Double Trouble: interactions between deoxynivalenol and the pathogenesis of *Salmonella* Typhimurium infections in pigs

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www.mytox.be

TABLE OF CONTENTS

ABBREVIATION KEY

ABC	ATP binding cassette
ANOVA	analysis of variance
AP-1	activated protein-1
ATP	adenosine triphosphate
BGA	brilliant green agar
BLAST	basic local alignment search tool
BW	bodyweight
C/EBP	CCAAT-enhancer-binding protein
Caco-2	human colorectal adenocarcinoma cell
Caspase	cysteine-dependent aspartate-directed protease
CAST	Council for Agricultural Science and Technology
CFU	colony forming units
COX	cyclooxygenase
CREB	cyclic AMP response element binding protein
Ct	threshold cycle
DABCO	1,4-diazabicyclo[2.2.2]octane
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOM-1	de-epoxy-deoxynivalenol
DON	deoxynivalenol
EFSA	European Food Safety Authority
EGR1	early growth response 1
eIF2a	eukaryote translation initiation factor 2a
ELISA	enzyme-linked immunosorbent assay
EMA	ethidium mono-azide
ERK	extracellular signal-regulated kinase
FB	fumonisin B
FCS	fetal calf serum
FHB	*Fusarium* head blight
FITC	fluoresceine isothiocyanate
gDNA	genomic DNA
gfp	green fluorescent protein

GM-CSF	granulocyte/monocytes-colony stimulating factor
Go	goblet cell
HCK	hematopoietic cell kinase
HIS	histone
HPRT	hypoxanthine-guanine phosphoribosyl transferase
IBD	inflammatory bowel disease
IC_{10}	inhibitory concentration of 10%
IFNγ	interferon gamma
Ig	immunoglobulin
IGF	insulin-like growth factor
IGFALS	insulin-like growth factor acid labile subunit
IL	interleukin
IPEC	intestinal porcine epithelial cell
IVET	*in vivo* expression technology
LB	luria broth
LDH	lactate dehydrogenase
LPS	lipopolysaccharide
LSD	least significant difference
MAPK	mitogen-activated protein kinase
M-cells	microfold cells
MCP-1	monocyte chemotactic protein-1
MIAME	minimum information about a microarray experiment
MIP	macrophage inflammatory protein
moi	multiplicity of infection
mRNA	messenger RNA
mφ	macrophage
NCBI	National Centre of Biotechnology Information
NF-κB	nuclear factor-kappa B
nm	nanometer
NOEL	no observed effect level
OD	optical density
Pa	paneth cell
PAK	p21 activated kinase
PAM	porcine alveolar macrophages
PAMPs	pathogen associated molecular patterns

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEEC	pathogen-elicit-epithelial-chemoattractant
PGE-2	prostaglandin E2
P-gp	P-glycoprotein
PI	plasmocyte secreting immunoglobulin A
PKR	RNA-activated protein kinase
PMA	phorbol myristate acetate
PMN	polymorphonuclear
PMTDI	provisional maximum tolerable daily intake
PP	Peyer's patch
PRRs	pattern recognition receptors
RNA	ribonucleic acid
rpm	rotations per minute
SAPK/JNK	stress-activated protein kinase/c-Jun N-terminal kinase
SCOOP	Scientific corporation on questions relating to food
SCV	*Salmonella* containing vacuole
SGLT1	D-glucose/D-galactose sodium dependent transporter
Sif	*Salmonella* induced filaments
SOCS	suppressors of cytokine signaling
sp	species
SPI	*Salmonella* pathogenicity island
T3SS	type three secretion system
TDI	tolerable daily intake
TEER	transepithelial electrical resistance
Th	T helper
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
v/v	volume/volume
VAP	vacuolar-associated actin polymerization
w/v	weight/volume
wt	wild type

GENERAL INTRODUCTION

1. **The mycotoxin issue in food and feed**

Mycotoxins are secondary metabolites of filamentous fungi, which can contaminate many types of food crops throughout the food and feed chain. Mycotoxin producing fungi can be divided into two groups: field fungi such as *Fusarium* species that produce mycotoxins in the field, whereas storage fungi (*Aspergillus* and *Penicillium*) occur after harvest. In some cases like unusually hot and dry conditions, *Aspergillus* and *Penicillium* sp. can also affect crops during the growing season. On the other hand, field fungi can continue growing and producing mycotoxins during transport and storage (www.mycotoxins.org).

Many factors are involved in the production of mycotoxins on cereal crops, with environmental conditions such as temperature and moisture being of utmost importance in both pre- and/or post-harvest stages (Figure 1).
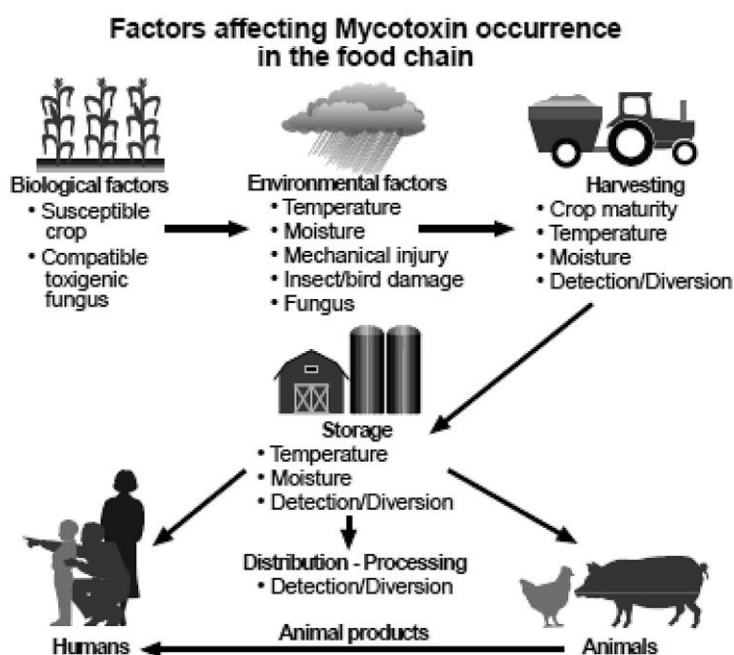
Figure 1: Factors affecting mycotoxin occurrence in the food chain (Council for Agricultural Science and Technology (CAST), 2003).

Their global importance as a potential risk factor for both animal and human health should not be neglected as it is estimated that one quarter of the world's crop production would be contaminated with mycotoxins (Fink-Gremmels, 1999; Oswald *et al.*, 2005).

In a recent paper, Binder *et al.* (2007) reported the results of a two-year survey program undertaken to evaluate the incidence of mycotoxins in feed and feed raw materials. A total of 2,753 assays were performed on 1,507 samples taken from European and Mediterranean markets for the determination of aflatoxin B1, ochratoxin A, deoxynivalenol, T-2 toxin, zearalenone and fumonisins. Fifty-two percent of these samples were tested positive, with DON, zearalenone and T-2 toxin as major contaminants, indicating that the incidence of mycotoxins is quite high in animal feed.

The growing awareness that mycotoxins are a significant concern to human and animal health, has in many countries led to regulations of levels of mycotoxins in food and animal feedstuffs (van Egmond *et al.*, 2007). In Europe, maximum allowed levels for food and feed have been set up by European Commission Regulations and Recommendations. An overview of the current legislation can be found at the website of the European Food Safety Authority (EFSA, http://efsa.europa.eu). The EFSA is the keystone of European Union risk assessment regarding food and feed safety. EFSA's Panel on Contaminants in the Food Chain (CONTAM) provides risk managers with scientific advice to inform their decision-making on the setting of maximum levels of mycotoxins (such as ochratoxin A, deoxynivalenol or zearalenone) in food and feed. It estimates the related risks posed to human and animal health and may set tolerable daily intake levels for various mycotoxins. With regard to mycotoxins in feed, the CONTAM Panel also considers the level of carry-over from feed to foods of animal origin and identifies feed materials which could be considered as sources of exposure.

A correct evaluation of mycotoxin contamination in food and feed is of utmost importance in determining the compliance with the acceptable safety standards. Because of the often highly heterogeneous distribution of mycotoxins in so-called 'hot-spots' in the food, the most critical stage is taking a representative sample. Therefore appropriate sample plans are essential to ensure that the analytically derived mean concentration of a sample is representative to the true mean concentration of a lot (Whitaker *et al.*, 2005; Milićević *et al.*, 2010; Chaytor *et al.*, 2011).

Mycotoxin contamination of food and feed chain has a major economic impact. In addition to crop losses and reduced animal productivity, costs are derived from the efforts made by producers and distributors to counteract their initial losses, the costs of improved technologies for production, storage and transport, analytical testing and sampling plans, especially as detection and regulations become more and more stringent (Whitaker *et al.*, 2005).

Fusarium species are the most prevalent mycotoxin producing fungi in Europe due to their preference for the weather conditions existing in this region. Infection of crops by these fungi can lead to *Fusarium* head blight (FHB) in small grain cereals and although this results in reduced yield, the major concern referred to FHB is the presence of mycotoxins. Trichothecenes, zearalenone, fumonisins and moniliformin are the most important mycotoxins produced by *Fusarium* fungi. Among the trichothecenes, deoxynivalenol (DON), which is mainly produced by *F. graminearum*, is the predominant mycotoxin (McMullen *et al.*, 1997). It is generally accepted that rainfall just before and during flowering of the crop, which is situated around June, favours FHB pathogens. Recently, it was demonstrated that the weather conditions during winter might also influence the survival of primary inoculums in soil, weeds and crop residues (Landschoot *et al.*, 2011a,b). As *Fusarium* mycotoxins are produced within the growing crop, agricultural practices such as maize as previous crop, minimum tillage after maize and use of a moderate sensitive or susceptible wheat variety, can affect the mycotoxin contamination of the crop (Obst *et al*., 2000; Edwards, 2004). Also climate changes might contribute to the *Fusarium* incidence and coinciding DON contamination in cereal grains (Paterson and Lima, 2010; Pestka, 2010). Although the implementation of good agricultural practices helps to reduce the risk for *Fusarium* epidemics, the application of fungicides remains the most important control measure to reduce *Fusarium* symptoms. Where the effect of fungicides on fungal outgrowth is quite straightforward, several reports suggest that the application of some fungicides not automatically results in a corresponding reduction in mycotoxin contamination (Edward, 2004; Medina *et al*., 2007; Zhang *et al.*, 2009a,b; Audenaert *et al*., 2011). Of particular interest is the fact that exposure of *Fusarium* to sub lethal concentrations of fungicides might stimulate mycotoxin production. Audenaert *et al.* (2010) demonstrated using an *in vitro* approach that exposing *F. graminearum* to sub lethal doses of prothioconazole resulted in proliferated production of DON by inducing an oxidative stress in the fungus. At long-term, the adaptation ability of fungi to stress, can lead to mutations or adaptations that can give rise to more aggressive crop pathogens (Audenaert *et al*., 2011).

A large-scale collaborative study by the Scientific Corporation on Questions relating to Food (SCOOP) assessed the frequency with which predominantly cereal-based foods (44,670 food items) from European Union members states were contaminated by *Fusarium* mycotoxins. Eleven countries provided data on the levels of DON on a subtotal of 11,022 food samples. Overall, DON was the most frequently observed mycotoxin, and 57% of all samples tested were contaminated. Moreover, the report suggested that for some individuals, particularly children, exposure may exceed the maximum tolerable daily intake (TDI) of 1 µg/kg bodyweight/day (European Commission, 2003; Schothorst and van Egmond, 2004). The maximum levels for DON set down by the European Commission for cereals and cereal based products, vary from 1.75 mg/kg for unprocessed wheat to less than 0.2 mg/kg in processed cereals based foods for infants and young children (European Commission, 2007).

The importance of DON in feed matrices was recently illustrated by the analysis of 82 samples of 3 different feed matrices in Europe (sow feed, wheat and maize). This resulted in the detection of 67 contaminated samples of which 52 contained DON in concentrations varying from 74 to 9528 µg/kg (Monbaliu *et al.*, 2010). In swine production, cereal grains account for 55 − 70% of the total feed mixture as they represent the main source of energy (NRC, 1998). With the pig being the most sensitive species to the effects of DON and *Fusarium* mycotoxins in general, contamination of swine feed can seriously affect the health and economic stability of swine production. The guidance value for DON in complementary and complete feeding stuffs for pigs was set at 0.9 mg/kg, as described in the Commission Recommendation of 17 August 2006 (2006/576/EG) (Table 1; European Commission, 2006).

Table 1. The guidance values on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding, as determined in the Commission Recommendation of 17 August 2006 (2006/576/EG).

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 cereals 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 A+B	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	50

(*)Particular attention has to be paid to cereals and cereal products fed directly to the animals that their use in a daily ration should not lead to the animal being exposed to a higher level of these mycotoxins than the corresponding levels of exposure where only the complete feedingstuffs are used in a daily ration.
(**)The term 'cereals and cereal products' includes not only the feed materials listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in part B of the Annex to Council Directive 96/25/EC of 29 April 1996 on the circulation and use of feed materials (OJ L 125, 23.5.1996, p.35) but also other feed materials derived from cereals in particular cereal forages and roughages.
(***)The term 'Maize and maize-byproducts' 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 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.

2. **The mycotoxin deoxynivalenol**

2.1.   Chemical structure

Trichothecene mycotoxins are a large group of structurally related sesquiterpenoid metabolites. All trichothecenes have a 9,10 double bond and a 12,13-epoxy group in common, the latter related to their toxicity. Depending on their functional groups, trichothecenes have been classified into A, B, C and D groups (Figure 2). Members of group A do not contain carbonyl on C-8 (e.g. T-2 toxin). Group B differs from group A by the presence of a carbonyl group on C-8 (e.g. DON). Group C members have another epoxy group between the C-7 and C-8 or C-8 and C-9 positions, respectively (e.g. crotocin). Compounds in group D include a macrocyclic ring between C-4 and C-15 (e.g. satratoxin) (Wu *et al.*, 2010).

Group A: T-2 toxin

Group B: Deoxynivalenol

Group C: Crotocin

Group D: Satratoxin G
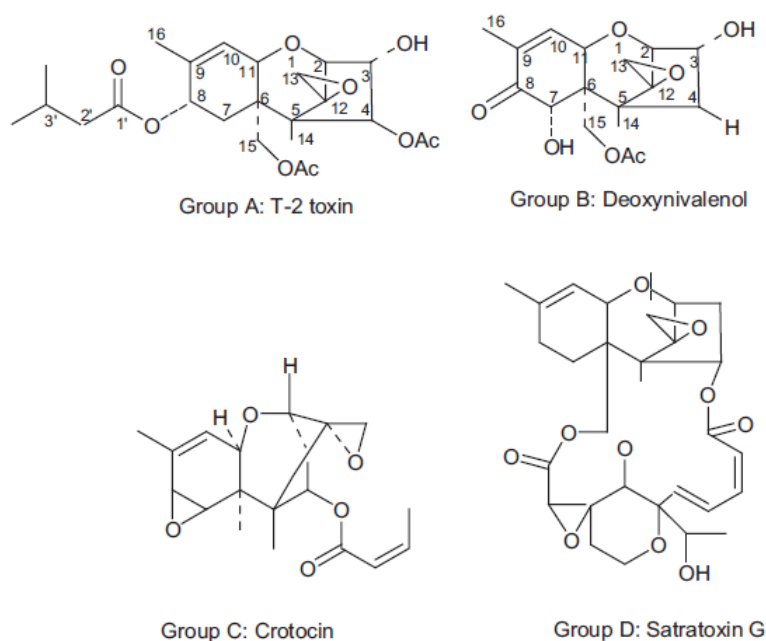
Figure 2: Chemical structures of trichothecenes with examples from groups A to D (Wu *et al*, 2010).

DON is very stable at temperatures up to 350 °C. Due to this stability during processing and cooking, DON can easily enter the food and feed chain with potential to cause human and animal toxicity (Pestka and Smolinski, 2005; Bullerman and Bianchini, 2007).

2.2.   Mechanisms of toxicity
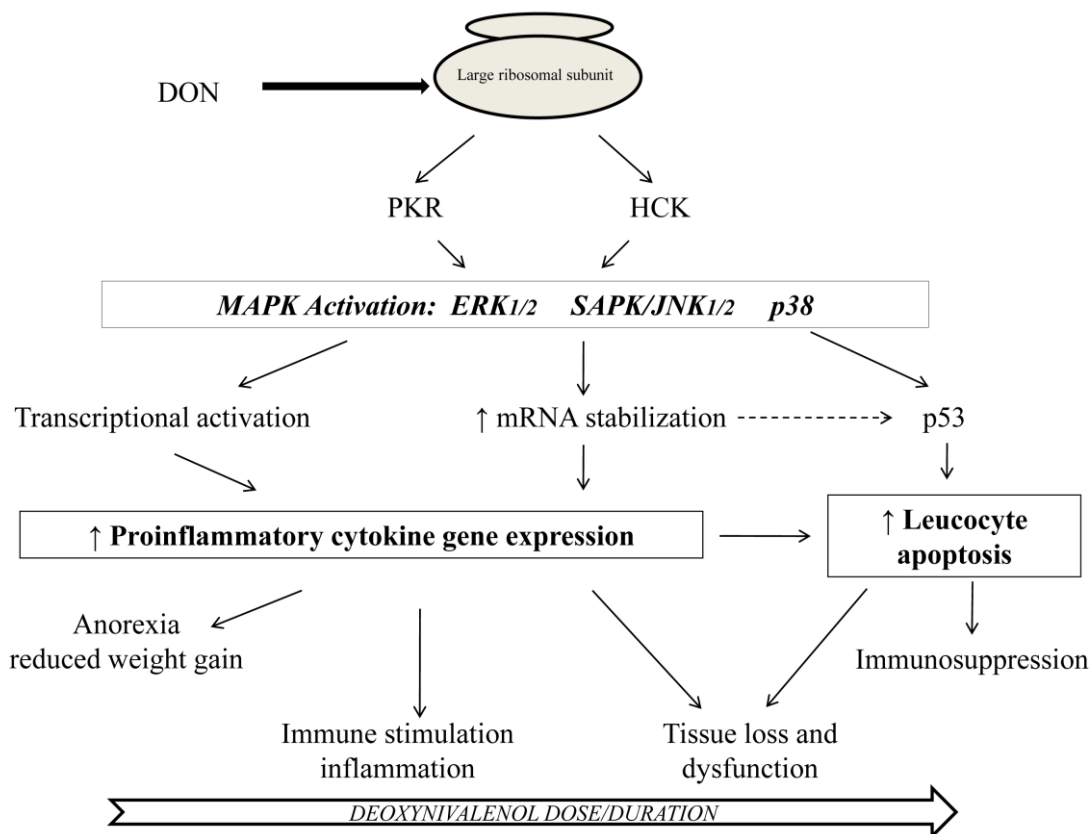
2.2.1.   *Molecular mode of action*

Trichothecenes to which DON belongs, have multiple inhibitory effects on eukaryote cells including inhibition of protein synthesis, DNA and RNA synthesis, inhibition of mitochondrial function, effects on cell division and membrane effects (Rocha *et al.*, 2005).

The molecular target of trichothecenes in leucocytes and actively dividing cells is the 60s ribosomal subunit. After diffusion through the cell membrane, trichothecenes are able to bind eukaryotic ribosomes and interfere with peptidyltransferase function with consequent impairment of initiation and elongation and inhibition of protein synthesis. Apart from this ribosome binding, other possible mechanisms involved in the translation inhibition by DON are suggested, with the ability of DON to promote degradation of 28S rRNA being the most studied one. The disruption of the function of the 3'-end of the 28S rRNA by DON is followed by an inhibition of the protein synthesis since this region of the ribosome is functional in aminoacyl-tRNA binding, peptidyltransferase activity and ribosomal translocation (Ueno, 1985, 1988; Uptain *et al.*, 1997).

The capacity of specific ribosome-directed stressors to damage 28S rRNA by interfering with its functioning during gene translation, can lead to what has been called 'ribotoxic stress response'. These ribosome-directed stressors cover ribosome-inactivating proteins including ricin, saporin from plants and shigatoxin from bacteria but also non-protein ribotoxic stressors including ultraviolet radiation, trichothecene mycotoxins and anisomycin antibiotics (Iordanov *et al.*, 1997; Laskin *et al.*, 2002; Moon, 2012). Most characteristic biochemical patterns of cellular responses to ribotoxic stress are the activation of mitogen-activated protein kinases (MAPKs) and their downstream targets. Activation by DON of the MAPKs extracellular signal-regulated kinases (ERK1/2), stress-activated protein kinase/c-Jun N-terminal kinase 1 and 2 (SAPK/JNK1/2) and p38 results in the subsequent activation of downstream transcription factors such as nuclear factor kappa B (NF-κB), Activating Protein (AP)-1, CCAAT-enhancer-binding protein (C/EBP), cyclic AMP Response Element Binding protein (CREB) and Early Growth Response 1 (EGR1) gene product, all of which are important in driving critical cellular processes such as cell proliferation and differentiation, inflammation and apoptosis but also for transcriptional activation of pro-inflammatory mediator expression (Iordanov *et al.*, 1997; Shifrin and Anderson, 1999; Chang and Karin,

2001; Zhou *et al.*, 2003a; Pestka, 2005; Bae and Pestka, 2008; Van De Walle *et al.*, 2010a; Moon, 2012).

