or inhibitors of P-gp, can give a first indication.

   Nevertheless, our results indicate there is the need to investigate the effect of mycotoxins on proteins involved in xenobiotic detoxifying pathways (i.e. active transporters, CYPs). A changed oral bioavailability of drugs due to a changed expression of P-gp or reduced activity of CYP enzymes, can have consequences for treatment efficacy and withdrawal time of the drug. Modulation of drug bioavailability through P-gp regulation by components in food is extensively described. The effect of mycotoxins on P-gp is only studied recently, indicating the awareness concerning the risk of mycotoxins and its effect on intestinal health.

4. The efficacy and safety of mycotoxin binders needs further to be investigated. In 2010, EFSA published a scientific opinion on the establishment of guidelines for the assessment of additives from the functional group ‘substances for reduction of the contamination of feed by mycotoxins’, proving to acknowledge the problem concerning the efficacy and safety of mycotoxin detoxifying agents currently available

at the market. At our department, *in vitro* and *in vivo* models for efficacy and safety testing of mycotoxin detoxifying agents are currently being developed.

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Summary

Mycotoxins are toxic secondary metabolites produced by fungi. Of importance for human and animal health are, amongst others, those toxins produced by fungi present on crops. Because mycotoxins are very stable, they are able to survive storage and processing and thus end up in the feed and food chain. In our temperate regions, fungi from the genus *Fusarium* are most frequently occurring with deoxynivalenol (DON), T2-toxin (T-2), zearalenone (ZEA) and fumonisin B1 (FB1) as main metabolites. These mycotoxins play a major role as feed contaminant in pig industry, since pigs are one of the most sensitive species to mycotoxins. Moreover, pigs have a cereal rich diet and are therefore frequently exposed to these mycotoxins. General symptoms after intake or exposure to relative high concentrations of these *Fusarium* mycotoxins are well known, namely weight loss, reduced feed intake, vomiting, diarrhea, fertility problems and skin irritations. By setting maximal permissible guidance limits of mycotoxins in raw materials and feed, problems due to exposure to high concentrations of mycotoxins are generally prevented. The influence of low and in practice more relevant concentrations of these mycotoxins is, however, hardly known. This thesis deals with the effects of low concentrations of common *Fusarium* toxins on the oral bioavailability of antibiotics in pigs, as a marker for potential damage to the intestinal barrier.

The first experiments were performed *in vitro* by using the intestinal porcine epithelial cell line derived from the jejunum of pigs (IPEC-J2). This cell line allows to mimic the intestinal situation of the pig. In the **first chapter** it was shown that *in vitro* exposure of the intestinal epithelial monolayer to the *Fusarium* toxins DON, T-2, ZEA or FB1 can result in cell death in both a time- and concentration dependent manner. Consequently, this was associated with an increased passage of the drugs doxycycline and paromomycin through these intestinal cell monolayer. Of the four mycotoxins tested, T-2 was the most toxic metabolite, with toxic concentrations in the ng/ml range, followed by DON, ZEA and FB1, which were only toxic in

the mg/ml range. T-2 was shown to be approximately 100 times more toxic than the other mycotoxins tested. It was also demonstrated that T-2 and DON both induce different mechanisms of cell death. After exposure of intestinal cells to cytotoxic concentrations of T-2, mainly necrotic cells are formed. Exposure to cytotoxic concentrations of DON results, on the other hand, in a higher proportion of apoptotic cells. The experiments described in chapter one reveal that, *in vitro*, mycotoxins can be harmful to intestinal epithelial cells resulting in increased passage of drugs.

In the **second chapter** these *in vitro* findings were tested *in vivo*. In addition, the possible effect of adding a mycotoxin binder to the feed was also evaluated. It is a common knowledge that completely mycotoxin-free feed cannot be guaranteed. Therefore, feed additives have been developed which claim to bind or degrade mycotoxins in the gastrointestinal tract, so that they are no longer available for absorption into the animal. Mycotoxin binders derived from the yeast *Saccharomyces cerevisiae* are frequently used. These feed additives consist mainly of α-D-mannans and β-D-glucans, the two main components of a yeast cell wall. In this research a commercially available modified glucomannan mycotoxin binder was included to evaluate its efficacy. Our *in vivo* research revealed that intake during 7 or 13 days of 100 µg/kg T-2 or 1 mg/kg DON contaminated feed combined with the mycotoxin binder, respectively, results in significant increased plasma concentrations of doxycycline. Nothwithstanding no significant effect of T-2 by itself could be demonstrated, this finding is of importance for the animal feed industry. As already claimed by the European Food Safety Authority (EFSA) there is a need for studies and models to prove the efficiency and safety of mycotoxin binders.

Since the increased absorption of doxycycline, seen in chapter two, cannot *stricto sensu* be attributed to the presence of T-2 in the feed, a possible time-dependent effect of T-2 was

investigated in **chapter 3**. Therefore, plasma concentrations of chlortetracycline were determined in pigs which received for 21 days feed artificially contaminated with 100 µg/kg T-2, whether or not combined with a glucomannan-based feed additive. Chlortetracycline (CTC) was chosen because it is a first generation tetracycline with a mean oral bioavailability of 5 % in pigs. Doxycycline, on the other hand, is a second generation tetracycline derivative with a mean oral bioavailability of approximately 20 % in pigs. It could be interesting to investigate the effect with a first generation tetracycline with a much lower oral bioavailability than doxycycline because, if the same effect is seen with CTC, i.e. increased oral bioavailability of CTC after intake of T-2 contaminated feed, this would have important implications for its efficacy, possible toxicity and the withdrawal time of the antibiotic. Results confirmed previous *in vivo* findings, i.e. intake of T-2 contaminated feed supplemented with a yeast-derived feed additive also results in significant increased plasma concentrations of chlortetracycline. Furthermore, even significant increased plasma concentrations of chlortetracycline were noted after intake of solely T-2 contaminated feed. This demonstrates clearly there is a time dependent effect of T-2 toxin on the intestinal barrier integrity of pigs, which results in increased plasma concentrations of orally administered tetracyclines.

In man and animals, drugs can be metabolized by cytochrome P450 (CYP) enzymes in the liver. Especially CYP3A enzymes are responsible for the phase I metabolism of a wide variety of drugs. In **chapter 4**, the effect of T-2 on the activity of these CYP3A enzymes was investigated. A changed activity or expression could affect the bioavailability of drugs which are a substrate for these enzymes. Our experiment revealed that intake during 14 days of feed artificially contaminated with 1 mg/kg T-2, results in a significant decreased activity of

CYP3A enzymes in the liver of pigs. Addition of a yeast-derived feed additive to T-2 contaminated feed did not change these observations.

In the last part of the experimental studies, **chapter 5**, the effect of DON on the expression of permeability glycoprotein (P-gp) was investigated. P-gp is a membrane glycoprotein which is expressed at the apical side of intestinal epithelial cells. It is involved in the active secretion (efflux) of drugs out of the epithelial wall back to the intestinal lumen. In this way, absorption of drugs after peroral administration can be limited. A changed activity or expression of this protein could result in changed plasma concentrations of drugs which are a substrate for this transporter. Our research revealed that intake of feed artificially contaminated with 1 mg/kg DON and 3 mg/kg DON contaminated feed results in reduced expression and even complete disappearance of P-gp at the level of the ileum of pigs, respectively. This could result in increased plasma concentrations of drugs which are a substrate for this permeability glycoprotein.

In general, the results of this thesis show that the *Fusarium* toxins DON and T-2 can influence the oral bioavailability of the antibiotics doxycycline and chlortetracycline in pigs. Increased plasma concentrations of these drugs could have consequences for public health with respect to the withdrawal time of the drug and tissue residues. However, it needs to be investigated whether increased plasma concentrations do result in increased tissue residues, taking into account the withdrawal time of the antibiotic. Since it is not known whether doxycycline and chlortetracycline are substrates for CYP3A and P-gp in pigs, the discussion concerning the role of P-gp and CYP3A in the increased plasma concentrations of these antibiotics is thus still open. Nevertheless, these findings are still important for drugs which are a substrate for P-gp and/or CYP3A. Next to the conclusions concerning the effect of DON and T-2 on the oral bioavailability of drugs, it can also be concluded that there is a clear need to establish

maximal permissible guidance limits for T-2 in the feed, as well as to perform research on the effect of low concentrations of mycotoxins on human and animal health.