A schematic summary of the molecular mode of action of DON is illustrated in Figure 3.

Figure 3: Molecular mode of action of deoxynivalenol (DON). After entry into the cell, DON binds to the large ribosomal subunit transducing a signal to PKR and HCK. As a result of this ribotoxic stress response, MAPKs, including ERK1/2, SAPK/JNK1/2 and p38, pathways are initiated. This MAPKs activation leads, depending on the DON dose, to an increased pro-inflammatory cytokine gene expression or leucocyte apoptosis resulting in anorexia and reduced weight gain, immune stimulation and inflammation, tissue loss and dysfunction, and immunosuppression, respectively (adapted from Riley and Pestka, 2005).

It was shown that trichothecenes are able to induce the phosphorylation of two putative kinases identified as upstream transducers of the MAPK activation, namely RNA-activated protein kinase (PKR) and hematopoietic cell kinase (HCK) (Pestka *et al.*, 2004). Both play a potential role in the early steps in the ribotoxic stress response as they associate constitutively with ribosomes and possibly sense the damage induced by DON to rRNA (Zhou *et al.*, 2003b; Pestka *et al.*, 2004; Pestka, 2010).

2.2.2. *Neuropharmacological effects*

The reduction of the feed consumption (anorexia) at low concentrations and the induction of vomiting (emesis) at higher acute doses are two biological activities associated with the intake of DON, especially in pigs. An altered neuroendocrine signaling within the nervous system at the central and enteric level appears to be involved in these effects. The serotoninergic system has been demonstrated to play a role in both the anorectic response and the emetic response. Intragastric dosing of pigs with DON led to a significant and prolonged increase in cerebral fluid concentration of the major metabolite of serotonin, indicating an enhanced turnover of this neurotransmitter, which is known to be implicated in neural processes underlying feeding behavior at central level. The possible link between DON-induced emesis and the serotoninergic mechanism was proven as pretreatment with selective serotonin receptor blockers indeed blocked the DON-induced vomiting in pigs (Prelusky and Trenholm, 1993). Apart from the direct effect of DON on the central nervous system, it was speculated that a part of DON's mechanism of action is mediated through the action on the peripheral serotonin receptors found in the gastrointestinal tract (Fioramonti *et al.*, 1993; Prelusky, 1993). Indeed much of the body's serotonin is produced and released by enterochromaffin cells in the gut where it acts in a paracrine way on the terminals of vagal efferent neurons of the enteric nervous system (Li, 2007). Recently it was described using a mouse model that in addition to a peripheral action, after *per os* administration DON can reach the brain and act centrally resulting in impairment of anorexigenic balance by interfering with central neuronal networks involved in food regulation (Girardet *et al.*, 2011b). In addition, the upregulation of IL-1β, IL-6 and TNFα mRNA within central structures involved in food intake control, seen after *per os* administration of DON might explain the DON-induced anorexia (Girardet *et al.*, 2011a). The DON-induced conditioned taste aversion to other constituents present in feed was studied in rats and it was found that this was mediated by the area postrema, a circumventricular organ on the fourth ventricle of the brain which has been functionally associated in a range of physiological and behavioral processes (Borison, 1989; Ossenkopp *et al.*, 1994). Using crystalline DON, complete feed refusal was seen at levels of 12 mg DON per kg feed and vomiting at 20 mg/kg feed (Forsyth *et al.*, 1977; Young *et al.*, 1983). However, several studies indicate that naturally infected feed led to more pronounced effects on feed intake and weight gain than pure toxin. A possible explanation could be the presence of other toxins or other compounds in the material affecting the toxicity of trichothecenes and inducing taste aversion (Rotter *et al.*, 1996).

2.3.   Effects at the gastrointestinal level

The gastrointestinal epithelium functions as a dynamic barrier regulating on the one hand the uptake of nutrients and water but on the other hand preventing the entry of luminal antigens into the underlying tissues (Oswald, 2006). DON usually enters the body through the ingestion of contaminated food or feed and therefore intestinal epithelial cells can be exposed to high concentrations of the toxin (Maresca *et al.*, 2002; Bouhet and Oswald, 2005; Sergent *et al.*, 2006). Since DON is known to be an effective protein synthesis inhibitor, the constant renewing intestinal epithelium may be particularly sensitive to the toxic effects of DON (Instanes *et al.*, 2004).
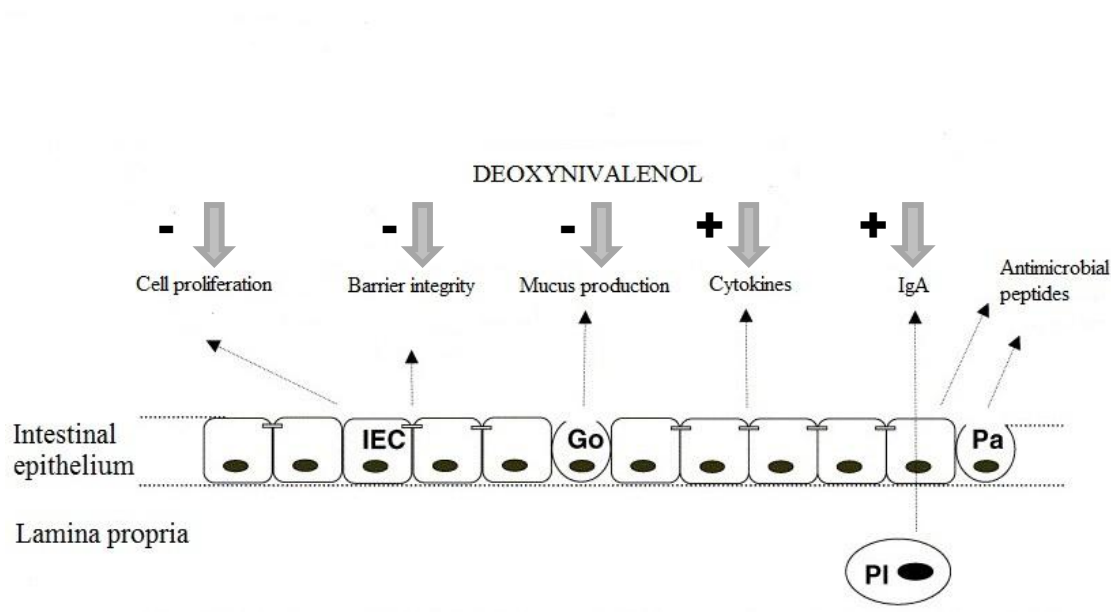
2.3.1.   *Effects on the intestinal epithelial cell-derived innate defence mechanisms*

Intestinal epithelial cells are known to participate in the intestinal innate immune system by different mechanisms, which can be classified as intrinsic or extrinsic of nature. A continuous monolayer of intestinal epithelial cells firmly joined together by intercellular structures including tight junctions, adherence junctions and desmosomes at the apical region of cells accounts for the intrinsic component of the intestinal immunity (Gumbiner, 1993). In addition to this physical barrier, intestinal epithelial cells have developed a number of extrinsic defense mechanisms including mucus secretion, defensins and antibacterial peptide synthesis and secretion of cytokines and chemokines. Each of them is of great importance in reducing the risks of exposure of intestinal epithelial cells to both invasive pathogens and damaging toxic compounds (Oswald, 2006). The diverse effects of DON on intestinal epithelial cells have been studied using both *in vitro* diverse intestinal epithelial cell lines as *ex vivo* and *in vivo* experimental setups.

Enterocytes may be exposed to DON either from the luminal or from the basolateral side. The luminal exposure especially accounts for the duodenum and the first parts of the jejunum but when absorbed, DON can reach the enterocytes in the more distal regions via the systemic circulation (Danicke *et al*., 2010). In contrast with *in vitro* cell culture experiments, where the cells are exposed continuously to DON, enterocytes *in vivo* appear thus not to be exposed to DON in a continuous manner but rather to time-dependent fluctuations as the absorption of DON is nearly completed 3 to 4 hours after the consumption of a DON containing meal (Danicke *et al.*, 2004; Goyarts *et al.*, 2006). It is thus clear that the effects of DON *in vitro* might not completely reflect the situation *in vivo* because of the much larger number of

processes interacting in processing the ingesta (Danicke *et al.*, 2010; Maresca and Fantini, 2011).

Figure 4 gives an overview of the effects of DON on the local defence mechanisms of intestinal epithelial cells. Deregulation of this immune homeostasis of the gut may contribute to the development of intestinal inflammation.

Figure 4: The effects of DON on the local defence mechanisms of intestinal epithelial cells. IEC : intestinal epithelial cells, PI: plasmocyte secreting immunoglobulin A (= IgA), Go: goblet cell; Pa: paneth cell (adapted from Bouhet and Oswald, 2005).

Cell viability, proliferation and intestinal morphology

Considering the effects of DON on intestinal cell proliferation and intestinal morphology, alteration of the cell differentiation with structural and functional characteristics after exposure to low concentrations of DON was shown in the human colonic adenocarcinoma cell lines Caco-2 and T84 (Kasuga *et al.*, 1998). Using intestinal porcine epithelial cells (IPEC)-1 and IPEC-J2 cell culture models, the toxic effects of DON appeared to be time- and dose-dependent, with high concentrations DON inducing toxic effects compromising the intestinal barrier integrity whereas low concentrations modulated the cellular regulation (Diesing *et al.*, 2011). Dose-dependent alterations in the histology of pig jejunal explants were observed after exposure to DON, including shortening and coalescence of villi, lysis of enterocytes and edema (Kolf-Clauw *et al.*, 2009).

Cell homeostasis and viability can also be severely compromised by oxidative damage. Although DON treatment inhibited cell proliferation in human colon carcinoma cells, the toxin did not induce heat shock protein expression nor oxidative damage (Bensassi *et al.*, 2009). On the other hand, a genotoxic potential for DON at low non-cytotoxic concentrations was demonstrated in human cells lines and this was caused by direct DNA fragmentation leading to p53 and caspase-dependent apoptosis (Bony *et al.*, 2006; Bensassi *et al.*, 2009).

Mucus production

Goblet cells present in the crypt and villus of the intestinal epithelium form a continuous mucus layer lining the apical side of intestinal epithelial cells. This mucus, which consists of mucins associated with proteins and lipids, protects the epithelium not only against the adhesion and invasion by pathogens but also to physical and chemical attacks (Montagne *et al.*, 2004). A reduction of the number of mucus-producing goblet cells and of the tightness of the intestinal glycocalyx was seen in pigs exposed to low concentrations of mixtures of DON, T-2 toxin and zearalenone (Obremski *et al.*, 2008).

Production of antimicrobial substances

Another extrinsic mechanism of mucosal defence occurs by the secretion of agents exhibiting antimicrobial properties by paneth cells and intestinal epithelial cells (Pitman and Blumberg, 2000). Most of these antimicrobial molecules are cationic and act by disrupting the integrity of the microbial membranes (Bouhet and Oswald, 2005). They include bactericidal peptides or proteins such as defensins, cathelicidins, angiogenins as well as the antimicrobial enzyme lysozyme and phospholipase A2 (Müller *et al*., 2005). There are no studies available describing the effect of mycotoxins on the secretion of intestinal antimicrobial peptides.

The secretory immunoglobulin (Ig) pathway represents a further form of extrinsic resistance to foreign pathogens present within the lumen. IgA is secreted from mucosal plasma cells present in the sub-epithelial space across mucous membranes of the intestinal, respiratory, genital and biliary tract. Following its release into the lumen, secreted IgA can participate in immune exclusion by inhibiting bacteria to associate with the epithelial surface (Pitman and Blumberg, 2000; Bouhet and Oswald, 2005). It is known that trichothecenes can modulate the serum concentration of IgA (Pestka, 2003), but their effect on intestinal secreted IgA has not been investigated yet.

Barrier function

An important aspect of the toxic effects of DON on the enterocyte border is the toxin's ability to disrupt the epithelial barrier integrity, resulting in an enhanced permeability of the cell layer with potential intestinal disorders as a consequence. The determination of the transepithelial electrical resistance (TEER) in *in vitro* cell monolayers serves as a good indication of epithelial integrity and of the degree of organization of the tight junctions (Hashimoto and Shimizu, 1993).

Several studies using human cell monolayers indicate that long term treatment with cytotoxic doses of DON compromises tight junctions which is indicated by a drop in TEER and an increase in the passage of paracellular tracers such as FITC-dextran (Kasuga *et al*., 1998; Maresca *et al*., 2002, 2008; Sergent *et al.*, 2006; Pinton *et al.*, 2009, 2010). Consequently this increased intestinal permeability caused by high doses of DON (up to 100 µM) may allow the transepithelial passage of both non-invasive commensal bacteria (Maresca *et al.*, 2008) as of pathogenic *Escherichia coli*, as was demonstrated using IPEC-1 cell monolayers (Pinton *et al.*, 2009). Apart from the DON induced cytotoxic effect and the inhibition of the protein synthesis described in human enterocytes (Kouadio *et al.*, 2005, 2007; Van De Walle *et al.*, 2010b), these alterations in the barrier function may coincide with a specific reduction in the expression of claudins which was demonstrated *in vitro* using IPEC-1 and Caco-2 cells and confirmed *in vivo* using jejunal explants of pigs exposed to DON contaminated feed (Pinton *et al.*, 2009, 2010). It is described that the structure and function of tight junctions such as claudin can be regulated by signaling molecules involved in MAPKs cascades (Matter and Balda, 2003). Since DON is known to activate these MAPKs pathways (Pestka *et al.*, 2004), exposure to the toxin may explain the decreased expression of tight junction proteins with subsequent reduction of the barrier function. However, Maresca *et al.* (2008) also showed a significant increase in bacterial passage through Caco-2 cells exposed to lower concentrations of DON (10 µM) that did not compromise the barrier function. In this context, it could be speculated that, exposure to apparently non-cytotoxic concentrations of DON could render the intestinal epithelium more vulnerable for enteropathogenic bacteria, including invasive pathogens such as *Salmonella* Typhimurium.

Secretion of cytokines and chemokines

As reviewed by Oswald (2006) intestinal epithelial cells are able to secrete cytokines and to respond to exogenous chemokines which are crucial elements for the recruitment and the activation of immune cells.

In response to mucosal ribotoxic stress, as encountered after intake of DON contaminated food or feed, the intestinal epithelium transmits the danger signals to the underlying lymphocytes and antigen-presenting cells by releasing pro-inflammatory chemokines such as IL-8. Induction of epithelial pro-inflammatory cytokines by trichothecenes appears to be independent on NF-κB signaling pathway in the intestinal epithelia. Indeed, promotion of IL-8 production and its positive modulator early growth response gene 1 (EGR1) was seen in human epithelial intestinal 407 cells after treatment with DON, indicating that EGR1 is involved in the pro-inflammatory IL-8 gene expression in the DON treated intestinal epithelial cells (Moon *et al.*, 2007). EGR1-mediated IL-8 production could also be addressed in association with the transcription factors NF-κB, AP-1 and C/EBP since these partially contribute to ribotoxin-induced cytokine transcriptional activation (Moon, 2012). In Caco-2 cells a dose-dependent increase in IL-8 production and prostaglandin E2 (PGE-2) synthesizing capacity was described (Van De Walle *et al.*, 2008, 2010a), and this was linked to the activation of ERK1/2 (Moon *et al*., 2007) and PKR, p38 and NF-κB inflammatory cascades (Maresca *et al.*, 2008).

IL-8 is a pro-inflammatory chemokine that regulates the attraction and trafficking of neutrophils during the acute phase of inflammation. Prostaglandins such as PGE-2 regulate vascular dilatation, mucosal secretion and generate fever and are thus important mediators in inflammatory responses (Borish and Steinke, 2003). The NF-κB transcription factor activates the transcription of many inflammatory genes including cytokines and chemokines such as TNFα and interleukins (Ahn *et al*., 2007; Magalhaes *et al.*, 2007).

In addition to its direct effect on IL-8 secretion, DON is also able to potentiate the action of other pro-inflammatory stimuli as described for IL-1β in human enterocytes (Maresca *et al*., 2008) and lipopolysaccharide (LPS) in the mouse (Islam and Pestka, 2006).

The ability of DON and mycotoxins in general to induce intestinal inflammation can thus be either indirect through alterations of the intestinal epithelial barrier and the crossing of luminal antigens, or direct by the secretion of pro-inflammatory cytokines by the intestinal epithelium (Maresca *et al.*, 2008).

2.3.2.   *Effects on epithelial transport systems*

After exposure to mucosal ribotoxic xenobiotics, such as trichothecenes, gut epithelia employ defensive transporters to excrete the toxins. Indeed, efflux transporters play a key role in disposition and excretion of many substances, including dietary contaminants such as

mycotoxins. The known efflux transporters in the intestine mostly belong to the ATP-binding cassette (ABC) transporter superfamily which includes the multidrug resistance protein-1 (MDR1) or P-glycoprotein (P-gp), multidrug resistance-associated proteins (MRPs) and the breast cancer resistance protein (BRCP). Intestinal epithelial cells can use ABC transporters such as P-gp and MRP2 to export ribotoxic trichothecenes (Tep *et al.*, 2007; Videmann *et al.*, 2007). Conflicting reports have been published on the role of ABC transporters in DON transfer. Sergent *et al.* (2006) suggested that DON crossed the intestinal mucosa in a paracellular way which was not affected by P-gp nor MRP inhibitors. On the other hand, Videmann *et al.* (2007) demonstrated that DON represented a substrate for both P-gp and MRP2, leading to a transcellular transport through the intestinal epithelial barrier.

Moreover, ribotoxic xenobiotics also modulate nutrient transporting by interfering with various nutrient transporter systems which might account for the malnutrition and weight loss in exposed animals. The effect of DON on the uptake of nutrients was studied in the human epithelial intestinal cell line HT-29-D4 and indicated that DON selectively affected the activities of the intestinal transporters including the D-glucose/D-galactose sodium-dependent transporter (SGLT1), the passive D-fructose transporter GLUT5 and the active and passive L-serine transporter and that this may be due to the ability of DON to induce apoptosis through protein synthesis inhibition, as was shown by mimicking these effects using cycloheximide (Maresca *et al.*, 2002). Studies in broilers fed low to moderate dietary concentrations of DON indicated a specific inhibitory effect of the toxin on glucose transport in addition to a concurrent decrease of intestinal surface area with shortening and thickening of villi (Awad *et al.*, 2007, 2008b, 2011). It is rather surprising that poultry performance is not or only moderately affected by the intake of DON contaminated feed. As it is known that chickens are able to absorb D-glucose and amino acids even from the large intestine, it is possible that by the decrease of nutrient absorption in the proximal small intestine, a part of the uptake is transferred to the more distal parts, where the absorptive functions may be more protected against the deleterious effects of DON (reviewed by Awad *et al.*, 2008a).

2.3.3. *Effects on intestinal microflora*

An enormous number of micro-organisms are known to colonize the intestine and form complex communities or microbiota. The host-microbiota associations usually evolve into beneficial relationships, where bacteria present in the gastrointestinal tract supply key nutrients and prevent colonization by opportunistic pathogens. The stability of the intestinal

microflora is thus of great importance as an impaired balance could have many adverse effects on the health of the host.

Ribosome-inactivating stressors, such as trichothecene mycotoxins are generally known to be effective in blocking eukaryotic 28S rRNA and thus, theoretically do not interfere with bacterial protein translation. Although many *in vitro* screening and microbial analyses have identified intestinal bacterial strains that contribute to trichothecene detoxification (He *et al.*, 1992; Eriksen *et al.*, 2002; Niderkorn *et al.*, 2006; Young *et al.*, 2007), only a few studies have looked at the action of ribotoxic stress on the gut bacterial population and its activity. In pigs and rats, it was shown that administration of T-2 toxin for one week caused a substantial increase in the aerobic intestinal bacteria count (Tenk *et al.*, 1982). A recent investigation using DON demonstrated a slight but statistically significant increase in the aerobic mesophilic bacteria count in DON-exposed piglet colon. Although there was no effect of DON on microbial diversity, the richness index, which was based on the microbial numbers, was significantly increased by DON exposure (Wache *et al.*, 2009b). The mechanisms by which T-2 toxin, DON and other ribotoxic stressors affect the gut bacterial composition are however still unclear.

2.4. Effects on immune system

Numerous studies, both *in vitro* and *in vivo* have determined that the immune system is extremely sensitive to trichothecenes and for DON this effect can be either immunostimulatory or immunosuppressive, depending on the dose of DON, the exposure frequency, and the type of the functional immune assay (Pestka and Smolinski, 2005). Inflammation is a rapidly occurring non-specific response which leads to the activation of macrophages and neutrophils and subsequent secretion of cytokines, arachidonic acid metabolites and active oxygen and nitrogen compounds by these activated phagocytes.