Samenvatting

Mycotoxines zijn toxische, secundaire metabolieten die geproduceerd worden door schimmels. Van belang voor de gezondheid van mens en dier zijn o.a. deze toxines die geproduceerd worden door schimmels die voorkomen op gewassen. Mycotoxines zijn immers zeer stabiele verbindingen, die niet afgebroken worden tijdens bewerkings- en verwerkingsprocessen, en bijgevolg terechtkomen in de eindproducten bestemd voor zowel humane- als dierconsumptie. In onze gematigde streken komen schimmels van het genus *Fusarium* het vaakst voor met deoxynivalenol (DON), T2-toxine (T-2), zearalenone (ZEA) en fumonisine B1 (FB1) als voornaamste metabolieten. Deze toxines spelen een belangrijke rol in de varkenssector, aangezien het varken één van de meest gevoelige diersoorten is voor deze mycotoxines. Bovendien worden vaak granen of graanbijproducten verwerkt in varkensmengvoeder, waardoor het varken frequent aan deze mycotoxines blootgesteld wordt. Algemeen voorkomende symptomen na inname of blootstelling aan relatief hoge concentraties van deze toxines zijn wel bekend namelijk gewichtsverlies, verminderde eetlust, braken, diarree, vruchtbaarheidsstoornissen en eventueel huidirritaties. Door het instellen van maximaal toelaatbare concentraties in grondstoffen en veevoeders worden problemen als gevolg van te hoge concentraties mycotoxines in principe voorkomen. De invloed van lage, voor de praktijk meer relevante, concentraties van deze mycotoxines zijn echter nauwelijks gekend. In deze thesis werd daarom het effect van lage concentraties *Fusarium* toxines op de biologische beschikbaarheid van antibiotica, als merker voor schade aan de darmbarrière bij het varken, nagegaan.

De eerste experimenten werden *in vitro* uitgevoerd. Hiervoor werd gewerkt met een intestinale porciene epitheliale cellijn afkomstig van het jejunum van varkens (IPEC-J2). Deze cellijn laat toe de intestinale situatie van het varken na te bootsen. In een **eerste hoofdstuk** werd aangetoond dat *in vitro* blootstelling van de intestinale epitheliale monolayer

aan de *Fusarium* toxines DON, T-2, ZEA of FB1 zowel op een tijds- en concentratie afhankelijke manier kan leiden tot celdood. Dit gaat bijgevolg gepaard met een verhoogde passage van de geneesmiddelen doxycycline en paromomycine doorheen deze *in vitro* darmcellen. Van de vier onderzochte mycotoxines kon T-2 als meest toxische metaboliet bestempeld worden, gevolgd door DON, ZEA en FB1. Daarnaast kon ook aangetoond worden dat T-2 en DON beiden een verschillend mechanisme van celdood induceren. Na blootstelling van intestinale cellen aan cytotoxische concentraties van T-2 konden voornamelijk necrotische cellen teruggevonden worden. Blootstelling aan cytotoxische concentraties DON resulteerde daarentegen in een groter aandeel van apoptotische cellen. Uit de experimenten besproken in het eerste onderdeel van dit onderzoek kan besloten worden dat mycotoxines schadelijk kunnen zijn voor cellen van de darmwand. Dit leidt *in vitro* ook tot verhoogde passages van geneesmiddelen, wat ook voor de *in vivo* situatie van belang kan zijn.

In een **tweede hoofdstuk** werden deze *in vitro* bevindingen verder *in vivo* getoetst. Bijkomende factor die hierbij onderzocht werd, was de mogelijke invloed van toevoeging van een mycotoxine binder aan het voeder. Zoals algemeen bekend kan volledig mycotoxine-vrij voeder niet gegarandeerd worden. Om die reden werden voederadditieven ontwikkeld die claimen mycotoxines in de darm af te breken of te binden, zodat deze niet langer beschikbaar zijn voor absorptie in het dier. Mycotoxine binders afgeleid van de gist *Saccharomyces cerevisiae* worden hiertoe frequent gebruikt. Deze voederadditieven bestaan voornamelijk uit α-D-mannanen en β-D-glucanen, de twee voornaamste componenten van de gistcelwand. In dit onderzoek werd dan ook een commercieel verkrijgbare gewijzigde glucomannaan mycotoxine binder ingesloten om de efficiëntie ervan na te gaan. De *in vivo* experimenten toonden aan dat inname gedurende 7 of 13 dagen van voeder artificieel gecontamineerd met respectievelijk 100 µg/kg T-2 of 1 mg/kg DON gecombineerd met de mycotoxine binder,