Macrophages play an essential role in both the innate and acquired immune response. The effect of DON on macrophages has been described in several *in vitro* and *in vivo* studies. Exposure of macrophages to low doses of DON may lead to an increase in the production and secretion of inflammatory cytokines (Wong *et al.*, 1998; Doll *et al.*, 2009), whereas high doses of DON induce macrophage apoptosis (Zhou *et al.*, 2005). DON also inhibits IFNγ mediated human macrophage activation and this could contribute to a decrease of host resistance to several pathogens after exposure to DON (Wache *et al.*, 2009a).

The immune stimulating effect of low doses of DON relates to the ability of the mycotoxin to induce gene upregulation (Pestka, 2008) which can be due to either an increased mRNA stability (Wong *et al.*, 2001) or an increased transcription factor activity linked to phosphorylation of MAPKs (Zhou *et al.*, 2003a; Pestka *et al.*, 2004). *In vitro* and *in vivo* experiments on murine immune cells exposed to DON showed an activation of critical transcription factors including e.g. NF-κB and activator protein-1 (AP-1) (Ouyang *et al.*, 1996; Li *et al.*, 2000; Wong *et al.*, 2002) and upregulation of their downstream targets such as cyclooxygenase-2 (COX-2) (Moon *et al.*, 2003), IL-1β, IL-2, IL-4, IL-5, IL-6 (Dong *et al.*, 1994; Azcona-Olivera *et al.*, 1995; Wong *et al.*, 1998) and TNFα (Wong *et al.*, 1998; Chung *et al.*, 2003). Exposure of human lymphocytes to DON also led to an increased expression of IL-2, IL-4, IL-6, IL-8 and TNFα (Meky *et al.*, 2001; Sugita-Konishi and Pestka, 2001). In pigs, after an acute intravenous exposure to DON, a temporary recruitment of neutrophils in the peripheral blood by IL-8 and subsequent activation of the bactericidal function was seen, together with the transient increase in pro-inflammatory cytokines and acute-phase proteins (Mikami *et al.*, 2011).

The effect of DON on the antibody synthesis is manifested by an elevation in serum immunoglobulin (Ig) A and concurrent depression of IgM and IgG. Concomitantly with the increase in serum IgA, an elevation in IgA immune complex and polymeric IgA appears and this may be mediated by T-lymphocytes and macrophages especially through the superinduction of cytokine genes such as IL-2, IL-5 and IL-6 (Pestka, 2003; Oswald *et al.*, 2005). In mice this DON-induced immunopathology closely mimics the human glomeronephritis IgA nephropathy (Pestka, 2003).

High doses of DON promote the rapid onset of leucocyte apoptosis with concomitant immune suppression. Using macrophage and monocyte models it was shown, that in analogy with the gene upregulation, DON-mediated apoptosis correlates with activation of the MAPKs families (Yang *et al.*, 2000).

The consequences of this DON-induced suppressed immune function may involve decreased resistance to infectious diseases, possible reactivation of chronic infections and decreased vaccine and drug efficacy (reviewed by Oswald *et al.*, 2005). It was shown using a pig model that chronic consumption of DON contaminated feed impaired the immune response during a vaccination protocol as demonstrated by the alteration of the antibody response, the lymphocyte proliferation and the cytokine expression in the lymph nodes (Pinton *et al.*, 2008).

In mice acute DON exposure impaired the immune response to respiratory reovirus infection by suppression of IFN-mediated responses, upregulation of pro-inflammatory cytokine and chemokines expression and elevated IgA responses in the lung (Li *et al.*, 2006).

2.5. Implications for pig's health

In farm animals including poultry and ruminants, intoxication following the consumption of cereals and cereal-derived products contaminated with DON results in feed refusal and reduced weight gain, leading to growth retardation with great economic consequences (Girardet *et al.*, 2011b).

Numerous toxicological studies have been performed in diverse animal species in order to elucidate the toxin's mode of action and potential hazardous effects. Differences in absorption, distribution, metabolism and excretion of DON among the animal species may account for the species sensitivity to toxin's effects (Trenholm *et al.*, 1984). Among all animal species evaluated, the pig is shown to be the most susceptible species to the effects of DON, following the ranking order pigs > mice > rats > ruminants ≈ poultry (Rotter *et al.*, 1996).

2.5.1. *Toxicokinetics of DON in pigs*

In pigs, the absorption of DON after oral administration is rapid with an oral bioavailability around 80% and reaching peak plasma concentration within 15 − 30 minutes after ingestion (Prelusky *et al.*, 1988). Although the toxin is extensively absorbed only a small proportion of the dose can be found in the blood. Depending on the studies the plasma elimination half-life was found to vary between 1.2 and 3.9 hours (Prelusky *et al.*, 1991; European Commission, 2004).

In a study by Prelusky *et al.* (1988) ^{14}C-labeled DON was given intragastrically to pigs at a dose of 0.6 mg/kg bodyweight or intravenously at a dose of 0.3 mg/kg of bodyweight and blood, urine, bile and feces were collected over 24 hours. Urine is the major excretory route for both intravenously and orally applied DON. About 95% of the administered dose was recovered as unchanged DON, and no glucuronide conjugates of DON were found, although a glucuronide-conjugate metabolite was found with the treatment of urine and bile samples with β-glucuronidase. Two and a half percent of the dose was found to be excreted by the bile, 20% through the feces and 68% with urine. An efficient urinary excretion indicates indeed a

high gastro-intestinal absorption while a high fecal elimination might be due to an efficient biliary excretion or a lack of systemic absorption (Goyarts and Danicke, 2006).

The main metabolites of DON in pigs are de-epoxy-DON (DOM-1) and glucuronide-conjugated DON (Figure 6).
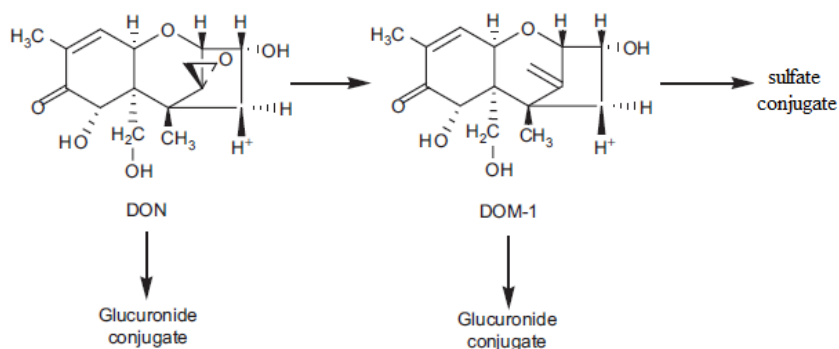
Figure 6. Metabolism of DON in animals (adapted from Wu *et al.*, 2010).

Prelusky *et al.* (1988) concluded from the small increase in the DON level seen after incubation with β-glucuronidase that only small amounts of the toxin are present as the glucuronide conjugate metabolite in pigs. Conjugation with activated glucuronic acid proceeds in the endoplasmatic reticulum, particularly in the liver as a reaction to enhance the water solubility and to facilitate the excretion with the urine or bile. However, there appeared to be a difference between the route of administration, as following oral administration, 9.1 – 60% of total DON was found to be conjugated in the serum whereas after intravenous administration no DON glucuronide conjugates were present (Goyarts and Danicke, 2006). As phase II enzymes accounting for the conjugation are also widespread in extrahepatic tissues e.g. gastrointestinal tract, it could be hypothesized that DON is conjugated in the intestine before absorption and that glucuronide-conjugated DON is not as toxic as free DON possibly because it does not fit into the activation centre of the 60S ribosomal subunit and is eliminated faster with the urine (Goyarts and Danicke, 2006).

DON can be metabolized to the non-toxic DOM-1 in the gut, especially in the caudal segments. This was illustrated by anaerobic incubation of DON with suspensions of intestinal contents (duodenum, jejunum, cecum, colon, rectum) from porcine origin for 24 hours. The caudal segments and the colon content in particular showed stronger metabolic activity whereas the microorganisms of the cranial segments exhibited no transforming activity

(Kollarczik *et al.*, 1994). These findings were confirmed *in vivo* in pigs that were fed a diet containing 4.2 mg/kg DON over a period of 7 days (Danicke *et al.*, 2004). No DOM-1 was detected in serum and only traces in the stomach and small intestine, whereas notable amounts of the metabolite were found in the distal segments of the gut. It was suggested that this de-epoxidation probably does not contribute much to the toxin's detoxification in pigs as it mainly occurs in the large intestine and the majority of the ingested DON is already absorbed in the proximal part of the small intestine (Danicke *et al*., 2004).

Residue analysis of DON in tissues from pigs fed DON concentrations ranging between 0.7 to 7.6 mg DON/kg feed revealed in most cases low (< 20 µg/kg) or undetectable DON concentrations (European Commission, 2004).

2.5.2. *Acute toxic effects*

Although DON is one of the less toxic trichothecenes compared to e.g. T-2 toxin, extremely high doses of DON can cause shock-like death. In sensitive animal species, symptoms of acute DON toxicity include abdominal distress, increased salivation, malaise, diarrhea, emesis and anorexia (Pestka, 2007).

A single dose of DON as little as 0.05 mg/kg bodyweight intraperitoneally and 0.1 – 0.2 mg/kg bodyweight orally was described to cause vomiting in pigs (Forsyth *et al*., 1977). Another study showed that emesis in pigs occurs within minutes after ingestion of feed containing 19.7 mg/kg, which correlates with the intake of 150 µg/kg bodyweight/day of DON. On the other hand, emesis was not detected when the pigs were fed 11.9 mg/kg suggesting that the no observed effect level (NOEL) for emesis in pigs was 120 µg/kg bodyweight/day based on average daily intake data (Young *et al.*, 1983). For this effect to occur via a contaminated feed, it is likely that the DON dose must be presented in a single meal rather than small amounts ingested throughout a day.

2.5.3. *Chronic toxic effects*

In most monogastric species, subchronic and chronic exposure to DON leads to growth and weight gain suppression.

Porcine experimental models showed that prolonged dietary exposure to DON results in decreased weight gain, anorexia and altered nutritional efficiency (Rotter *et al.*, 1996). It was reported that DON at 1 – 2 mg/kg caused partial feed refusal in pigs ingesting naturally

contaminated feedstuffs, whereas 12 mg/kg caused complete feed refusal (Forsyth *et al.*, 1977; Young *et al.*, 1983; Rotter *et al.*, 1994). A dose dependent decrease of weight gain in the first eight weeks was described when pigs were fed a diet containing 2 and 4 mg/kg of DON, with the highest concentration causing a decreased feed intake and feed utilization efficiency throughout the experiment (Bergsjo *et al.*, 1992). Similar effects on feed intake and weight gain were seen in castrated male pigs fed diets with 3 mg/kg of DON. However there appeared to be a difference between the intake of naturally contaminated feed ingredients versus feed spiked with purified DON which might be due to the presence of additional toxins and fungal components in naturally contaminated grains contributing additionally or synergistically to the effect of DON (Prelusky *et al.*, 1994).

When pigs were fed 3.5 mg/kg of DON, an increase in liver weight, decreased serum protein and albumin levels and temporary drop in serum calcium and phosphorus was seen, in addition to a significantly decreased bodyweight, decreased slaughter weight and reduced feed utilization efficiency (Bergsjo *et al.*, 1993).

Other clinical effects that were described when DON was incorporated in pig's diets are reduction in thyroid size, increased thyroxine levels and morphological changes in the esophageal region of the stomach (Rotter *et al.*, 1994). DON fed in combination with zearalenone, was reported to significantly reduce oocyte quality with possible implications for reproduction (Alm *et al.*, 2006). Changes in the histology of the liver (glycogen decrease, hemosiderin enhancement, interlobular collagen uptake, increased fatty and autophagic vacuoles) were also described although changes in serum transaminases levels, indicating liver damage, were not observed (Tiemann *et al.*, 2006).

2.6. Implications for men's health

The potential impact of DON on human health may occur after ingestion of contaminated foods from oats, barley, wheat, maize or other grains but also flour, bread, noodles, beer and malt may contain DON. Indeed, several epidemiological studies have reported acute illnesses including vomiting, abdominal pain, diarrhea, dizziness in populations who have consumed *Fusarium* contaminated grains. Outbreaks of human food poisoning associated with the ingestion of DON contaminated food such as scabby wheat, barley and moldy maize have been described in Japan, China and India (Bhat *et al.*, 1989; Pestka and Smolinski, 2005). As illustrated by the large scale results from the SCOOP report, the levels and patterns of human

food commodities contamination justify that DON consumption constitutes a major issue for global public health. Wheat and wheat containing products like bread and pasta represent the major source of intake for DON. Estimated daily intake of DON ranges from 0.3 to 2 µg/kg bodyweight/day in European Union countries (European Commission, 2003; Schothorst and van Egmond, 2004).

Given the capacity of DON to evoke toxicity in animals, accurate exposure assessment is necessary to understand the potential for adverse health effects in both farm animals and humans (Riley *et al.*, 2011). In this context, the development of adequate biomarkers is an emerging issue. Biomarkers are defined as measurable biochemical or molecular indicators of either exposure or biological response to a specific mycotoxin. Mechanism-based biomarkers often include changes in the level of specific proteins, cellular metabolites, or gene expression resulting from specific alterations in metabolic or signaling pathways. Exposure-based biomarkers, on the other hand, are most often the mycotoxin itself or the metabolized mycotoxin by-product such as glucuronide conjugates (Baldwin *et al.*, 2011; Riley *et al.*, 2011). Based on the observation that DON is conjugated with glucuronic acid in the liver, DON-glucuronide can be used as a biomarker of exposure in human populations (Meky *et al.*, 2003; Turner *et al.*, 2008). Urinary DON levels were also shown to be a sensitive exposure biomarker and a valuable tool for biomonitoring as part of surveillance strategies and in etiologic studies of DON and human disease risk. Moreover, there appeared to be a positive correlation between the urinary levels of DON and the cereal intake, particularly bread consumption (Turner *et al.*, 2008). Relative to these biomarkers of effect, other approaches in developing biomarkers for DON exposure were proposed, as illustrated in Figure 7. Although pro-inflammatory cytokines seem an obvious choice, they can be induced by many stressors and chemical agents, making them rather non-selective relative to DON. However, these pro-inflammatory cytokines are known to induce several suppressors of cytokine signaling (SOCS) of which some impair the growth hormone signaling, leading to growth retardation. Hepatic SOCS3 mRNA is suggested as a particular sensitive indicator for DON exposure although the need for an invasive procedure such as a liver biopsy might hamper the practical use. The hepatic upregulation for SOCS3 co-occurs with a downregulation of hepatic insulin-like growth factor acid labile subunit (IGFALS) and a reduction in circulating insulin-like growth factor 1 (IGF1) and IGFALS levels, suggesting the latter could serve as biomarkers of effect in populations exposed to DON (Amuzie *et al.*, 2010; Pestka, 2010; Riley *et al.*, 2011).
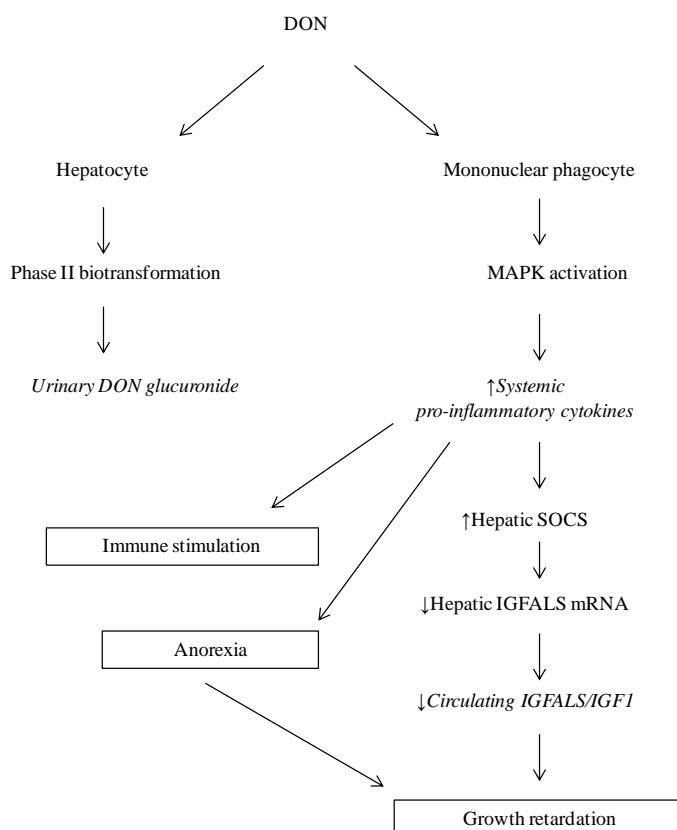
Figure 7: Metabolism and aspects of the mode of action of DON and potential biomarkers of exposure and effect (adapted from Amuzie *et al*., 2010; Riley *et al*., 2011).

Interestingly, there appears to be mechanistic evidence that ribotoxic stress-induced cellular response are implicated as critical etiological factors of mucosa-associated diseases, in particular epithelial inflammatory diseases (Moon, 2011, 2012). The term "chronic intestinal inflammatory diseases" covers many human diseases characterized by a dysregulated immune response in the gut of genetically predisposed patients. Celiac disease and inflammatory bowel diseases such as ulcerative colitis and Crohn's disease are the best known. Although apart from a genetic predisposition, the definite causes of the diseases are still unclear, there are three widely accepted etiological hypotheses in terms of the epithelial pathogenesis: 1) chronic persistent microbial activation and imbalance of microbial diversity and dominance; 2) disruption of mucosal barrier including epithelial junction and mucosal defence; 3) abnormal immune regulation such as excessive activation of mucosal immune response and insufficient suppressor function (Moon, 2012). In this context, a potential link between exposure to some food-associated mycotoxins including DON and human chronic intestinal inflammatory diseases, such as celiac and Crohn's disease or ulcerative colitis has been put forward. At realistic concentrations, DON has been shown to modulate key intestinal

functions involved at the onset and during the progression of chronic intestinal inflammatory diseases, as is illustrated in Figure 8 (Maresca and Fantini, 2010).

One can thus state that there is solid evidence for the potential health implications of fairly low exposure to DON and that there is an urgent need to define the human risk from this foodborne contaminant.
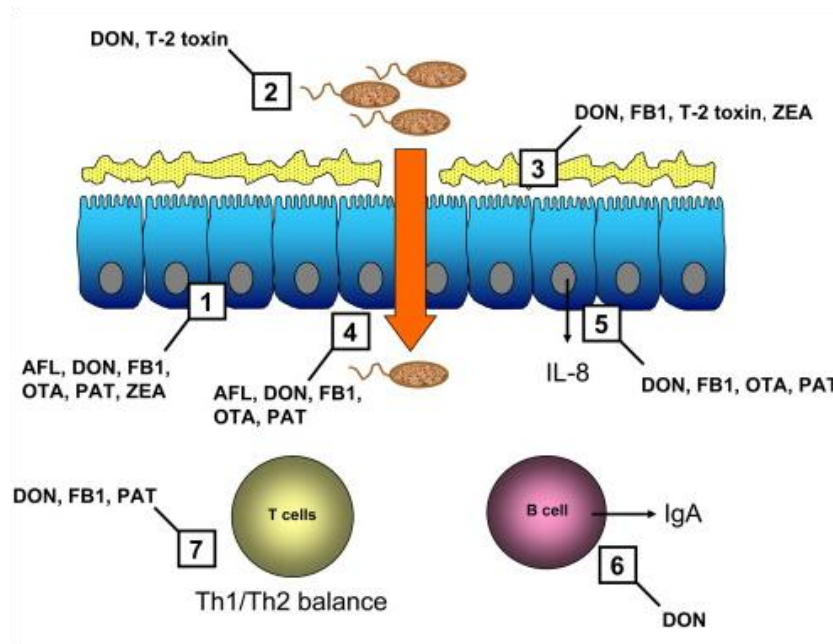
Figure 8: Intestinal alterations caused by the exposure of mycotoxins in relation to the induction and/or persistence of human chronic intestinal inflammatory diseases. Mycotoxins can 1) cause gene mutations in the enterocytes, 2) modify the intestinal flora, 3) alter the production and the composition of the mucus layer, 4) increase the transepithelial bacterial passage through an increased transcellular passage and/or to an increase in the intestinal permeability due to tight junction defects, 5) modulate the pro-inflammatory cytokine secretion by the intestinal epithelium e.g. IL-8, 6) increase the secretion of IgA directed against microflora and autoantigens, 7) disturb the Th1/Th2 immune response balance (adapted from Maresca and Fantini, 2010).

3.   ***Salmonella* Typhimurium infections in pigs**

3.1.   The genus *Salmonella*

Salmonella is a Gram-negative facultative intracellular pathogen that causes a spectrum of clinical diseases depending on the infecting bacterial serovar and the underlying host susceptibility. *Salmonella* spp. are ubiquitous in nature recovered from nearly all vertebrates from amphibians, reptiles to birds and mammals, posing both an animal health and a food safety problem. The genus *Salmonella* is divided into two species: *Salmonella bongori* and *Salmonella enterica*.

Salmonella bongori resides primarily in reptiles and infrequently causes disease in endothermic animals. The species *Salmonella enterica* contains over 2,500 serovars and there are two main clinical syndromes associated with *Salmonella enterica* infection: non-typhoidal salmonellosis or a gastrointestinal disease also known as enteritis, and typhoid fever, a systemic disease characterized by fever, intestinal perforation and hemorrhage, enlargement of the mesenteric lymph nodes, spleen and liver (Valdez *et al.*, 2009).