leidt tot significant verhoogde plasma concentraties van doxycycline. Niettegenstaande er geen significant effect van T-2 op zich aangeduid kon worden, is deze bevinding van belang voor de veevoedersector. Zoals reeds aangekaart door het Europese Agentschap voor Voedselveiligheid (EFSA) is er duidelijk nood aan studies en modellen om de efficiëntie en veiligheid van mycotoxine binders te toetsen.

Aangezien in hoofdstuk twee de verhoogde absorptie van doxycycline niet *stricto sensu* toegeschreven kon worden aan de aanwezigheid van T-2 in het voeder werd in **hoofdstuk drie** het mogelijks tijdsafhankelijk effect van T-2 toxine nagegaan. Varkens kregen hiervoor gedurende 21 dagen voeder artificieel gecontamineerd met 100 µg/kg T-2 toegediend, al dan niet in combinatie met een gelijkaardig, van de gistcelwand afgeleid, voederadditief bestaande uit α-D-mannanen en β-D-glucanen. In tegenstelling tot eerdere experimenten, waarbij bij varkens doxycycline, een antibioticum met een gemiddelde orale biologische beschikbaarheid van ongeveer 20 %, gebruikt werd, werd in dit experiment een eerste generatie tetracycline, namelijk chloortetracycline, met een gemiddelde orale biologische beschikbaarheid van amper 5 % aangewend. Indien een gelijkaardig effect, namelijk verhoogde plasmaconcentraties na inname van toxine gecontamineerd voeder, verkregen werd met een geneesmiddel met veel lagere biologische beschikbaarheid zou dit belangrijke gevolgen kunnen hebben voor de klinische efficaciteit, mogelijks toxiciteit en de wachttijd van het geneesmiddel. De resultaten bevestigden de bevindingen met doxycycline, namelijk inname van 100 µg/kg T-2 gecontamineerd voeder gesupplementeerd met een glucomannaan voederadditief resulteert ook in significant verhoogde plasma concentraties voor chloortetracycline. Bijkomend konden zelfs significant verhoogde plasma concentraties van chloortetracycline aangeduid worden na inname van T-2 gecontamineerd voeder, zonder aanwezigheid van het additief. Hiermee werd aangetoond dat er duidelijk een tijdsafhankelijk

effect van T-2 toxine op de darmbarrière van varkens is, wat resulteert in verhoogde plasmaconcentraties van oraal toegediende tetracyclines.

In mens en dier kunnen geneesmiddelen, na passage doorheen de darmwand, gemetaboliseerd worden door cytochroom P450 (CYP) enzymen in de lever. Voornamelijk CYP3A enzymen zijn hierbij belangrijk en verantwoordelijk voor de fase I metabolisatie van een groot aantal geneesmiddelen. In **hoofdstuk 4** werd daarom het effect van T-2 op de activiteit van deze CYP3A enzymen onderzocht. Een gewijzigde activiteit of expressie kan immers een invloed hebben op de plasmaconcentraties van geneesmiddelen die substraat zijn voor deze enzymen. In dit onderzoek werd aangetoond dat inname van voeder artificieel gecontamineerd met 1 mg/kg T-2 gedurende 14 dagen resulteert in een significant en duidelijk verminderde activiteit van hepatische CYP3A enzymen in het varken. Toevoeging van mycotoxine binder aan T-2 gecontamineerd voeder heft dit nadelige effect van het mycotoxine niet op.