Depending on their host restriction, *Salmonella* serotypes can be divided in: (1) serotypes capable of causing typhoid-like disease in a single host species (host-restricted serotypes, e.g. *Salmonella* Typhi in humans); (2) serotypes associated with one host species but also able to cause disease in other hosts (host-adapted serotypes, e.g. *Salmonella* Choleraesuis in pigs); (3) the vast majority of the remaining serotypes which rarely produce systemic infections but are able to colonize the alimentary tract of a wide range of animals (broad host range serotypes, e.g. *Salmonella* Typhimurium).

Salmonella enterica can cause fatal disease in domestic animals, with severe economic losses and can persist undetected in food animals causing a serious problem for the food industry because of food poisoning (Mastroeni and Grant, 2011).

3.2.   Importance of salmonellosis for human and animal health

Salmonella species have been recognized as human and animal pathogens for over a century. A recent study estimated that approximately 94 million human cases of gastroenteritis and 155,000 deaths occur due to *Salmonella* infections around the world each year (Majowicz *et al.*, 2010). Farm animals and foods of animal origin form an important source of human *Salmonella* infections.

Salmonella Enteritidis and *Salmonella* Typhimurium are the most frequently reported serovars constituting around 75% of all confirmed cases of human salmonellosis. In the period 2006 to 2009, the porcine reservoir, next to the laying hen reservoir was estimated to be the second largest contributor to human salmonellosis in the EU responsible for 26.9% of the reported cases. Moreover it was shown that in eight countries, including Belgium, pigs were even the most important source of infection (Biohaz, 2010; Pires *et al.*, 2011). The *Salmonella* Report 2010 of CODA-CERVA, which acts as Belgian reference laboratory for *Salmonella*, indicated that in pigs *Salmonella* Typhimurium isolates were found in 67.5% of the *Salmonella* positive samples analyzed (Anonymous, 2010). Pigs can thus be a major reservoir of *Salmonella* and the potential of *Salmonella* infection in slaughter pigs to translate into *Salmonella* contamination of pig meat can lead to transmission through the food chain resulting in human infection and disease (European Commission, 2008).

A variety of clinical manifestations have been observed in *Salmonella* infected pigs, ranging from no disease to peracute disease. Infections with *Salmonella* Typhimurium usually cause mild or no disease, however these subclinically infected animals may shed the bacteria for considerable periods of time with stress factors such as transport and lairage even enhancing this excretion (Berends *et al.*, 1996; Boyen *et al.*, 2006).

Salmonellosis in pigs poses thus a double threat since it not only causes economic losses due to animal weakening and underproduction but also due to the public health risk commercializing *Salmonella* infected pork products (Boyen *et al.*, 2008; Collado-Romero *et al.*, 2010).

3.3.   The pathogenesis of *Salmonella* Typhimurium infections in the pig

An infection with *Salmonella* Typhimurium generally occurs in three stages: 1) the colonization of the gut and adhesion to the intestinal wall, 2) the invasion of the gut wall and 3) the dissemination to mesenteric lymph nodes and other organs.

A schematic overview of the pathogenesis is given in Figure 9.
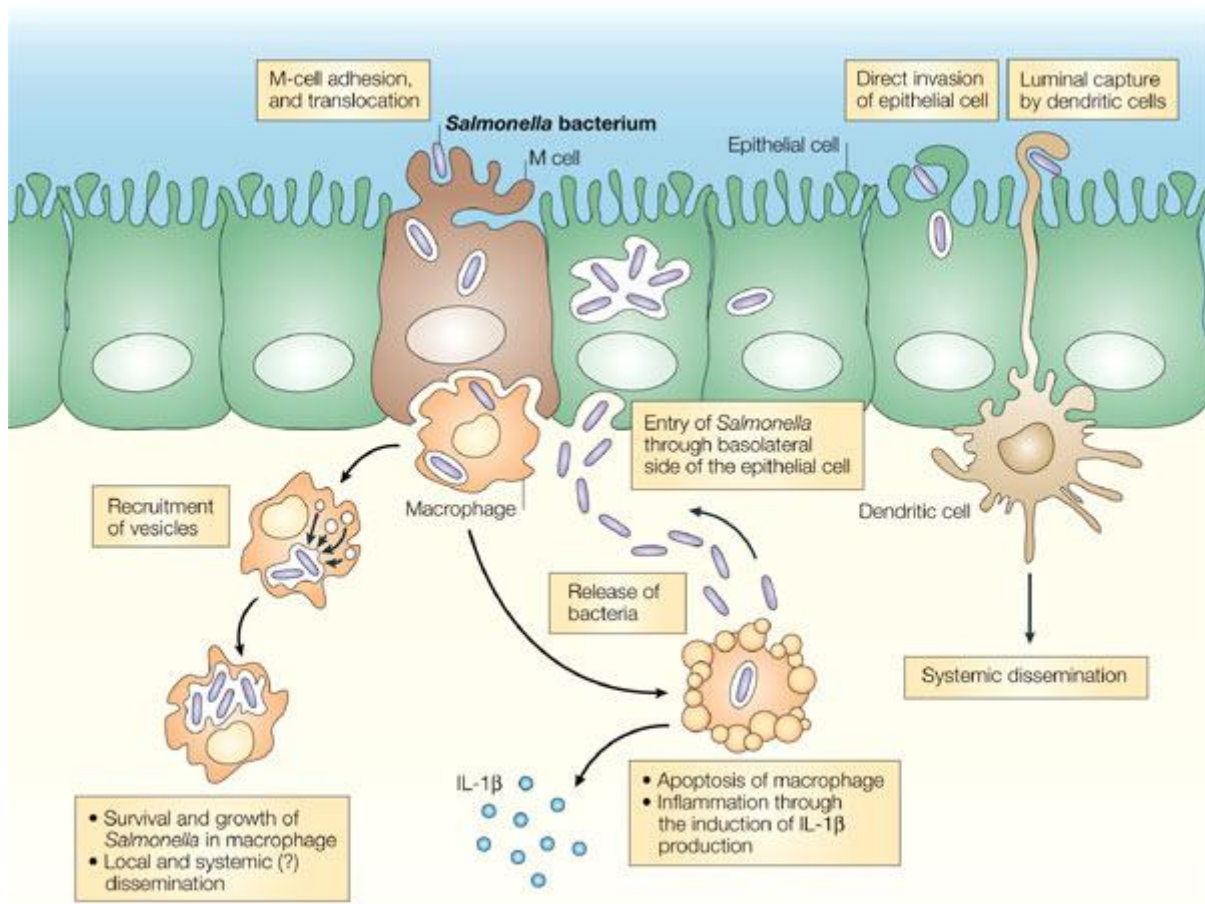
Figure 9: Pathogenesis of a *Salmonella* Typhimurium infection (Sansonetti, 2004).

3.3.1. *The intestinal phase of infection*

Several routes of transmission of *Salmonella* have been described, including airborne transmission, but the fecal - oral route of infection appears to be the most important one in pigs. In order to reach the small intestines, *Salmonella* must overcome various antimicrobial barriers with the porcine epithelial beta-defensin 1 expressed at the dorsal tongue surface and oral epithelium being the first one (Shi *et al.*, 1999). Subsequently, the acidic environment of the stomach represents a significant bottleneck to infection although salmonellae dispose of an adaptive acid-tolerance response when exposed to low pH (Giannella *et al.*, 1972; Ohl and Miller, 2002). After emerging from the stomach and entering the small intestine, the presence of high concentrations of bile in the duodenum may repress the invasion pathway of *Salmonella*, although the bacterium is able to adapt to high bile concentrations (Prouty and Gun, 2000). The presence of resident microbial flora might also provide benefit to the host, including niche protection from pathogens, although *Salmonella* appears to be able to create specific growth conditions in the intestinal lumen which enables it to outcompete the resident microbiota (Santos *et al.*, 2009).

After entering the small intestines, salmonellae must traverse the intestinal mucus layer before encountering and adhering to the intestinal epithelial cells. The adhesion, generally accepted as the first step in the pathogenesis of *Salmonella* infections in pigs, is a complex mechanism and several fimbriae, mainly type 1 fimbriae, contribute to the ability of *Salmonella* to adhere to intestinal epithelial cells (Ohl and Miller, 2002; Althouse *et al*., 2003). Moreover, a recent study showed that also the *Salmonella* Pathogenicity Island (SPI)-1 encoded type III secretion system (T3SS) effectors SipB, SipC and SipD act in concert to mediate the intimate association of *Salmonella* Typhimurium with host cells required for T3SS mediated protein delivery (Lara-Tejero and Galan, 2009).

Following the adhesion, the invasion into host cells is a next critical step in the pathogenesis of *Salmonella* and this can occur through several routes, as illustrated in Figure 9 (Sansonetti, 2004). *Salmonella* can invade non-phagocytic epithelial cells by a process called bacterial-mediated endocytosis after which the bacterium becomes enclosed within an intracellular phagosomal compartment known as the *Salmonella*-containing vacuole (SCV) allowing bacterial survival and multiplication (McGhie *et al.*, 2009). Being located subepithelial, *Salmonella* can then either invade enterocytes basolaterally or disseminate systemically. *Salmonella* can also cross the Microfold (M-) cells of the follicle-associated epithelium mainly located in the Peyer's patches (PP) followed by the uptake in dendritic cells (DC) and

macrophages in the subepithelial compartment. An alternative route of invasion that doesn't involve M-cells is the direct capture of *Salmonella* from the luminal content by a population of phagocytes that protrude pseudopods between the enterocytes. Some bacteria can even bypass the PPs and disseminate to the spleen and liver directly from the intestinal lumen, being transported by the blood within CD18^{+} cells (Mastroeni and Grant, 2011).

In addition to invasion of the intestinal epithelial barrier, *Salmonella* induces a secretory response in the intestinal epithelium and initiates recruitment and transmigration of neutrophils into the intestinal lumen, which is mainly regulated by the epithelial cell production of IL-8 (Ohl and Miller, 2002). The massive neutrophil influx in the intestine is the histopathological hallmark of *Salmonella* Typhimurium induced enterocolitis in humans and animals (Tukel *et al.*, 2006). The possible mechanism leading to neutrophil infiltration in the intestinal mucosa is not clear yet. One hypothesis states that by injecting effector proteins into the host cytosol, *Salmonella* Typhimurium triggers this host response, which induces a pro-inflammatory gene expression profile in the intestinal epithelium. The second hypothesis proposes that as a result of the bacterial invasion of the intestinal epithelium, pathogen-associated molecular patterns (PAMPs) are recognized through pathogen recognition receptors (PRRs) expressed by epithelial cells and mononuclear cells in the lamina propria, which leads to the activation of the innate pathways of inflammation. As reviewed by Tukel *et al*. (2006), it appears that while PRRs are clearly involved in eliciting neutrophils influx during *Salmonella* Typhimurium infection, a direct contribution of effectors in triggering pro-inflammatory host cell response cannot be ruled out.

3.3.2. *The systemic phase of infection*

After the invasion process is completed, *Salmonella* serotypes associated with systemic illness, spread from the gastrointestinal tract to the bloodstream using the host macrophage to establish the systemic infection. In pigs however, the systemic part of a *Salmonella* Typhimurium infection is poorly documented and colonization in pigs is mostly limited to the gastrointestinal tract (Boyen *et al.*, 2008). Being located inside the macrophage, *Salmonella* is shielded from the elements of the humoral immunity although the phagosome also exposes the bacterium to a nutrient poor and antimicrobial environment, since the phagosomal environment is acidic and contains antimicrobial peptides, oxygen and nitrogen radicals that can damage the bacterial cell (Rathman *et al*., 1996; Mastroeni *et al.*, 2000; Haraga *et al*., 2008). These phagosomal conditions activate many of the regulators that are implicated in

salmonellae virulence, permitting the bacterium's survival and replication in the intracellular environment (Ohl and Miller, 2002; Bader *et al*., 2003; Haraga *et al*., 2008). Using the blood stream and lymphatic fluids, the infected macrophages can then migrate throughout the body and colonize other organs of the reticuloendothelial system such as mesenteric lymph nodes, spleen and liver initiating prominent systemic and local immune response.

3.3.3. *Carrier state of infection and prolonged fecal shedding*

Although *Salmonella* Typhimurium generally causes no or mild disease in pigs, asymptomatic carriers are thought to represent the most important source of *Salmonella* introduction onto pig farms (Hoelzer *et al.*, 2011). In subclinical carrier pigs it was demonstrated that the tonsil is the primary site of colonization suggesting that, although the presence of low numbers of *Salmonella* Typhimurium within the tonsil may provide a continuous stimulation of the immune system, its response may not be sufficient to clear the bacteria from the host resulting in the development of a carrier state (Wood *et al.*, 1989, Wood and Rose, 1992; Gray *et al.*, 1995). The difficulty to detect the carrier state in pigs by bacteriological and serological methods may affect the monitoring programs (Boyen *et al.*, 2008). Moreover, it is known that stress factors such as lairage and transport not only induce *Salmonella* carriers to start shedding again but also turn *Salmonella*-free pigs more susceptible to infection (Williams and Newell, 1970; Berends *et al.*, 1996). Although in mice *Salmonella* virulence genes were identified that play a role in the development of the carrier state and long-term shedding, the role of these genes could not be confirmed in the pig, leaving underlying mechanisms unclear (Boyen *et al*., 2006).

Using *in vivo* expression technology (IVET), Van Parys *et al*. (2011) were the first to identify 37 genes specifically induced in porcine tonsils, ileum and ileocaecal lymph nodes at 3 weeks post-oral inoculation as an indication of genes involved in *Salmonella* persistence in these 3 different organs of pigs.

3.3.4. Salmonella *pathogenicity islands*

The ability of *Salmonella* to invade in non-phagocytic host cells and then to survive and replicate within the SCV determines the bacterium's virulence and is mediated by chromosomally encoded virulence genes, which are clustered in several SPIs (Steele-Mortimer, 2008; Valdez *et al.*, 2009). The two primary SPIs are SPI-1 and SPI-2.

Both SPI-1 and SPI-2 encode T3SS which are specialized needle-like devices used to inject bacterial effector proteins directly into the host cell cytoplasm. These proteins have the capacity to modulate a variety of cellular functions, including cytoskeleton dynamics, vesicular trafficking, cell cycle progression, programmed cell death and transcription (Lara-Tejero and Galan, 2009).

The translocation of effectors via the SPI-1 encoded T3SS allows *Salmonella* Typhimurium to enter non-phagocytic cells of the intestinal epithelium through modulation of the actin cytoskeleton dynamics. The T3SS encoded by SPI-2, which is structurally and functionally distinct from that of SPI-1, and whose expression is induced upon bacterial internalization, modulates vesicular trafficking and is essential for the bacterial growth and replication within a variety of host cells, including macrophages and dendritic cells. By delivering proteins inside the SCV or through the vacuolar membrane into the host cytosol, the SPI-2 T3SS effector proteins appear to interfere with the maturation of the SCV, preventing phagosome-lysosome fusion and allowing *Salmonella* to physically avoid the microbiocidal effectors within the macrophage (Marcus *et al.*, 2000; Ohl and Miller, 2002; Steele-Mortimer, 2008; McGhie *et al.*, 2009).

3.3.5. *Role of the actin cytoskeleton in* Salmonella *pathogenesis*

The actin cytoskeleton mediates a variety of essential biological functions in all eukaryotic cells. Apart from determining shape and motility of the cell, it is involved in crucial cellular processes.

3.3.5.1. Involvement of actin cytoskeleton in *Salmonella* invasion

The ability of *Salmonella* spp. to invade into M-cells and cells that are normally non-phagocytic such as intestinal epithelial cells, involves a complex series of cytoskeletal changes induced by translocated effector proteins (Guiney and Lesnick, 2005).

The organization of the host actin cytoskeleton is controlled by the Rho-family of small GTPases. As illustrated in Figure 10, the activation of the host Rho GTPases Cdc42 and Rac by the SPI-1 T3SS effector proteins SopB, SopE and SopE2 leads to the stimulation of downstream signaling pathways which not only activate the actin cytoskeleton reorganization but also lead to the stimulation of the transcription factors NF-κB and AP-1 and to the production of pro-inflammatory cytokines (Haraga *et al.*, 2008).

The formation of membrane ruffles, which is a typical characteristic of *Salmonella* invasion, is the result of the interaction between the bacterial effector proteins SopB, SopE and SopE2 and actin filaments, promoting macropinocytosis and engulfment of *Salmonella* in the SCV. Together with this G protein activation, two actin-binding proteins SipA and SipC are translocated into the host cell to promote actin polymerization and bundling of F-actin at the site of bacterial invasion. The cellular responses stimulated by *Salmonella* are transient and after bacterial entry, the cell regains its normal architecture. This process is mediated by SptP which exerts its function by serving as a GTPase activating protein for Cdc42 and Rac, turning these G proteins off after their stimulation by the bacterial effectors SopE and SopB, helping in restoring the actin cytoskeleton to its normal dynamics (Galan and Zhou, 2000; Galan, 2001; Murli *et al.*, 2001).

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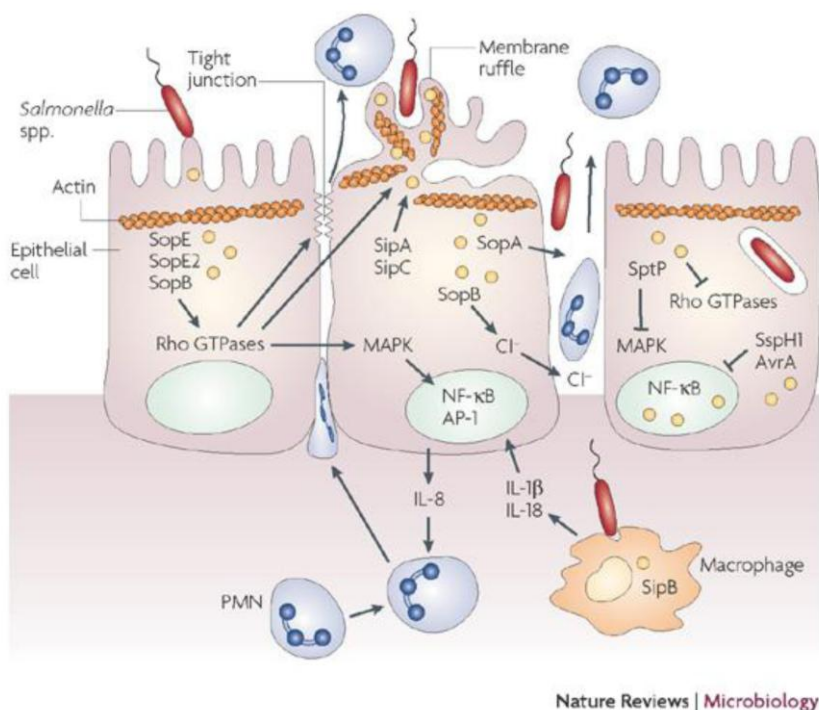
Figure 10: Model for *Salmonella* Typhimurium interaction with cytoskeleton of host cells (Haraga *et al*., 2008).

3.3.5.2.   Actin cytoskeletal events associated with intracellular infection

Within several hours after infection of host cells, a F-actin network assembles around the replicative SCV (Steele-Mortimer, 2008). This focal condensation of actin filaments (VAP or vacuolar-associated actin polymerizations) around the vacuole appears a morphological feature of the SCV and is induced by a SPI-2 dependent mechanism (Guiney and Lesnick,

2005). Although the role of these VAPs during the intracellular pathogenesis is not clear yet, they may help in creating a more favorable environment for the bacteria. The migration of the SCV during its maturation towards the perinuclear region of the host cells appears of great importance for promoting bacterial replication. Once the SCV is positioned, the intracellular replication of *Salmonella* is characterized by the formation of specialized tubulovesicular structures known as *Salmonella*-induced filaments (Sifs) (Steele-Mortimer, 2008).

Taken together, *Salmonella* is able to target cellular actin by a series of interactions, promoting bacterial uptake into host cells and the subsequent proliferation and intercellular spread of the bacteria.

3.3.6. *The interaction of* Salmonella *Typhimurium with macrophages*

The persistence of *Salmonella* within cells of the reticuloendothelial system has long been accepted as a key stage in the pathogenesis of the bacterium (Watson *et al.*, 2000). Salmonellae can enter macrophages by several endocytic processes, including SPI-1 and non-SPI-1 T3SS-induced macropinocytosis. Once intracellular, salmonellae remain inside a vacuolar compartment, which has been named the spacious phagosome. The spacious phagosome shrinks to form an adherent membrane around one of more bacteria, and is then referred to as the SCV (reviewed by Haraga *et al*., 2008). Apart from using macrophages to spread throughout the host's body during the systemic phase of infection by establishing an intracellular niche within the phagocytic vacuole, *Salmonella* Typhimurium can also induce host-cell death, providing a potential mechanism by which *Salmonella* can escape from an infected cell to infect neighboring cells.