In een laatste hoofdstuk, **hoofdstuk 5**, werd het effect van DON op de expressie van het permeabiliteits glycoproteine (P-gp) nagegaan. P-gp is een membraan glycoproteïne dat ondermeer apicaal in intestinale epitheliale cellen tot expressie komt. Het is betrokken in de actieve terugsecretie (efflux) van farmaca uit het darmepitheel naar het intestinaal lumen. Op die manier kan de intestinale absorptie van farmaca na perorale toediening verminderd worden. Een gewijzigde expressie van dit membraangebonden eiwit kan resulteren in gewijzigde plasmaconcentraties van geneesmiddelen die substraat zijn voor deze transporters. Ons onderzoek toonde aan dat inname van 1 mg/kg DON en 3 mg/kg DON gecontamineerd voeder resulteert in respectievelijk verminderde expressie tot zelfs volledige afwezigheid van P-gp ter hoogte van het ileum van varkens. Dit zou kunnen resulteren in hogere plasmaconcentraties van geneesmiddelen die substraat zijn voor dit glycoproteine.

De resultaten van deze thesis tonen aan dat de *Fusarium* toxines DON en T-2 een effect kunnen hebben op de biologische beschikbaarheid van de antibiotica doxycycline en chloortetracycline in varkens. Verhoogde plasmaconcentraties van deze geneesmiddelen kunnen gevolgen hebben voor de volksgezondheid met betrekking tot de wachttijd van het geneesmiddel en weefselresiduen. Het moet echter verder onderzocht worden of verhoogde plasmaconcentraties inderdaad resulteren in verhoogde weefselconcentraties, rekening houdende met de wachttijd van het geneesmiddel. Daarenboven, aangezien het niet bekend is dat doxycycline en chloortetracycline substraten zijn voor CYP3A en P-gp in varkens, staat de discussie betreffende de rol van deze eiwitten in de verhoogde plasmaconcentraties van deze geneesmiddelen eveneens open. Al deze bevindingen kunnen zeker van belang zijn voor geneesmiddelen die wel substraat zijn voor P-gp en/of CYP3A.

Naast de conclusies die getrokken kunnen worden betreffende het effect van DON en T-2 op de biologische beschikbaarheid van geneesmiddelen, kan er ook besloten worden dat de resultaten nopen tot het opstellen van richtwaarden voor T-2 toxine in het voeder, alsook tot het voeren van onderzoek naar de invloed van lage concentraties mycotoxines op de gezondheid van mens en dier.

Curriculum Vitae

Joline Goossens werd geboren op 18 juni 1985 te Geraardsbergen. Na het beëindigen van haar middelbare studies Moderne Talen-Wetenschappen in het Sint-Jozefsinstituut te Geraardsbergen, startte ze in 2003 de studie Biomedische Wetenschappen aan de Universiteit Gent. In 2007 studeerde ze af als Licentiaat in de Biomedische Wetenschappen, met onderscheiding. Aansluitend op deze studie vatte ze de opleiding tot Master in Laboratory Animal Science aan. In oktober 2008 trad zij in dienst als doctoraatsstudent bij de vakgroep Farmacologie, Toxicologie en Biochemie van de faculteit Diergeneeskunde. Zij verrichtte er onderzoek naar de invloed van de belangrijkste *Fusarium* mycotoxines op de biologische beschikbaarheid van farmaca in varkens. Dit in het kader van het IWT landbouwonderzoeksproject waar de invloed van *Fusarium* toxines op de darmgezondheid van het varken in het kader van de ontwikkeling van bestrijdingsmaatregelen onderzocht werd. Dit onderzoek gebeurde in samenwerking met de dienst Bacteriologie van de Vakgroep Pathologie, Bacteriologie en Pluimveeziekten. Tijdens dit doctoraatsonderzoek behaalde zij in 2009 het diploma van Master of Laboratory Animal science (FELASA categorie D).

In het kader van haar onderzoek is ze auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Ze nam deel aan verschillende nationale en internationale congressen en presenteerde de resultaten van haar onderzoek in de vorm van voordrachten en posters.

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…een thesis schrijf je nooit alleen…

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had ik graag in 'vakjargon' een aantal mensen willen bedanken…

Algemeen kan dit doctoraat vergeleken worden met een meerdaagse etappewedstrijd (een soort Tour De France tussen de wetenschappers zeg maar). Het begon met een aantal vlakke etappes, die qua moeilijkheidsgraad zeker niet te vergelijken waren met de lastige klimwedstrijden die op het programma stonden. Onderweg zijn we een paar keer gevallen, maar steeds opnieuw terug overeind geklommen om die eindmeet toch maar te bereiken. En dat hebben we! Toch zou dit niet gelukt zijn zonder de steun van een groot aantal mensen onderweg.