Programmed cell death or apoptosis is a fundamental mechanism to eliminate infected or damaged cells. It is characterized by the condensation of the nucleus and the cytoplasm, and fragmentation of the cell. This leads to the formation of apoptotic bodies which are then eliminated by the macrophages in order to prevent the release of noxious cellular contents (Fink and Cookson, 2007). *Salmonella* has been described to trigger two general mechanisms leading to different types of programmed cell death in macrophages, although the biological and immunological importance of these different types of cell death and their contribution to the pathogenesis of *Salmonella* or host defense is not clear yet (Guiney, 2005; Figure 11).
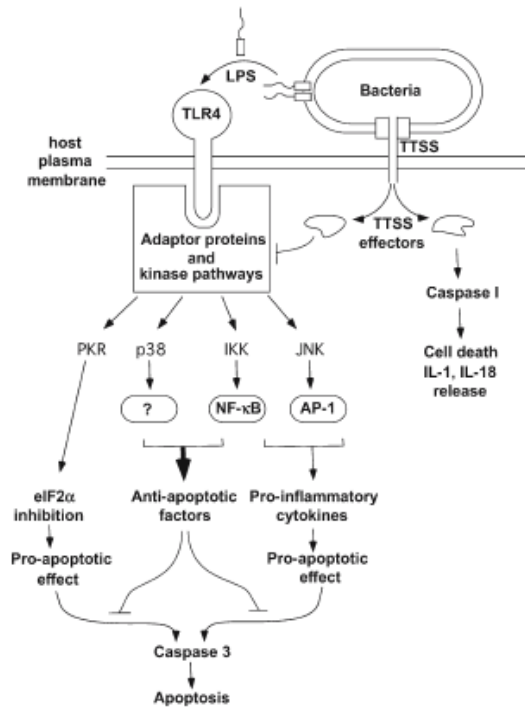
Figure 11: Pathways leading to the induction of macrophage cell death by *Salmonella* (Guiney, 2005).

The first pathway is mediated both by effector proteins delivered by SPI-1 TTSS and SipB which leads to the activation of caspase-1 inducing rapid cell death by a mechanism that shares features of both necrosis and apoptosis, and the release of IL-1β and IL-18. Recently Miao *et al*. (2010) demonstrated for the first time that caspase-1 clears intracellular bacteria *in vivo* independent of IL-1β and IL-18. The resulting pyroptosis, which is pro-inflammatory due to the rapid loss of cell membrane integrity and release of cytosolic content, appears an efficient mechanism of bacterial clearance by the innate immune system.

The second pathway involves the activation of Toll-like receptor (TLR) 4 by PAMPs leading to the initiation of a complex signal transduction cascade (Hsu *et al.*, 2004). Downstream activation of kinase pathways leads to balanced pro- and anti-apoptotic regulatory factors in the cell. NF-kB and p38 MAPK are particularly important for the induction of anti-apoptotic factors, whereas the kinase PKR which is required for bacterial-induced apoptosis, phosphorylates the eukaryote translation initiation factor 2A (eIF2a), leading to inhibition of protein synthesis. The balance of all these apoptosis regulatory factors determines whether the cell will survive or enter the programmed cell death pathway, with caspase-3 as the major executioner of the characteristic apoptotic changes.

Salmonella that are not causing a SPI-1 dependent rapid cell death can still trigger a delayed SPI-2 dependent cell death. The *Salmonella* SPI-2 T3SS is essential for altering the balance in

favor of apoptosis during intracellular infection. The infected, apoptotic cells are targeted for engulfment by incoming macrophages, thus perpetuating the cycle of cell-to-cell spread that is the hallmark of systemic *Salmonella* infection. Although the effectors involved in this delayed apoptosis are poorly characterized, SpvB has been suggested to play a role in apoptosis in human macrophages by depolymerizing the actin cytoskeleton (Lesnick *et al.*, 2001; Kurita *et al.*, 2003). Also the stimulation of TLR4 and the dsRNA responsive kinase PKR may be involved in this delayed from of salmonella-induced macrophage cell death (Hsu *et al*., 2004). It is however not clear whether macrophage cell death is triggered by salmonella to counteract host defence mechanisms or whether it constitutes a host response to halt bacterial replication (Hueffer and Galan, 2004).

3.3.7. *Innate immune control of* Salmonella *Typhimurium*

The outcome of a *Salmonella* infection is determined by both bacterial and host factors, including the virulence of the infecting *Salmonella* serotype, and the ability of the host to establish an efficient inflammatory and immune response in order to destroy the pathogen (Eckman and Kagnoff, 2001).

Intestinal epithelial cells play an important role in the innate immune response. Not only do they serve as a physical barrier, they are also able to produce signaling molecules through recognition of PAMP (*e.g.* LPS, peptidoglycan, mannose and flagellin) by toll-like receptors (Zeng *et al*., 2003; Arce *et al.*, 2010; Collado-Romero *et al.*, 2010). Most of these signaling molecules are chemokines which attract diverse cell types to the subepithelial regions after *Salmonella* infection. These cells include leucocytes such as neutrophils, monocytes and macrophages (*e.g.* by IL-8 and monocyte chemotactic protein-1, MCP-1) but also specialized subsets of T-cells and immature DC (Eckmann and Kagnoff, 2001). In addition to this basolateral release of chemokines, hepoxilin A3, formerly known as pathogen-elicited-epithelial-chemoattractant (PEEC), is released apically by the epithelial cells, attracting neutrophils across the epithelium into the gut lumen (McCormick *et al.*, 1998; Mrsny *et al.*, 2004). The intestinal epithelium also releases other cytokines that can enhance the local host defense by activating phagocytic cells (*e.g.* by granulocyte/monocyte-colony stimulating factor or GM-CSF) or mediating other pro-inflammatory functions (*e.g*. IL-6, TNFα) (Eckmann and Kagnoff, 2001).

In addition to the epithelial cells, phagocytic cells such as macrophages and monocytes are critical components of the innate immune response to *Salmonella* Typhimurium (Wick, 2004). The key role of macrophages and monocytes is to control and clear *Salmonella* infection from the host and this is characterized by a delicate balance between the macrophage effector mechanisms to kill the pathogen and the bacterial defence to resist this killing. Exposure to *Salmonella* promotes monocytes and macrophages to produce a series of cytokines including pro-inflammatory cytokines (e.g. IL-1, TNFα, IL-6), chemokines (e.g. macrophage inflammatory protein, MIP) and hematopoietic growth and survival factors (e.g. GM-CSF) each of them playing a role in attracting phagocytic cells to the infection site and in inducing acute inflammatory events (Eckmann and Kagnoff, 2001).

The host senses the presence of *Salmonella* Typhimurium in tissues by detecting PAMPs (*e.g.* LPS, flagellin) or patterns of pathogenesis (*e.g.* T3SS-1-dependent delivery of proteins) through PRRs which are located in the cell membrane, the cytosol or the humoral compartment.
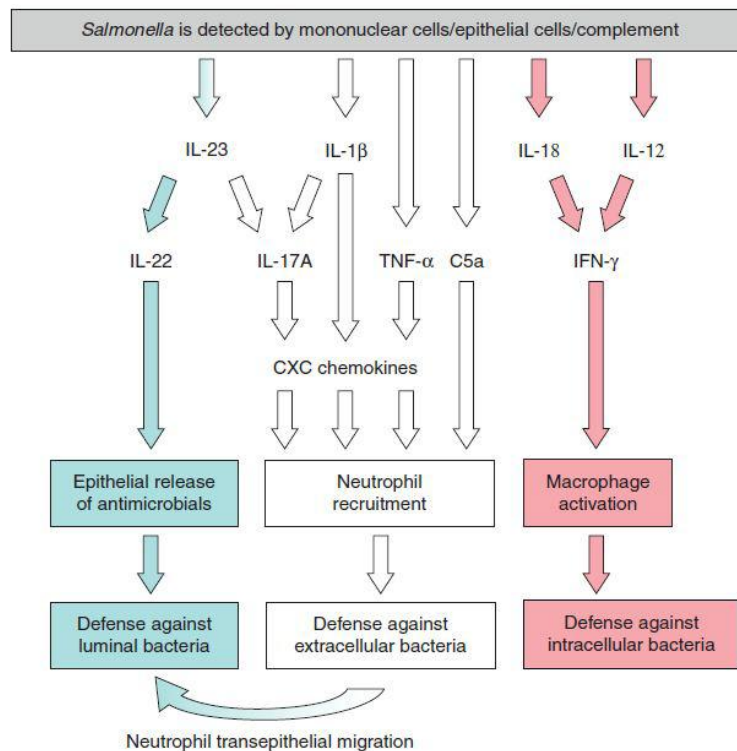
Figure 12: Schematic overview of the antibacterial inflammatory response as a result the detection of *Salmonella* Typhimurium (Thiennimitr *et al.*, 2012).

As illustrated in Figure 12, signaling through these PRRs will result in the production of IL-1β, IL-12, IL-18, IL-23, TNFα, IFNγ and C5a, initiating three important antibacterial responses in tissues: the epithelial release of antimicrobials, the recruitment of neutrophils and the activation of macrophages (reviewed by Eckman and Kagnoff, 2001; Santos *et al*., 2009; Thiennimithr *et al*., 2012).

IFNγ plays a central role in controlling *Salmonella* infection by activating the ability of macrophages to kill intracellular *Salmonella*. The production of IFNγ is largely induced by IL-12 and IL-18. IL-23 contributes to the production of IL-22, a cytokine that stimulates epithelial cells to release antimicrobials into the intestinal lumen. In synergy with IL-1β, IL-23 also induces the production of IL-17, the latter acting in concert with IL-1β and TNFα to induce the release of CXC chemokines from epithelial cells. Together with the complement factor C5a, these CXC chemokines play an important role in the recruitment of neutrophils to the intestinal lumen.

4. **Crosstalk between *Salmonella* and deoxynivalenol: potential mode of action?**

Exposure to ribotoxic stressors such as the trichothecene mycotoxin DON results in a coordinated signaling cascade linked to the activation of MAPKs. This not only leads to alterations of intestinal mucosal integrity but can also trigger intestinal and systemic inflammation (Moon, 2012).

The pathogenesis of a *Salmonella* Typhimurium infection is characterized by a dynamic and complex interplay between the host and the bacterium (Haraga *et al*., 2008). *Salmonella* is able to subvert the host cell cytoskeleton, signal transduction pathways, membrane trafficking and pro-inflammatory responses by using its specialized effector proteins (McGhie *et al*., 2009).

A potential mode of action for the crosstalk between DON and *Salmonella* Typhimurium could be the MAPKs signal transduction cascade since this pathway is not only involved in actin dynamics and subsequent regulation of the host cell cytoskeleton, but also plays a role in the induction of pro-inflammatory cytokines.

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SCIENTIFIC AIMS

Worldwide, the contamination of cereal-based commodities by mycotoxins is an emerging issue with a considerable impact on human and animal health. In pig industry, the contamination of feed with deoxynivalenol (DON) is of great concern due to the high sensitivity of pigs to the toxic effects of this mycotoxin, and the high exposure level resulting from their cereal-rich diet. Most studies have focused on the effects of high concentrations of DON in pig feed. However, the impact of DON at low and in practice more relevant concentrations is poorly understood.

Salmonella Typhimurium is one of the leading serotypes causing human non typhoid salmonellosis. Pork meat, derived from mostly asymptomatic *Salmonella* carrier pigs constitutes an important source of *Salmonella* Typhimurium infections for humans. The course of a *Salmonella* infection in pigs depends on numerous factors. However, whether highly prevalent mycotoxins in feed affect the course of an infection with *Salmonella* Typhimurium in pigs has not been studied.

The intake of DON contaminated feed leads to a mucosal ribotoxic stress which specifically affects the highly dividing intestinal epithelial cells and the underlying mucosa-associated leucocytes. Considering the importance of these cells in the pathogenesis of *Salmonella* Typhimurium infections, co-exposure to DON may result in altered interactions between *Salmonella* Typhimurium and its porcine host. Therefore, the main goal of the present study was to investigate the effects of exposure to low concentrations of DON on the pathogenesis of a *Salmonella* Typhimurium infection in the pig.

The specific objectives of this thesis were as follows:

- ✓ To examine the effects of low and non-cytotoxic concentrations of DON on the intestinal phase of a *Salmonella* Typhimurium infection, using both an *in vitro* model of porcine intestinal epithelial cells and an *ex vivo* porcine ligated ileal loop model.
- ✓ To investigate whether exposure to low and non-cytotoxic concentrations of DON could affect the interaction of *Salmonella* Typhimurium with the macrophage, using an *in vitro* model of primary porcine alveolar macrophages.

EXPERIMENTAL STUDIES

Chapter 1.

Effect of DON on the intestinal phase of a *Salmonella* Typhimurium infection in the pig

The mycotoxin deoxynivalenol potentiates intestinal inflammation by *Salmonella* Typhimurium in porcine ileal loops

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ABSTRACT

Background and aims: Both deoxynivalenol (DON) and nontyphoidal salmonellosis are emerging threats with possible hazardous effects on both human and animal health. The objective of this study was to examine whether DON at low but relevant concentrations interacts with the intestinal inflammation induced by *Salmonella* Typhimurium.

Methodology: By using a porcine intestinal ileal loop model, we investigated whether intake of low concentrations of DON interacts with the early intestinal inflammatory response induced by *Salmonella* Typhimurium.

Results: A significant higher expression of IL-12 and TNFα and a clear potentiation of the expression of IL-1β, IL-8, MCP-1 and IL-6 was seen in loops co-exposed to 1 µg/mL of DON and *Salmonella* Typhimurium compared to loops exposed to *Salmonella* Typhimurium alone. This potentiation coincided with a significantly enhanced *Salmonella* invasion in and translocation over the intestinal epithelial IPEC-J2 cells, exposed to non-cytotoxic concentrations of DON for 24 h. Exposure of *Salmonella* Typhimurium to 0.250 µg/mL of DON affected the bacterial gene expression level of a limited number of genes, however none of these expression changes seemed to give an explanation for the increased invasion and translocation of *Salmonella* Typhimurium and the potentiated inflammatory response in combination with DON.

Conclusion: These data imply that the intake of low and relevant concentrations of DON renders the intestinal epithelium more susceptible to *Salmonella* Typhimurium with a subsequent potentiation of the inflammatory response in the gut.

INTRODUCTION

The contamination of food and feed with mycotoxins poses a worldwide problem with an acknowledged negative effect on both human and animal health and significant economic and international trade implications. Deoxynivalenol (DON) is a trichothecene mycotoxin frequently contaminating maize and small grain cereals in temperate regions of Europe, North America and Asia (Bottalico and Perrone, 2002). The intake of DON contaminated food or feed can lead to adverse health effects in both humans and animals. For humans, a large-scale European study on the occurrence of *Fusarium* toxins and the dietary intake showed that 57% of the tested cereals samples such as wheat were positive for DON and based on the intake estimates, it is clear that the presence of trichothecenes can pose a public health concern (Schothorst and van Egmond, 2004; Gonzalez-Osnaya *et al*., 2011). A study on the prevalence and level of urinary DON in the United Kingdom population indicated that urinary levels of DON were positively correlated with cereal intake suggesting that the European Union recommended maximum tolerable daily intake of 1 µg DON/kg BW may be exceeded. Urinary DON levels can be a valuable tool as exposure biomarker for biomonitoring in etiological studies of DON and human disease risks (Turner *et al*., 2008). For human food products, the European Union sets maximum limits for DON in cereals and cereal-based products, ranging from 200 to 1750 µg/kg (European Commission, 2007). Anorexia, altered feed intake, reduced weight gain and immunologic alterations are associated with chronic low-doses ingestion of DON whereas acute high-dose exposure is characterized by emesis, diarrhea, vomiting and rectal bleeding. Among farm animals, pigs are considered particularly sensitive to the dietary intake of DON resulting in substantial economical losses (Böhm, 1992).

Nontyphoidal *Salmonella* represents an important human and animal pathogen worldwide. Each year approximate 93.8 million human cases of gastroenteritis occur due to *Salmonella* Typhimurium around the world (Majowicz *et al*., 2010). In 2009, *Salmonella* was the most commonly reported bacteriological agent of human food borne diseases in the USA, causing approximate 44% of confirmed food borne bacterial infections (Prevention CfDCA, 2009). *Salmonella* Typhimurium is, together with *Salmonella* Enteriditis, the most common serotype associated with human nontyphoidal salmonellosis in Europe and USA (Eurosurveillance website, 2011; Doyle *et al*., 2009). In pigs, clinical salmonellosis is not a common problem as *Salmonella* infections are mostly subclinical. However, these carrier pigs are an important contaminating source for the environment, other pigs and carcasses in the slaughterhouse and

as such they pose a serious public health problem being the most important reservoir of *Salmonella* Typhimurium for humans (Wray, 2000; Donné *et al*., 2005; Boyen *et al*., 2008).

Several studies already described the direct effects of the intake of DON contaminated food or feed on the gastrointestinal tract (Kasuga *et al*., 1998; Maresca *et al*., 2002; Sergent *et al*., 2006; Obremski *et al*., 2008; Kolf-Clauw *et al*., 2009; Pinton *et al*., 2009, 2010; Danicke *et al*., 2010; Van De Walle *et al*., 2010b; Diesing *et al*., 2011), however only few examined the possible link between DON ingestion and intestinal inflammation using *in vitro* intestinal cells line models (Bouhet and Oswald, 2005; Mbandi and Pestka, 2006; Maresca *et al*., 2008; Van De Walle *et al*., 2008, 2010a; Maresca and Fantini, 2010). The general effects of mycotoxins on the local intestinal immune response were reviewed by Bouhet and Oswald (2005), whereas Mbandi and Pestka (2006) examined the capacity of DON to potentiate chemokine and pro-inflammatory cytokine production in murine macrophages induced by killed irradiated suspensions of *Salmonella* Typhimurium, concluding that the induction of IL-1β, IL-6, TNFα and MIP-2 by the pathogen was potentiated by DON. Maresca *et al*. (2008) indicated that besides the direct pro-inflammatory effect and potentiation of an existing inflammation, DON could also cause intestinal inflammation indirectly through alteration of the intestinal barrier function. Van De Walle *et al*. (2008, 2010a) suggested that high concentrations of DON could trigger intestinal inflammation and worsen inflammation related parameters.

Recently a potential link between food-associated exposure to certain mycotoxins including DON, and the induction and/or persistence of Inflammatory Bowel Disease (IBD) in genetically predisposed patients has been suggested (Maresca and Fantini, 2010). At realistic doses, mycotoxins are able to cause immune and intestinal alterations comparable with those involved in human chronic intestinal inflammatory diseases (Bouhet and Oswald, 2005). In addition, infections with enteric pathogens such as nontyphoidal *Salmonella* have been implicated in the etiology of IBD (Gradel *et al*., 2009; Jess *et al*., 2011).

With DON and salmonellosis being emerging issues with possible deleterious consequences for both animal and human health, and with the gastrointestinal tract being the primary target for both, we aimed to test whether DON at low but relevant concentrations interacts with the intestinal inflammation induced by *Salmonella* Typhimurium using a porcine model of infection, as representative of the human gastrointestinal gut (Almond, 1996; Rothkotter *et al*., 2002; Niewold *et al*., 2005).

MATERIALS AND METHODS

Ethics statement

The *in vivo* experimental protocols and care of the animals were approved by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University, Belgium (EC 2010/065, May 3, 2010).

Chemicals

DON stock solution of 1 mg/mL (Sigma-Aldrich, Steinheim, Germany) was prepared in anhydrous methanol and stored at -20 °C. Serial dilutions of DON were prepared in cell medium or Luria-Broth (LB, Sigma-Aldrich), depending on the experiment.

Bacterial strains and growth conditions

Salmonella enterica subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) strain 112910a was used as wild type strain. To obtain highly invasive late logarithmic cultures for invasion assays, 2 µL of a stationary phase culture was inoculated in 5 mL LB and grown for 5 h at 37 °C without aeration.

The inocula for the intestinal loop model were prepared according to the temperature shift method for *Salmonella*. Cultures in LB were shaken at 130 rpm for 24 h at 25 °C. After diluting threefold and adjusting the OD_{600nm} to 1, the bacteria were incubated for 2 h at 37 °C, with shaking at 130 rpm. The actual number of bacteria/mL was assessed by plating tenfold dilutions on Brilliant Green agar (BGA, Oxoid, Hampshire, UK).

Cell cultures

The IPEC-J2 cell line, a non-transformed intestinal cell line, which was originally derived from jejunal epithelium of an unsuckled piglet was used for all the experiments (Roads *et al*., 1994; Schierack *et al*., 2006). IPEC-J2 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F-12 (1:1) (Invitrogen[TM] Life Technologies, Carlsbad, CA, USA) supplemented with 5% (v/v) FCS (Hyclone, Cramlington, England, UK), 1% (v/v) insulin/transferrin/Na-selenite (Gibco, Life Technologies, Paisley, Scotland), 1% (v/v) penicillin/streptomycin (Gibco) and 1% (v/v) kanamycin (Gibco).

Cytotoxicity assay

IPEC-J2 cells were seeded into a 96-well plate at a concentration of 1 x 10^5 cells/mL and cultured for 1 day or 21 days, representing undifferentiated proliferating and highly differentiated IPEC-J2 cells respectively, after which the cells were exposed to DON at a concentration of 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1, 5 or 10 µg/mL during 24 h. The cells were maintained in an atmosphere of 5% CO_2 at 37 °C. To assess cytotoxicity, 150 µL of freshly prepared neutral red solution (33 mg/L in DMEM without phenol red) prewarmed to 37 °C was added to each well and the plate was incubated at 37 °C for an additional 2 hours. The cells were then washed two times with Hanks Buffered Saline Solution (HBSS) and 150 µL of extracting solution (ethanol/Milli-Q water/acetic acid, 50/49/1 (v/v/v)) was added in each well. The plate was shaken for 10 min. The absorbance was determined at 540 nm using a microplate ELISA reader (Multiscan MS, Thermo Labsystems, Helsinki, Finland). The percentage of viable cells was calculated using the following formula:

$$\% \text{ viability} = 100 \text{ x } \frac{(a-b)}{(c-b)}$$

In this formula a = OD_{540nm} derived from the wells incubated with DON, b = OD_{540nm} derived from blank wells, c = OD_{540nm} derived from untreated control wells.

Invasion and translocation assays of *Salmonella* Typhimurium in porcine intestinal epithelial cells

To examine whether the ability of *Salmonella* Typhimurium to invade in and translocate through a monolayer of IPEC-J2 cells was altered after pre-exposure of the IPEC-J2 cells to DON, invasion and translocation assays were performed.

For the invasion assays, IPEC-J2 cells were seeded in 24-well plates at a density of 1 x 10^5 cells per well and were cultured for 1 day or 21 days depending on the experimental setup. The 1-day-old cells were then exposed either to non-cytotoxic concentrations of DON up to 0.1 µg/mL, and to 0.25 µg/mL which decreased the percentage of viable cells to 62.3% as determined by the cytotoxicity assay described above. The 21-days-old IPEC-J2 cells were exposed to non-cytotoxic concentrations of DON up to 1 µg/mL. The invasion assay was performed as described by Boyen *et al*. (2009). To quantify the transepithelial passage of *Salmonella* Typhimurium through IPEC-J2 cells exposed to DON, IPEC-J2 cells were seeded on Transwell® inserts with a pore size of 3.0 µm and membrane diameter of 6.5 mm (Corning Costar Corp., Cambridge, MA) at a density of 5 x 10^5 cells/mL and cultured until 21 days

after reaching confluence. Cell medium was refreshed every 3 days. DON was added at concentrations of 0.1, 0.25, 0.5, 0.75 or 1 µg/mL to the apical side in 200 µL of culture medium. The basolateral side received 1 mL of blank culture medium. After 24 h of treatment with DON, the Transwell® inserts were washed three times with HBSS. Then *Salmonella* Typhimurium was added to the apical compartment at a concentration of 2×10^7 colony forming units (CFU) per mL suspended in IPEC-J2 medium with the respective concentrations of DON but without supplementation of antibiotics. The basolateral compartment was filled with antibiotic-free IPEC-J2 medium. After 60 min at 37 °C and 5% CO_2, the number of bacteria (CFU/mL) was determined in the basolateral compartment by plating tenfold dilutions on BGA plates. Transepithelial electrical resistance (TEER) measurements were performed before and after the incubation with DON in order to evaluate the cell barrier integrity.

Porcine intestinal ileal loop experiments

Considering the fact that the gastrointestinal tract and the immune system of pigs are very similar to that of humans (Almond, 1996; Rothkotter *et al.*, 2002; Niewold *et al.*, 2005), porcine intestinal loops were used to reproduce *Salmonella* Typhimurium induced intestinal inflammation.

Two 5-week-old pigs were used in the gut loop experiments. Feed was deprived for 16 h before surgery. The pigs were anesthetized with a mixture of tiletamine+zolazepam (Zoletil 100, Virbac, Carros, France) and xylazine (Xyl-M 2%, VMD, Arendonk, Belgium), given intramuscularly at 0.2 mL/kg bodyweight after which they were intubated intratracheally. Anesthesia was maintained with 1-3% isoflurane in conjunction with 1% pure oxygen using a semiclosed circuit. Intramuscular injection of fentanyl (5 µg/kg BW/h) was used for pain management. The porcine ligated loop model was performed as described before with slight modifications (Boyen *et al.*, 2009). After cleaning and disinfection of the abdomen, a ventral line laparotomy was performed aseptically and twelve loops were produced commencing at the distal ileum, each 6 cm in length with a short intervening segment in between. Ligation was done by an intestinal circumferential ligature through the mesentery without damaging grossly visible mesenteric arcades and thus maintaining full blood supply for both loops and inter-loop segments.

A volume of 1 mL of each test condition was injected into each loop. In each pig, the treatments were randomly assigned and performed in triplicate. Following test conditions were included: negative control (LB), 1 µg/mL of DON in LB, 4×10^8 CFU/mL of

Salmonella Typhimurium and 1 µg/mL of DON in combination with 4 x 10^8 CFU/mL of *Salmonella* Typhimurium. Piglets were maintained under anesthesia for 6 h after which they were euthanized using T61 intravenously (0.3 mL/kg bodyweight) (Intervet, Ukkel, Belgium) while still anesthetized. The pieces of the ligated intestine were then quickly excised from each loop and processed for RNA isolation.

Analysis of intestinal cytokine response

For RNA extraction, pieces of tissue samples were collected from the ileal loops, immediately frozen in liquid nitrogen and stored at -70 °C until analysis. Total RNA from the intestinal samples was isolated using RNAzol®RT (MRC Inc., Cincinnati, USA) according to the manufacturer's instructions. The RNA concentration was measured by absorbance at 260 nm using Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) and the purity of the RNA samples was checked using an Experion RNA StdSens Analysis kit (Biorad Laboratories, Hercules, CA, USA).

Reverse transcription was carried out using the iScript cDNA Synthesis Kit (Biorad Laboratories). Briefly, reverse transcription was carried out in a 20 µL final volume that included 4 µL of 5x iScript Reaction Mix, 1 µL of iScript Reverse Transcriptase, 1 µL of RNA template (1 µg), and nuclease-free water to complete the final volume. The reverse transcription mix was incubated at 25 °C for 5 min, heated to 42 °C for 30 min, and inactivated at 85 °C for 5 min. The resultant cDNA was stored (-20 °C) until further use.

Primers used for the amplification were designed using Primer3 software (available at http://frodo.wi.mit.edu/primer3) using the GeneBank sequences (Rozen and Skaletsky, 2000). The primers for IL-8, TNFα and IL-12 were adopted from Volf *et al*. (2007, 2010). The specificity of the primers was tested by performing a BLAST search against the genomic NCBI database. To optimize the amplification procedure, all primer pairs were designed to be used at the same annealing temperature (60 °C). The list of genes and sequences of the primers used for quantitative PCR analysis are listed in Table 1. HPRT and HIS were used as housekeeping genes. Both genes had a stable expression, for all the samples and test conditions tested, as calculated using the geNorm software (data not shown).

To quantify the products of interest (IL-1β, IL-6, IL-8, IL-12, IL-18, TNFα, IFNγ and MCP-1) real-time quantitative PCR was utilized. The PCR reaction was carried out in 96-well plates with the appropriate forward and reverse primers (500nM), 5 µL of iQ™ SYBR®Green Supermix (Biorad) and 1 µL of the fivefold diluted cDNA template. Thermocycling parameters were used according to the manufacturer's instructions and included 40 cycles of

20 s at 95 °C, 30 s at 60 °C, 30 s at 73 °C. The threshold cycle values (Ct) were first normalized to the geometric means of appropriate reference mRNAs and the normalized mRNA levels were calculated according to $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

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

Gene name	Forward primer (5'→3')	Reverse primer (3'→5')	Accession number
HPRT	GAGCTACTGTAATGACCAG TCAACG	CCAGTGTCAATTATATCTTCAACAA TCAA	NM_001032376
HIS	AAACAGATCTGCGCTTCC	GTCTTCAAAAAGGCCAAC	NM_213930
IL-1β	GGGACTTGAAGAGAGAAG TGG	CTTTCCCTTGATCCCTAAGGT	NM_001005149
IL-6	CACCGGTCTTGTGGAGTTT C	GTGGTGGCTTTGTCTGGATT	M86722
IL-8	TTCTGCAGCTCTCTGTGAG GC	GGTGGAAAGGTGTGGAATGC	M86923
IL-12	CACTCCTGCTGCTTCACAA A	CGTCCGGAGTAATTCTTTGC	U08317
IL-18	ATGCCTGATTCTGACTGTT C	CTGCACAGAGATGGTTACTGC	AB010003
TNFα	CCCCCAGAAGGAAGAGTTT C	CGGGCTTATCTGAGGTTTGA	NM_214022
IFNγ	CCATTCAAAGGAGCATGGA T	GAGTTCACTGATGGCTTTGC	AY188090
MCP-1	CAGAAGAGTCACCAGCAG CA	TCCAGGTGGCTTATGGAGTC	NM_214214

Micro-array analysis of *Salmonella* Typhimurium gene expression

To test whether DON affected the gene expression of *Salmonella* Typhimurium, a microarray analysis was performed on RNA isolated from cultures of *Salmonella* Typhimurium grown to logarithmic and stationary phase in the presence or absence of 0.250 µg/mL of DON. This concentration was chosen based on the results of the *in vitro* assays on IPEC-J2 cells.

Stationary phase cultures were obtained by aerated, overnight culture at 37 °C in 5 mL LB in 50 mL flasks. To obtain highly invasive late logarithmic culture, 2 µL of a stationary phase culture was inoculated in 5 mL LB and grown for 5 hours at 37 °C without aeration (Lundberg *et al*., 1999).

Of the stationary and logarithmic culture respectively, 2.00 OD_{600} units were harvested and RNA was extracted and purified using SV Total RNA Isolation Kit (Promega Benelux bv, Leiden, The Netherlands) according to manufacturers' instructions. The quality and purity of the isolated RNA was determined using a Nanodrop spectrophotometer and Experion RNA StdSens Analysis kit (Biorad). The SALSA microarrays and protocols for RNA labeling, microarray hybridization and subsequent data acquisition have been described previously (Nagy *et al.*, 2006). RNA (10 μg) from 3 independent biological replicates of DON treated and untreated (control) logarithmic and stationary phase cultures was labeled with Cy5 dCTP and hybridized to SALSA microarrays with 400 ng of Cy3 dCTP labeled gDNA, as a common reference. Genes were assessed to be statistically significantly differently expressed between the DON treated and untreated controls at each growth phase by an analysis of variance test with a Benjamini and Hochberg false discovery rate of 0.05 and with a $\geq$ 1.5-fold change in the expression level.

Micro array accession number

The microarray data discussed in this publication are MIAME compliant and have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE29399 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE29399)

Statistical analysis

All *in vitro* experiments were conducted in triplicate with three repeats per experiment, unless otherwise noted. The data were analyzed using Student's t-test to address the significance of difference between mean values with significance set at 0.05.

The differences in mRNA expression among groups were assessed by performing two-factor ANOVA after determination of normality and variance homogeneity. Multiple comparisons were performed using LSD post-hoc test. Not normally distributed data were analyzed using the non parametric Kruskal-Wallis analysis, followed by Mann-Whitney test using SPSS 17.0 Software (SPSS Inc., Chicago, IL, USA). Significance level was set at 0.05.

RESULTS

DON is more toxic to undifferentiated than to differentiated IPEC-J2 cells

The cytotoxic effect of DON on IPEC-J2 cells as determined using the neutral red assay is shown in Figure 1. The viability of 1-day-old IPEC-J2 cells was significantly decreased by exposure to concentrations of DON higher than 0.1 µg/mL (Figure 1A). DON concentrations up to 10 µg/mL did not significantly affect the viability of differentiated IPEC-J2 cells (Figure 1B). However, although not significant, exposure of differentiated IPEC-J2 cells to 5 and 10 µg/mL of DON during 24 h reduced the viability to 86 +/- 7% and 80 +/- 4% respectively.
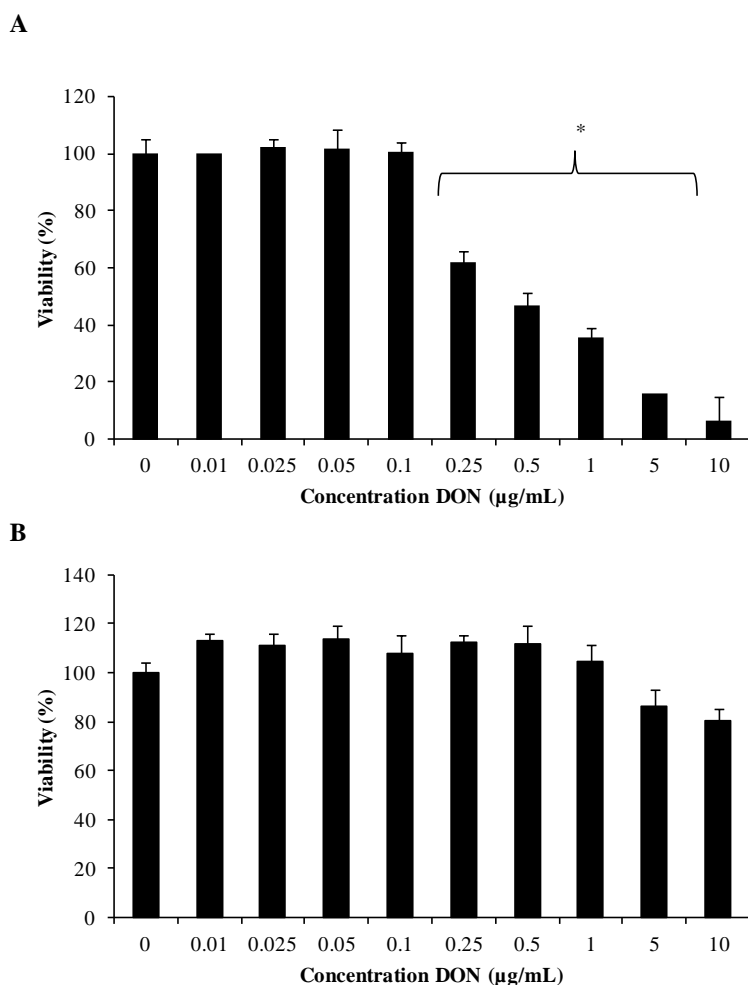
Figure 1: Percentage viability (%) of IPEC-J2 cells exposed to different concentrations of DON (0.005 – 10 µg/mL). IPEC-J2 cells were grown to a confluent monolayer during 1 day (A) or grown until differentiation during 21 days (B). Twenty-four hours after incubation with DON, the cytotoxic effect was determined using neutral red assay. Results represent the means of 3 independent experiments conducted in triplicate and their standard error of the mean. * refers to a significantly higher cytotoxic effect compared to the unexposed control cells ($p < 0.05$).

DON promotes the invasion of *Salmonella* Typhimurium in IPEC-J2 cells

The invasion of *Salmonella* Typhimurium was higher in undifferentiated proliferating IPEC-J2 cells exposed to concentrations of DON higher than 0.025 µg/mL in comparison to non treated IPEC-J2 cells, with a significant increased invasion when exposed to 0.1 and 0.25 µg/mL of DON during 24 h ($p < 0.05$), although the latter (0.25 µg/mL) reduced the percentage of viable IPEC-J2 cells to 62.3% (Figure 2A). The same tendency was seen in highly differentiated 21-days-old IPEC-J2 cells, where exposure to non-cytotoxic concentrations of DON led to a dose-dependent increase in the invasion of *Salmonella* Typhimurium in the cells. Exposure to concentrations of DON ≥ 0.75 µg/mL resulted in a significantly higher bacterial count in IPEC-J2 cells when compared to non treated control cells (Figure 2B).

A

B
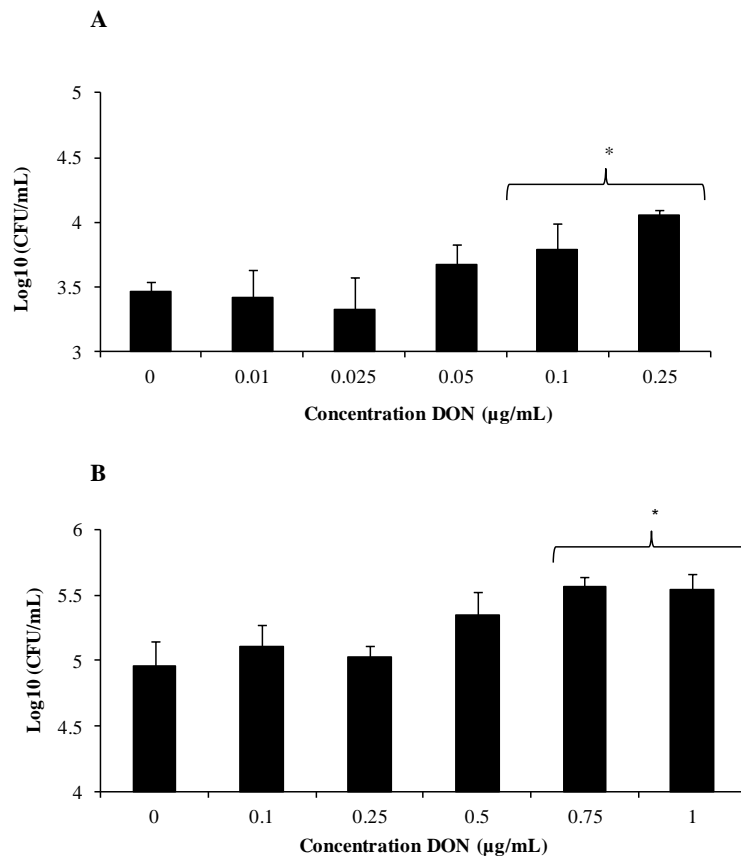
Figure 2: The invasiveness of *Salmonella* Typhimurium in IPEC-J2 cells after exposure to different concentrations of DON. The number of invaded bacteria in actively dividing 1-day-old (A) or differentiated 21-days-old (B) IPEC-J2 cells exposed to different concentrations of DON is shown. The 1-day-old cells were exposed either to non-cytotoxic concentrations of DON up to 0.1 µg/mL, and to 0.25 µg/mL of DON. The 21-days-old IPEC-J2 cells were exposed to non-cytotoxic concentrations of DON up to 1 µg/mL. The results show a representative of three independent experiments conducted in triplicate + standard deviation. * refers to a significantly higher invasiveness compared to unexposed control cells ($p < 0.05$).

DON increases the transepithelial passage of *Salmonella* Typhimurium through the intestinal epithelium

We assessed the passage of *Salmonella* Typhimurium through 21-days-old IPEC-J2 cells treated for 24 h with non-cytotoxic concentrations of DON varying from 0.1 to 1 µg/mL (Figure 3). Exposure to DON for 24 h did not lead to a decrease in TEER (data not shown) indicating no loss of integrity of the epithelial monolayer. However, there was a significant increase in the passage of *Salmonella* Typhimurium bacteria ($p < 0.05$) after exposure to 0.5, 0.75 and 1 µg/mL of DON in comparison to non treated IPEC-J2 cells.
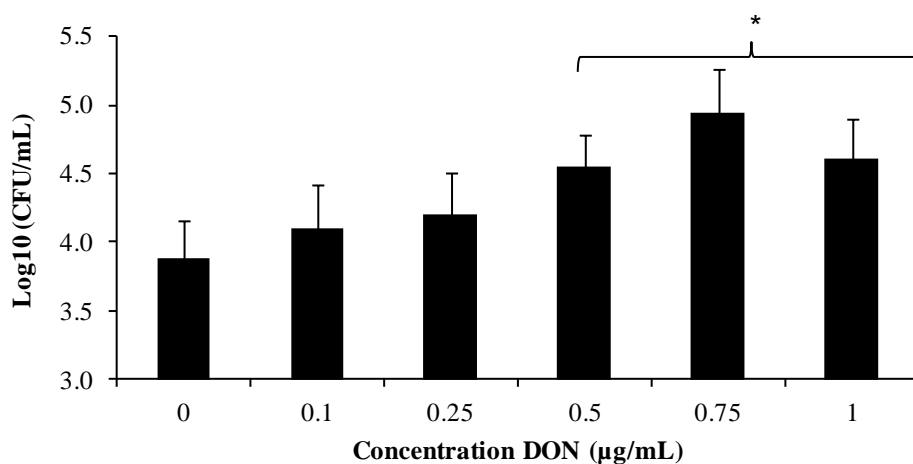
Figure 3: Impact of DON on transepithelial passage of *Salmonella* Typhimurium. IPEC-J2 cells seeded onto inserts until differentiation were either exposed to blank medium or treated with different concentrations of DON (0.1; 0.25, 0.5; 0.75 or 1 µg/mL) for 24 h, prior to measuring the transepithelial passage of *Salmonella* Typhimurium. The results show the means of four independent experiments conducted in triplicate with their standard error of the mean. * refers to a significantly higher translocation of the bacteria compared to the unexposed control cells ($p < 0.05$).