Een dikke paragraaf van dit dankwoord wordt eerst en vooral besteed aan Anja! Anja, de voorbije vier jaar heb jij een ongelooflijk grote en onmisbare rol gespeeld in mijn werk! Geen enkel experiment(je) of dierproef(je) was jou teveel. Jij was tegelijk soigneur, ploegmaat, supporter, waterdrager en voedingsdeskundige ;-) Ik mis de quality-time in de stallen, het liedjes (of mensen ;-)) raden aan de flow, onze bourgondische levensstijl, de spannende verhalen na de weekends :-p en zoveel meer! Toch ben ik ervan overtuigd dat het verlaten van de vakgroep niet het einde van onze vriendschap betekent. We horen elkaar zeker nog, en oh ja, ik ben nog steeds onze afspraak van 4 jaar geleden niet vergeten hoor!!!

Lief en leed werd het meest gedeeld met de teamgenoten. Virginie, wanneer ik pas op de vakgroep begon kon ik op jou rekenen om me de knepen van het *in vitro* werk te leren. Je was steeds bereid voor een woordje uitleg of hulp bij een dierproefje ;-). Ik zal niet gauw die bewuste dag voor de Omloop het Nieuwsblad vergeten! Ook aan ons uitstapje naar Lyngby en de workshop in Brussel denk ik met een lach terug :-). Ik wens je veel succes en voldoening op jouw job en veel liefde en plezier op het thuisfront! Ann, voor jou is het einde van je doctoraat ook bijna in zicht. Ik ben er zeker van dat je er een mooi geheel zal van maken. Als je tijdens het schrijven eens even nood hebt aan ontspanning, laat maar iets weten hé, dan voegen we toch gewoon een deeltje toe aan 'klussen met Ann' :-). Aan onze man die enige tijd aan de andere kant van het water vertoefde, de immer gedreven en ambitieuze Mathias, jou wens ik eveneens veel succes toe bij het afwerken van je onderzoek. Je was een fijne collega! Enneuh, ik hou je eraan hé, maar binnenkort trekken we echt wel eens naar 'Destelbeurgen'! De nieuwkomers, Nathan en Thomas, veel succes met jullie onderzoek en tot op één of andere sportieve activiteit. De Ekiden staat reeds in mijn agenda!

De mycotoxers die geen deel uitmaakten van de mycotox-bureau, maar daarom niet minder tot ons team behoorden. Elin, voor jou is het allemaal al achter de rug. Geen paniek, je hebt

het er schitterend vanaf gebracht! Tis een doctoraat geworden om U tegen te zeggen! Bedankt voor alle hulp tijdens de dierproeven, het ineenpassen van onze 6-maandelijkse verslagen, en de raad en daad bij *in vitro* werk! Je bent een enthousiaste meid wat me zelf steeds opnieuw de moed gaf om er terug in te vliegen. Gunther, jou wens ik veel succes met je onderzoek. Misschien komen we elkaar wel nog eens tegen in Viane-city. De vreemde eend in de bijt, Sandra, gewezen lid van de mytox bureau maar LPS-er in hart en nieren, ik denk met plezier terug aan de tijd dat wij de bureau mochten delen. Ik wens je een mooie toekomst toe!

Het LPS team! Heidi, Elke en Anneleen! Bij jullie kon ik steeds terecht om eens te ventileren ;-) Bedankt om mental coach te spelen! Mijn eerste stappen op de vakgroep deelde ik met Heidi, die op exact dezelfde dag startte met wetenschappelijk onderzoek op de vakgroep Farmacologie. Ik zie ons nog staan… toen nog niets vermoedend dat we Wilibrord, Adelheid, Amadeus, Isidoor, knorretje, zwelletje, vlekje, en alle anderen gemeen zouden hebben ;-). Veel succes met het verdere verloop van je onderzoek. Dat komt allemaal snor! De immer opgewekte Anneleen! Ik bewonder jouw enthousiasme, positieve ingesteldheid en gezonde dosis relativeringsvermogen. Het zijn zeker en vast troeven om jouw onderzoek tot een goed einde te brengen. Elke, jou wens ik heel veel succes met de ELISA's, de kalfjes en alle toxicologisch onderzoek dat je nog voorgeschoteld zal krijgen. Ik duim voor die eerste publicatie! Ex-LPS-bureaulid Eva, bedankt om mij in het prille begin de knepen de sputterende HPLC-UV aan te leren en, wat minder werkgerelateerd, bedankt om de koekjespauze te introduceren. Dat is alleszins iets wat tot op de dag van vandaag nog steeds gesmaakt wordt!