DON potentiates the intestinal inflammatory response to *Salmonella* Typhimurium in the porcine intestinal ileal loop model

Using the porcine intestinal loop model, the effect of 1 µg/mL of DON, *Salmonella* Typhimurium and co-exposure of *Salmonella* Typhimurium with 1 µg/mL of DON on the intestinal mRNA expression levels of the cytokines (IL-1β, IL-6, IL-12, IL-18, IFNγ and TNFα) and chemokines (IL-8 and MCP-1) 6 hours post administration was examined. The results are illustrated in Figure 4. Exposure to 1 µg/mL of DON did not significantly affect the mRNA expression level of any cytokine and chemokine tested. Co-exposure of the ileal loops to 1 µg/mL of DON and *Salmonella* Typhimurium for 6 hours, resulted in a potentiated

intestinal immune response in comparison to loops incubated with *Salmonella* Typhimurium but without the presence of DON. Although not significant, except for TNFα and IL-12 ($p < 0.05$), there was a higher increase in fold change in mRNA expression compared to the *Salmonella* Typhimurium inoculated loops for IL-1β (10.9 to 6.1 respectively), IL-12 (6.5 to 2.3 respectively), IL-8 (6.2 to 3.0 respectively), MCP-1 (2.5 to 1.2 respectively), TNFα (1.8 to 0.9 respectively) and IL-6 (1.6 to 1.1 respectively). However, no increase in mRNA expression level was seen for IL-18 and IFNγ.

Gene expression of *Salmonella* Typhimurium is affected by exposure to 0.250 μg/mL of DON

In the stationary phase cultures of *Salmonella* Typhimurium, exposure to 0.250 μg/mL of DON did not affect the gene expression level of any gene tested in the microarray analysis.

In the logarithmic culture, 159 genes were significantly ($p < 0.05$) up-or downregulated ≥ 1.5-fold by exposure to 0.250 μg/mL of DON (Table 2). A general reduction in expression of genes involved in energy production as well as an increased expression of *emrAB* multidrug efflux systems was seen. Six genes known to play a role in the pathogenesis of a *Salmonella* infection were affected by exposure to 0.250 μg/mL of DON. Three *Salmonella* Pathogenicity Island-3 (SPI-3) genes were either upregulated (*marT* and *fidL*) or downregulated (*cigR*), as well as the gene encoding for the SipA protein and *fliD* and *flgL*, two genes encoding for hook-associate proteins, which were downregulated.
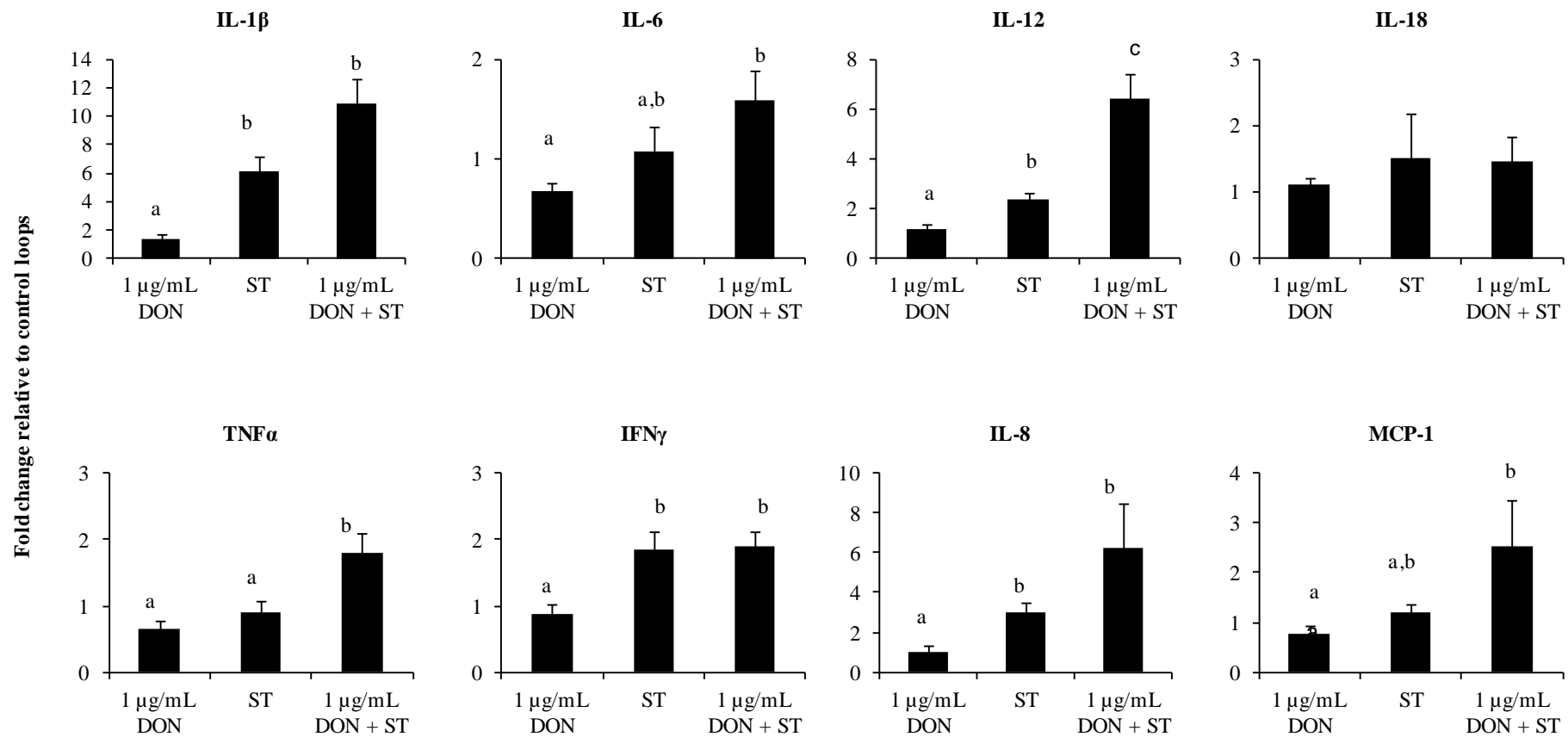
Figure 4: Fold change in cytokine gene expression level of the porcine ileum relative to control loops. Ileal loops were exposed to 1 µg/mL DON, *Salmonella* Typhimurium or 1 µg/mL DON + *Salmonella* Typhimurium respectively. The intestinal mRNA expression levels of the cytokines (IL-1β, IL-6, IL-12, IL-18, IFNγ and TNFα) and chemokines (IL-8 and MCP-1) 6 hours post administration was examined. Data represent the normalized target gene amount relative to control which is considered 1. Data were presented as means + standard error of the mean for a total of 6 loops per test condition. Bars marked with different letters indicate significantly different responses (p < 0.05).

Table 2. Results of the micro-array analysis of the logarithmic phase culture of *Salmonella* Typhimurium exposed to 0.250 µg/mL of deoxynivalenol. The genes in red are upregulated, the genes in blue are downregulated.

Gene		Fold Change	Function
Amino acid transport and metabolism			
STM0426	phnV	2.229546	Putative RBS for phnV; RegulonDB:STMS1H000774
STM0532	arcC	1.877833	similar to E. coli putative carbamate kinase (AAC73623.1); Blastp hit to AAC73623.1 (297 aa), 86% identity in aa 1 - 297; putative carbamate kinase [Salmonella typhimurium LT2].
STM0663	gltK	0.6583	Putative RBS for gltK; RegulonDB:STMS1H000984
STM1358	aroD	0.6383225	similar to E. coli 3-dehydroquinate dehydratase (AAC74763.1); Blastp hit to AAC74763.1 (252 aa), 80% identity in aa 1 - 252; 3-dehydroquinate dehydratase [Salmonella typhimurium LT2].
STM1743	oppD	1.654658	ABC superfamily (atp-binding); oligopeptide transport ATP-binding protein OPPD. (SW:OPPD_SALTY); oligopeptide transport protein [Salmonella typhimurium LT2].
STM2163	yehX	2.5853953	Putative RBS for yehX; RegulonDB:STMS1H002307
STM2355	argT	0.5622683	ABC superfamily (bind_prot); lysine-arginine-ornithine-binding periplasmic protein precursor(LAO-binding protein). (SW:ARGT_SALTY); lysine/arginine/ornithine transport protein [Salmonella typhimurium LT2].
STM2970	sdaC	0.39894575	similar to E. coli probable serine transporter (AAC75838.1); Blastp hit to AAC75838.1 (429 aa), 95% identity in aa 1 - 429; putative HAAAP family serine transport protein [Salmonella typhimurium LT2].
STM2984	csdA	3.559786	similar to E. coli orf, hypothetical protein (AAC75852.1); Blastp hit to AAC75852.1 (401 aa), 89% identity in aa 1 - 401; putative selenocysteine lyase [Salmonella typhimurium LT2].
STM3022		1.6945873	
STM4326	aspA	0.43755987	similar to E. coli aspartate ammonia-lyase (aspartase) (AAC77099.1); Blastp hit to AAC77099.1 (493 aa), 97% identity in aa 16 - 493; aspartate ammonia-lyase; aspartase [Salmonella typhimurium LT2].
Carbohydrate transport and metabolism			
STM0007	talB	0.3914382	similar to E. coli transaldolase B (AAC73119.1); Blastp hit to AAC73119.1 (317 aa), 94% identity in aa 1 - 317; transaldolase B [Salmonella typhimurium LT2].
STM0491	gsk	1.9748945	Ortholog of E. coli inosine-guanosine kinase (AAC73579.1); Blastp hit to AAC73579.1 (434 aa), 94% identity in aa 1 - 434
STM0968	ycaD	1.6483	similar to E. coli putative transport (AAC73984.1); Blastp hit to AAC73984.1 (382 aa), 88% identity in aa 1 - 382; putative MFS family transport protein [Salmonella typhimurium LT2].
STM1467	manA	0.6342271	mannose-6-phosphate isomerase. (SW:MANA_SALTY); mannose-6-phosphate isomerase [Salmonella typhimurium LT2].
STM2300		0.3928249	
STM2546	suhB	4.462645	similar to E. coli enhances synthesis of sigma32 in mutant; extragenic suppressor, may modulate RNAse III lethal action (AAC75586.1); Blastp hit to AAC75586.1 (267 aa), 97% identity in aa 1 - 267; inositol monophosphatase [Salmonella typhimurium LT2].
STM2815	emrB	2.457679	similar to E. coli multidrug resistance; probably membrane translocase (AAC75733.1); Blastp hit to AAC75733.1 (512 aa), 95% identity in aa 1 - 512; putative MFS superfamily multidrug transport protein [Salmonella typhimurium LT2].
STM4087	glpF	0.26459196	similar to E. coli facilitated diffusion of glycerol (AAC76909.1); Blastp hit to AAC76909.1 (281 aa), 92% identity in aa 1 - 281; glycerol diffusion MIP channel [Salmonella typhimurium LT2].
STM4230	malK	0.4964398	bifunctional; ABC superfamily (atp_bind); maltose/maltodextrin transport ATP-binding protein malk. (SW:MALK_SALTY); maltose transport protein; phenotypic repressor of mal operon [Salmonella typhimurium
STM4290	proP	2.875156	Ortholog of E. coli low-affinity transport system; proline permease II (AAC77072.1); Blastp hit to AAC77072.1 (500 aa), 95% identity in aa 1 - 500
Cell motility and secretion			
STM1184	flgL	0.46746355	S. typhimurium flagellar hook-associated protein 3 (HAP3) (hook-filament junctionprotein). (SW:FLGL_SALTY)
STM1626	trg	0.3163375	ribose and galactose sensor receptor; similar to E. coli methyl-accepting chemotaxis protein III, ribose sensor receptor (AAC74503.1); Blastp hit to AAC74503.1 (546 aa), 79% identity in aa 6 - 526; methyl-accepting
STM1960	fliD	0.55615443	flagellar biosynthesis; enables filament assembly; flagellar hook-associated protein 2 (HAP2) (filament CAP protein)(flagellar CAP protein). (SW:FLID_SALTY); filament capping protein [Salmonella typhimurium LT2].
STM2894	invC	0.4258712	secretory proteins; probable ATP synthase SPAL. (SW:SPAL_SALTY); surface presentation of antigens [Salmonella typhimurium LT2].
Cell envelope biogenesis and OM			
STM0125	mraY	1.6941427	similar to E. coli phospho-N-acetylmuramoyl-pentapeptide transferase? (AAC73198.1); Blastp hit to AAC73198.1 (360 aa), 97% identity in aa 1 - 360; phospho-N-acetylmuramoyl-pentapeptide transferase
STM0666	lnt	2.135983	copper homeostasis protein; inner membrane; similar to E. coli apolipoprotein N-acyltransferase, copper homeostasis protein, inner membrane (AAC73758.1); Blastp hit to AAC73758.1 (512 aa), 89% identity in aa 1 - 512; apolipoprotein N-acyltransferase [Salmonella typhimurium LT2].
STM1732	ompW	0.379469	colicin S4 receptor; putative transporter; similar to E. coli putative outer membrane protein (AAC74338.1); Blastp hit to AAC74338.1 (212 aa), 88% identity in aa 1 - 212; outer membrane protein W [Salmonella Typhimurium LT2
STM2079	wzzB	1.5676478	Putative RBS for wzzB; RegulonDB:STMS1H002228
STM2080	udg	0.4551292	S. typhimurium UDP-glucose 6-dehydrogenase. (SW:UDG_SALTY)
STM2089	rfbJ	0.434289	Putative RBS for rfbJ; RegulonDB:STMS1H002236
STM2298	pmrF	0.62928617	similar to E. coli putative sugar transferase (AAC75314.1); Blastp hit to AAC75314.1 (322 aa), 87% identity in aa 1 - 318; putative glycosyl transferase [Salmonella typhimurium LT2].
STM2299	yfbG	0.33526444	hypothetical 73.6 Kda protein in ais-pmrD intergenic region. (SW:YFBG_SALTY); putative transformylase [Salmonella typhimurium LT2].
STM2301	pqaB	0.42729312	PqaB; affects polymycin B resistance and lipopolysaccharide synthesis; similar to E. coli orf, hypothetical protein (AAC75317.1); Blastp hit to AAC75317.1 (550 aa), 71% identity in aa 1 - 550; putative melittin resistance protein [Salmonella typhimurium LT2].
STM2439	yfeL	2.118134	penicillin-binding protein; hypothetical 20.5 Kda protein in pdxK-cysM intergenic region. (SW:YFEL_SALTY); putative membrane carboxypeptidase [Salmonella typhimurium LT2].
STM3031		0.654256	
STM3724	kdtA	2.4534893	KDO transferase; similar to E. coli 3-deoxy-D-manno-octulosonic-acid transferase (KDO transferase) (AAC76657.1); Blastp hit to AAC76657.1 (425 aa), 96% identity in aa 1 - 425; 3-deoxy-D-manno-octulosonic-acid transferase [Salmonella typhimurium LT2].
Coenzyme metabolism			
STM0422	dxs	1.5248274	Ortholog of E. coli 1-deoxyxylulose-5-phosphate synthase; flavoprotein (AAC73523.1); Blastp hit to AAC73523.1 (620 aa), 96% identity in aa 1 - 620
STM0806	moaE	1.73779	similar to E. coli molybdopterin converting factor, subunit 2 (AAC73872.1); Blastp hit to AAC73872.1 (150 aa), 91% identity in aa 1 - 150; molybdopterin converting factor, subunit 2 [Salmonella typhimurium LT2].
Drug/analog resistance			
STM2814	emrA	2.3524168	similar to E. coli multidrug resistance secretion protein (AAC75732.1); Blastp hit to AAC75732.1 (390 aa), 89% identity in aa 1 - 390; multidrug resistance secretion protein [Salmonella typhimurium LT2].
STM2815	emrB	2.457679	similar to E. coli multidrug resistance; probably membrane translocase (AAC75733.1); Blastp hit to AAC75733.1 (512 aa), 95% identity in aa 1 - 512; putative MFS superfamily multidrug transport protein [Salmonella typhimurium LT2].
Global regulatory function			
STM0398	phoR	1.62859	Ortholog of E. coli positive and negative sensor protein for pho regulon (AAC73503.1); Blastp hit to AAC73503.1 (431 aa), 90% identity in aa 1 - 431
STM2546	suhB	4.462645	similar to E. coli enhances synthesis of sigma32 in mutant; extragenic suppressor, may modulate RNAse III lethal action (AAC75586.1); Blastp hit to AAC75586.1 (267 aa), 97% identity in aa 1 - 267; inositol monophosphatase [Salmonella typhimurium LT2].
STM3466	crp	0.58347833	catabolite gene activator (CAMP receptor protein) (CAMP-regulatoryprotein). (SW:CRP_SALTY); catabolite activator protein (CAP); cyclic AMP receptor protein (CRP family) [Salmonella typhimurium LT2].
Inorganic ion transport and metabolism			
STM0425	thiI	1.977267	
STM0492	ybaL	1.935829	similar to E. coli putative transport protein (AAC73580.1); Blastp hit to AAC73580.1 (558 aa), 94% identity in aa 1 - 556; putative CPA2 family transport protein [Salmonella typhimurium LT2].
STM1483	ydgE	2.48227	similar to E. coli possible chaperone (AAC74671.1); Blastp hit to AAC74671.1 (109 aa), 84% identity in aa 3 - 109; putative membrane transporter of cations and cationic drugs [Salmonella typhimurium LT2].
Nucleotide transport and metabolism			
STM3167		0.536925	

pSLT

PSLT076	traY	0.5288665

Signal transduction

STM0398	phoR	1.62859	Ortholog of E. coli positive and negative sensor protein for pho regulon (AAC73503.1); Blastp hit to AAC73503.1 (431 aa), 90% identity in aa 1 - 431
STM1987		1.8766178	
STM2314		0.29318234	
STM2672	yfiN	2.66188	Ortholog of E. coli orf, hypothetical protein (AAC75653.1); Blastp hit to AAC75653.1 (408 aa), 75% identity in aa 2 - 405
STM3466	crp	0.58347833	catabolite gene activator (CAMP receptor protein) (CAMP-regulatoryprotein). (SW:CRP_SALTY); catabolite activator protein (CAP); cyclic AMP receptor protein (CRP family) [Salmonella typhimurium LT2].

Energy production and conversion

STM0152	aceE	0.36217234	similar to E. coli pyruvate dehydrogenase (decarboxylase component) (AAC73225.1); Blastp hit to AAC73225.1 (887 aa), 96% identity in aa 1 - 887; pyruvate dehydrogenase, decarboxylase component [Salmonella typhimurium LT2].
STM0153	aceF	0.3973649	dihydrolipoyltransacetylase component; similar to E. coli pyruvate dehydrogenase (dihydrolipoyltransacetylase component) (AAC73226.1); Blastp hit to AAC73226.1 (630 aa), 93% identity in aa 1 - 630; pyruvate dehydrogenase [Salmonella typhimurium LT2].
STM0732	sdhC	0.447988	similar to E. coli succinate dehydrogenase, cytochrome b556 (AAC73815.1); Blastp hit to AAC73815.1 (129 aa), 92% identity in aa 1 - 129; succinate dehydrogenase, cytochrome b556 [Salmonella typhimurium LT2].
STM0733	sdhD	0.28647473	Ortholog of E. coli succinate dehydrogenase, hydrophobic subunit (AAC73816.1); Blastp hit to AAC73816.1 (115 aa), 94% identity in aa 1 - 115
STM0735	sdhB	0.51159	Ortholog of E. coli succinate dehydrogenase, iron sulfur protein (AAC73818.1); Blastp hit to AAC73818.1 (238 aa), 96% identity in aa 1 - 238
STM0741	cydB	0.4453657	similar to E. coli cytochrome d terminal oxidase polypeptide subunit II (AAC73828.1); Blastp hit to AAC73828.1 (379 aa), 92% identity in aa 1 - 379; cytochrome d terminal oxidase polypeptide subunit II [Salmonella typhimurium LT2].
STM0973	pflB	0.3471967	Putative RBS for pflB; RegulonDB:STMS1H001259
STM1569	fdnH	0.6122913	nitrate-inducible; similar to E. coli formate dehydrogenase-N, nitrate-inducible, iron-sulfur beta subunit (AAD13439.1); Blastp hit to AAD13439.1 (294 aa), 93% identity in aa 1 - 294; formate dehydrogenase-N, Fe-S beta subunit [Salmonella typhimurium LT2].
STM1570	fdnG	0.4838479	similar to E. coli formate dehydrogenase-N, nitrate-inducible, alpha subunit (AAD13438.1); Blastp hit to AAD13438.1 (1015 aa), 93% identity in aa 1 - 1015; contains selenocysteine tRNA suppressible codon; putative molybdopterin oxidoreductases [Salmonella typhimurium LT2].
STM1749	adhE	0.524785	similar to E. coli CoA-linked acetaldehyde dehydrogenase and iron-dependent alcohol dehydrogenase; pyruvate-formate-lyase deactivase (AAC74323.1); Blastp hit to AAC74323.1 (891 aa), 97% identity in aa 1 - 891; iron-dependent alcohol dehydrogenase of the multifunctional alcohol dehydrogenase AdhE [Salmonella typhimurium LT2].
STM2316	nuoN	0.48786	similar to E. coli NADH dehydrogenase I chain N (AAC75336.1); Blastp hit to AAC75336.1 (425 aa), 95% identity in aa 1 - 425; NADH dehydrogenase I chain N [Salmonella typhimurium LT2].
STM2317	nuoM	0.44982365	similar to E. coli NADH dehydrogenase I chain M (AAC75337.1); Blastp hit to AAC75337.1 (509 aa), 96% identity in aa 1 - 509; NADH dehydrogenase I chain M [Salmonella typhimurium LT2].
STM2318	nuoL	0.4274567	similar to E. coli NADH dehydrogenase I chain L (AAC75338.1); Blastp hit to AAC75338.1 (613 aa), 94% identity in aa 1 - 613; NADH dehydrogenase I chain L [Salmonella typhimurium LT2].
STM2322	nuoH	0.4678277	nadh dehydrogenase I chain H. (SW:NUOH_SALTY); NADH dehydrogenase I chain H [Salmonella typhimurium LT2].
STM2323	nuoG	0.6153196	nadh dehydrogenase I chain G. (SW:NUOG_SALTY); NADH dehydrogenase I chain G [Salmonella typhimurium LT2].
STM2326	nuoC	0.5468371	nadh dehydrogenase I chain C/D. (SW:NUCD_SALTY); NADH dehydrogenase I chain C,D [Salmonella typhimurium LT2].
STM2327	nuoB	0.5123744	similar to E. coli NADH dehydrogenase I chain B (AAC75347.1); Blastp hit to AAC75347.1 (220 aa), 99% identity in aa 1 - 220; NADH dehydrogenase I chain B [Salmonella typhimurium LT2].
STM2337	ackA	0.49951687	propionate kinase 2; similar to E. coli acetate kinase (AAC75356.1); Blastp hit to AAC75356.1 (400 aa), 97% identity in aa 1 - 400; acetate kinase A [Salmonella typhimurium LT2].
STM3149	hybA	0.59847474	initially thought to be hydrogenase-2 small subunit which now identified as hybO; similar to E. coli hydrogenase-2 small subunit (AAC76032.1); Blastp hit to AAC76032.1 (328 aa), 95% identity in aa 1 - 328; unknown [Salmonella typhimurium LT2].
STM3500	pckA	0.3318487	phosphoenolpyruvate carboxykinase [ATP]. (SW:PPCK_SALTY); phosphoenolpyruvate carboxykinase [Salmonella typhimurium LT2].
STM3875	mioC	3.42172	similar to E. coli initiation of chromosome replication (AAC76765.1); Blastp hit to AAC76765.1 (147 aa), 87% identity in aa 1 - 147; initiation of chromosome replication [Salmonella typhimurium LT2].
STM4283	gltP	1.5683196	Ortholog of E. coli glutamate-aspartate symport protein (AAD13460.1); Blastp hit to AAD13460.1 (437 aa), 94% identity in aa 1 - 435

Surface structure

STM1184	flgL	0.46746355	S. typhimurium flagellar hook-associated protein 3 (HAP3) (hook-filament junctionprotein). (SW:FLGL_SALTY)
STM1960	fliD	0.55615443	flagellar biosynthesis; enables filament assembly; flagellar hook-associated protein 2 (HAP2) (filament CAP protein)(flagellar CAP protein). (SW:FLID_SALTY); filament capping protein [Salmonella typhimurium LT2].

Translation, ribosomal structure and biogenesis

STM0882	ybjF	1.7189455	similar to E. coli putative enzyme (AAC73946.1); Blastp hit to AAC73946.1 (375 aa), 87% identity in aa 1 - 372; putative tRNA (uracil-5-)-methyltransferase [Salmonella typhimurium LT2].
STM1167	rimJ	0.5644542	similar to E. coli acetylation of N-terminal alanine of 30S ribosomal subunit protein S5 (AAC74150.1); Blastp hit to AAC74150.1 (194 aa), 95% identity in aa 1 - 194; acetylation of N-terminal alanine of 30S ribosomal subunit protein S5 [Salmonella typhimurium LT2].
STM1187	rluC	1.8523933	similar to E. coli orf, hypothetical protein (AAC74170.1); Blastp hit to AAC74170.1 (319 aa), 97% identity in aa 1 - 319; 23S rRNA pseudouridylate synthase [Salmonella typhimurium LT2].
STM1334	infC	0.4772977	translation initiation factor IF-3. (SW:IF3_SALTY); protein chain initiation factor IF-3 [Salmonella typhimurium LT2].
STM1784	ychF	1.5313538	similar to E. coli putative GTP-binding protein (AAC74287.1); Blastp hit to AAC74287.1 (363 aa), 95% identity in aa 1 - 363; putative GTP-binding protein [Salmonella typhimurium LT2].
STM2222	rsuA	2.896299	similar to E. coli 16S pseudouridylate 516 synthase (AAC75244.1); Blastp hit to AAC75244.1 (231 aa), 94% identity in aa 1 - 230; 16S rRNA pseudouridylate 516 synthase [Salmonella typhimurium LT2].
STM2648	yfiF	1.614976	Putative RBS for yfiF; RegulonDB:STMS1H002741
STM3220	ygjO	1.628372	Putative RBS for ygjO; RegulonDB:STMS1H003248
STM3284	truB	0.3694835	similar to E. coli tRNA pseudouridine 5S synthase (AAC76200.1); Blastp hit to AAC76200.1 (314 aa), 93% identity in aa 1 - 314; tRNA pseudouridine 5S synthase [Salmonella typhimurium LT2].
STM3285	rbfA	0.3823642	role in processing of 10S rRNA; similar to E. coli ribosome-binding factor A (AAC76201.1); Blastp hit to AAC76201.1 (133 aa), 97% identity in aa 1 - 133; ribosome-binding factor [Salmonella typhimurium LT2].
STM3433	rplP	0.19943394	similar to E. coli 50S ribosomal subunit protein L16 (AAC76338.1); Blastp hit to AAC76338.1 (136 aa), 98% identity in aa 1 - 136; 50S ribosomal subunit protein L16 [Salmonella typhimurium LT2].

Islands

STM0276		0.68479455	
STM0426	phnV	2.229546	Putative RBS for phnV; RegulonDB:STMS1H000774
STM0859		1.733799	
STM1184	flgL	0.46746355	S. typhimurium flagellar hook-associated protein 3 (HAP3) (hook-filament junctionprotein). (SW:FLGL_SALTY)
STM1380	orf32	0.6215885	proline iminopeptidase like protein (gi|1526980); putative hydrolase or acyltransferase [Salmonella typhimurium LT2].
STM2038	pduA	0.3967658	polyhedral bodies; propanediol utilization protein PDUA. (SW:PDUA_SALTY); propanediol utilization protein [Salmonella typhimurium LT2].
STM2089	rfbJ	0.434289	Putative RBS for rfbJ; RegulonDB:STMS1H002236
STM2694		0.5831747	Putative RBS for STM2694; RegulonDB:STMS1H002780
STM2763		0.6447787	
STM2882	sipA	0.367162	SipA (gi|1172128); cell invasion protein [Salmonella typhimurium LT2].
STM2894	invC	0.4258712	secretory proteins; probable ATP synthase SPAL. (SW:SPAL_SALTY); surface presentation of antigens [Salmonella typhimurium LT2].
STM3031		0.654256	
STM3758	fidL	2.1544247	Pathogenicity island encoded protein: SPI3; FidL (gi|4324611); putative inner membrane protein [Salmonella typhimurium LT2].
STM3759	marT	2.29753	Pathogenicity island encoded protein: SPI3; putative transcriptional regulator MarT (gi|4324612); putative transcriptional regulatory protein [Salmonella typhimurium LT2].
STM3762	cigR	0.577116	Pathogenicity island encoded protein: SPI3; CigR (gi|4324614); putative inner membrane protein [Salmonella typhimurium LT2].
STM4200		0.6155267	
STM4215		0.36274347	Putative RBS for STM4215; RegulonDB:STMS1H004122

SPI1 to SPI5

STM1380	orf32	0.6215885	proline iminopeptidase like protein (gi|1526980); putative hydrolase or acyltransferase [Salmonella typhimurium LT2].
STM2882	sipA	0.367162	SipA (gi|1172128); cell invasion protein [Salmonella typhimurium LT2].
STM2894	invC	0.4258712	secretory proteins; probable ATP synthase SPAL. (SW:SPAL_SALTY); surface presentation of antigens [Salmonella typhimurium LT2].
STM3758	fidL	2.1544247	Pathogenicity island encoded protein: SPI3; FidL. (gi|4324611); putative inner membrane protein [Salmonella typhimurium LT2].
STM3759	marT	2.29753	Pathogenicity island encoded protein: SPI3; putative transcriptional regulator MarT (gi|4324614); putative transcriptional regulatory protein [Salmonella typhimurium LT2].
STM3762	cigR	0.577116	Pathogenicity island encoded protein: SPI3; CigR (gi|4324614); putative inner membrane protein [Salmonella typhimurium LT2].
STM3434	rpsC	0.17285423	similar to E. coli 30S ribosomal subunit protein S3 (AAC76339.1); Blastp hit to AAC76339.1 (233 aa), 100% identity in aa 1 - 233; 30S ribosomal subunit protein S3 [Salmonella typhimurium LT2].

Transcription

STM0516	allR	0.5464696	similar to E. coli putative regulator (AAC73608.1); Blastp hit to AAC73608.1 (271 aa), 91% identity in aa 1 - 268; putative regulatory protein [Salmonella typhimurium LT2].
STM0859		1.733799	
STM0959	lrp	0.5731178	S. typhimurium leucine-responsive regulatory protein. (SW:LRP_SALTY)
STM1588	yncC	1.8578427	similar to E. coli orf, hypothetical protein (AAC74532.1); Blastp hit to AAC74532.1 (240 aa), 58% identity in aa 20 - 238; putative gntR family regulatory protein [Salmonella typhimurium LT2].
STM3064	iciA	2.413778	LysR family; similar to E. coli replication initiation inhibitor, binds to 13-mers at oriC (AAC75953.1); Blastp hit to AAC75953.1 (297 aa), 97% identity in aa 1 - 297; inhibitor of replication initiation; transcriptional regulator of dnaA and argK [Salmonella typhimurium LT2].
STM3662	xylR	1.7719843	AraC/XylS family; similar to E. coli putative regulator of xyl operon (AAC76593.1); Blastp hit to AAC76593.1 (392 aa), 92% identity in aa 1 - 392; xylose operon regulatory protein [Salmonella typhimurium LT2].
STM3759	marT	2.29753	Pathogenicity island encoded protein: SPI3; putative transcriptional regulator MarT (gi|4324612); putative transcriptional regulatory protein [Salmonella typhimurium LT2].

Unknown function

STM0276		0.68479455	
STM1282	yeaK	2.13639	similar to E. coli orf, hypothetical protein (AAC74857.1); Blastp hit to AAC74857.1 (167 aa), 87% identity in aa 1 - 165; putative cytoplasmic protein [Salmonella typhimurium LT2].
STM1701	yciW	1.569138	similar to E. coli putative oxidoreductase (AAC74369.1); Blastp hit to AAC74369.1 (401 aa), 51% identity in aa 27 - 350; putative cytoplasmic protein [Salmonella typhimurium LT2].
STM2335	yfbU	0.67814	similar to E. coli orf, hypothetical protein (AAC75354.1); Blastp hit to AAC75354.1 (170 aa), 95% identity in aa 7 - 170; putative cytoplasmic protein [Salmonella typhimurium LT2].
STM3065	yggE	1.67294	similar to E. coli putative actin (AAC75959.1); Blastp hit to AAC75959.1 (246 aa), 88% identity in aa 1 - 245; putative periplasmic immunogenic protein [Salmonella typhimurium LT2].
STM3176	ygiW	0.5157943	similar to E. coli orf, hypothetical protein (AAC76060.1); Blastp hit to AAC76060.1 (130 aa), 89% identity in aa 1 - 130; putative outer membrane protein [Salmonella typhimurium LT2].
STM3332	yhcG	0.56955796	similar to E. coli orf, hypothetical protein (AAC76252.1); Blastp hit to AAC76252.1 (375 aa), 55% identity in aa 115 - 164; putative cytoplasmic protein [Salmonella typhimurium LT2].
STM3364	yhcP	1.6777942	similar to E. coli orf, hypothetical protein (AAC76272.1); Blastp hit to AAC76272.1 (655 aa), 92% identity in aa 1 - 655; putative inner membrane protein [Salmonella typhimurium LT2].
STM4288	phnB	1.8613836	Putative RBS for phnB; RegulonDB:STMS1H004185

DISCUSSION

Both DON and salmonellosis are worldwide emerging issues posing a threat for both animal and human health. To the best of our knowledge this is the first time that the combined effect of DON and an enteropathogenic pathogen such as *Salmonella* Typhimurium *in vivo* is demonstrated.

A porcine gut loop model was used to determine whether co-exposure to a physiologically relevant concentration of DON could potentiate the early intestinal immune response induced by *Salmonella* Typhimurium infection in the intestine. The choice for administering 1 µg/mL of DON was based mainly on the Commission Recommendation 2006/576/EC setting down 0.9 µg/g of DON in complementary and complete feeding stuff for pigs as guidance value (European Commission, 2006). Also, DON concentrations in human intestine are estimated between 0.160 µg/mL and 2 µg/mL depending on the contamination level of the food (Sergent *et al*., 2006). Moreover, 1 µg/mL was found non-cytotoxic to differentiated IPEC-J2 cells. Undifferentiated proliferating IPEC-J2 cells are more susceptible to the toxic effects of DON compared to highly differentiated cells, as shown by the cytotoxicity assay. These results correspond with the literature describing DON to be less cytotoxic for differentiated Caco-2 cells compared to dividing cells. No cytotoxicity was observed after 24 h exposure in differentiated cells whereas inhibitory concentrations of 10% (IC_{10}) value in the range 0.9 – 1.2 µM (0.260 – 0.356 µg/mL) were measured for dividing cells (Bony *et al*., 2006).

The results presented in this study provide evidence that DON at low but relevant intestinal concentrations enhances the intestinal inflammatory response to *Salmonella* Typhimurium. This is indicated by a significantly higher expression of IL-12 and TNFα ($p < 0.05$) and a clear potentiation of the expression of IL-1β, IL-8, MCP-1 and IL-6 in loops co-exposed to 1 µg/mL of DON and *Salmonella* Typhimurium. Whereas Maresca *et al*. (2008) described a dose-dependent increase in IL-8 mRNA levels in Caco-2 cells when exposed to concentrations of DON from 10 to 100 µM ($\approx$ 3 - 30 µg/mL), exposure of the ileal loops to 1 µg/mL of DON alone did not significantly affect the mRNA expression of any cytokine and chemokines tested. A possible explanation could be the lower dose of DON used in the ileal loop (1 µg/mL).

The enhanced intestinal inflammation seen in the co-exposed loops may be due to a stimulation of *Salmonella* Typhimurium invasion in and translocation through the intestinal epithelium, as indicated by the results of the assays performed on both dividing and differentiated IPEC-J2 cells. It has indeed been suggested that bacterial invasion stimulates

the innate pathways of inflammation by recognition of the pathogen-associated molecular patterns of *Salmonella* Typhimurium by Toll-like receptors (TLRs) present on epithelial cells and monocytes resulting in the production of several cytokines such as IL-8 (Tukel *et al*., 2006).

Microarray analysis on the *Salmonella* Typhimurium gene expression revealed that exposure to DON only changed the gene expression in logarithmic phase culture of *Salmonella* Typhimurium and among these, only six genes of possible importance in the pathogenesis of a *Salmonella* infection were affected. The *marT* gene, a non essential gene for virulence, encodes for a regulatory protein and could be involved in other aspects of pathogenesis, such as chronic infection and host specificity. The *fidl* and *cigR* gene products do not exhibit sequence similarity to proteins with known functions in the sequence database (Blanco-Potard *et al*., 1999). The genes *fliD* and *flgL* encode for proteins involved in flagellar biosynthesis (Yokoseki *et al*., 1995) and the effector protein SipA has been shown to modulate actin dynamics in order to promote *Salmonella* entry into epithelial cells (Higashide *et al*., 2002). The general reduction in expression of genes involved in energy production indicates a possible toxic effect of DON on *Salmonella*. The increased expression of *emrAB* multidrug efflux systems suggests that these could be involved in the removal of DON out of the bacterium. No changes in gene expression were seen in the stationary phase culture of *Salmonella* Typhimurium exposed to DON, indicating that the *Salmonella* virulence was not enhanced by DON. Since none of these bacterial gene expression alterations seem to offer a clear explanation for the increased invasion and translocation of *Salmonella* Typhimurium and the subsequent potentiated inflammatory response seen after coexposure to DON, we can thus conclude that the action of DON is more likely to occur on the epithelial cells than on *Salmonella*. This enhanced intestinal inflammation may be of importance for patients genetically predisposed for IBD since both DON and *Salmonella* are mentioned as factors of potential etiological importance in the development of this chronic intestinal disorder (Gradel *et al*., 2009; Maresca and Fantini, 2010; Jess *et al*., 2011).

The potency of non-cytotoxic concentrations of DON to enhance the susceptibility of porcine host cells and porcine macrophages in specific, to *Salmonella* invasion is also described in the experimental studies in chapter 2 (Vandenbroucke *et al*., 2009).

In pigs, intake of DON contaminated feed might result in a higher infection level in the herd and consequently a higher public health risk for salmonellosis from the consumption of contaminated pork meat.

In conclusion, our results indicate that the intake of DON contaminated food or feed at realistic contamination levels and after short-term exposure, could render the intestinal epithelium more susceptible for invasive food pathogens such as *Salmonella* Typhimurium with a subsequent amplification of the inflammatory processes in the gut. Considering the frequent occurrence of DON in cereal-based foods and feeds worldwide, the importance of these findings should not be underestimated.

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Chapter 2.

Effect of DON on the systemic phase of a *Salmonella* Typhimurium infection in the pig

The mycotoxin deoxynivalenol promotes uptake of *Salmonella* Typhimurium in porcine macrophages, associated with ERK1/2 induced cytoskeleton reorganization

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ABSTRACT

Both the mycotoxin deoxynivalenol (DON) and *Salmonella* Typhimurium are major issues in swine production. This study aimed at examining the interaction between DON and *Salmonella* Typhimurium at the level of the porcine innate immune system, represented by macrophages. First, we assessed the direct cytotoxic effect of DON on porcine macrophages. Incubation with 0.25 µg/mL of DON or higher resulted in a significant cytotoxic effect after 24 h of incubation. Secondly, the direct toxic effect of DON on the growth and on the expression of SPI-1 and SPI-2 virulence genes of *Salmonella* Typhimurium was determined. At low non-cytotoxic concentrations, as can be found in the serum of pigs, DON did not have any effect on either growth or virulence gene expression of *Salmonella* Typhimurium. However, when the invasion and intracellular survival of *Salmonella* Typhimurium in macrophages pre-exposed to 0.025 µg/mL of DON was examined, DON significantly promoted the uptake of *Salmonella* Typhimurium into macrophages. The enhanced uptake coincided with marked F-actin reorganization of the cells, which was due to the activation of extracellular signal-regulated protein kinase 1/2 (ERK1/2). These results suggest that low but relevant concentrations of DON modulate the innate immune system and could thus increase the susceptibility of pigs to infections with *Salmonella* Typhimurium.

INTRODUCTION

In the northern temperate regions the mycotoxin deoxynivalenol (DON) is one of the most frequent contaminants of maize and small grain cereal (Bottalico and Perrone, 2002). This contamination of cereal crops in the field is seen under low temperature and high humidity conditions. If critical concentrations of DON in diets for farm animals are exceeded, the health, growth and reproductive performance of animals may be impaired (Döll and Dänicke, 2004). The toxic effects of DON have been well documented and among farm animals, pigs seem to be particularly sensitive to the dietary intake of DON. DON has been associated with symptoms varying from partial feed refusal and decreased feed intake at feed concentrations as low as 1-2 mg/kg feed, to vomiting and complete feed refusal at concentrations of more than 20 mg/kg feed (Trenholm *et al*., 1988; Haschek *et al*., 2002). Substantial economic losses have therefore been attributed to DON contamination of pig feed (Böhm, 1992).

The mechanism of toxicity for trichothecenes, to which DON belongs, is complex, but the biochemical basis is non-competitive inhibition of different steps in the protein synthesis by interfering with peptidyltransferase at the active site on ribosomes (Cole and Cox, 1981). However, many of their toxic effects might also be related to a rapidly ensuing dysregulation of intracellular cell signaling and consequent alterations in downstream gene expression (Pestka, 2008). Exposure to low levels of trichothecenes appears to promote expression of a diverse array of cytokines and pro-inflammatory genes *in vitro* and *in vivo* via a mechanism known as the ribotoxic stress response that involves multiple intracellular signaling cascades (Zhou *et al*., 2003, 2005; Pestka *et al*., 2004). Trichothecenes such as DON are also known to rapidly activate mitogen-activated protein kinases (MAPKs) via a process termed the 'ribotoxic stress response'. These MAPKs modulate numerous physiological processes including cell growth, differentiation and apoptosis and are crucial for the signal transduction in the immune response. The members of the MAPKs family can be classified