Supporters aan de zijlijn, Jelle, An M., Marc, Kris D.K., Ann S. en Kris B.! Bedankt om interesse te tonen in mijn onderzoek, maar eveneens in mijn sportieve prestaties. Het was aangenaam om af en toe met jullie een babbeltje te slaan.

Grote dank gaat eveneens uit naar Siegrid. Zonder jouw werk zou het mij een pak meer tijd gekost hebben om dit doctoraat tot een goed einde te brengen. Bedankt om methodes op punt te stellen, stalen te analyseren en mijn wetenschappelijk schrijven kritisch te analyseren.

De mensen van de biochemie, Jella, Donna, Koen, Jorien, Kristel, Dieter en Bert… 't was leuk jullie in de gang, in de resto, tijdens de koekjespauze of in 't labo tegen te komen. Er kon al eens geklaagd en gezaagd worden, toch werden de pauzes grotendeels gereserveerd om eens goed te lachen. Veel succes in alles wat jullie doen!

…werken doe je niet alleen tijdens de werkuren, je neemt het ook vaak mee naar huis….

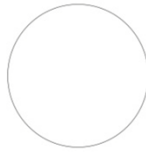
…een speciaal woordje van dank gaat dan ook uit naar mijn familie….

Samuel, mn lieve broer, bedankt dat je er altijd voor me bent! Ookal lijk je een stoere kerel, ik weet dat je een klein lief hartje hebt. Veel succes samen met Katrien in jullie nieuwe huis!
Mijn 'Madré' en 'Padré' ☺! Jullie kan ik niet genoeg bedanken! Bedankt om mij de kans te geven om te gaan studeren, om mij op kot te laten gaan, om me mijn hobby te laten uitoefenen (ookal was dat vaak tegen je zin hé mams ☺), om mij op te vangen, om me door dik en dun te steunen,… kortom, bedankt voor ALLES! Ik weet dat jullie wat extra grijze haartjes door mij gekregen hebben, maar kom geef toe, ge moogt toch ni klagen hé ;-)

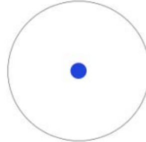
In jullie handen ligt alvast 1 verhaal die ik geschreven heb, het volgende schrijf ik graag samen met Jeroen. 'T heeft me wat tijd gekost om mijn ridder op zijn witte fiets te vinden, maar ik heb hem! Jeroen, mijn lieve Jerry, je geeft me een super en onbeschrijflijk gevoel. Je hebt een positieve kijk op alles, je begrijpt me, weet me te kalmeren wanneer nodig ;-), je bent gewoon alles wat ik zoek in iemand! Ik kijk uit naar onze toekomst samen!

- Joline -

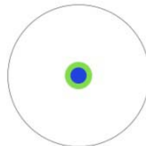
Imagine a circle that contains all of human knowledge:



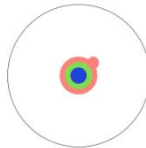
By the time you finish elementary school, you know a little:



By the time you finish high school, you know a bit more:

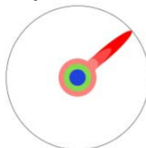


With a bachelor's degree, you gain a specialty:

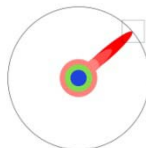


A master's degree deepens that specialty:

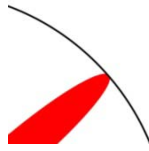
Reading research papers takes you to the edge of human knowledge:



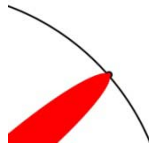
Once you're at the boundary, you focus:



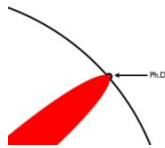
You push at the boundary for a few years:



Until one day, the boundary gives way:



And, that dent you've made is called a Ph.D.:



Of course, the world looks different to you now:

So, don't forget the bigger picture: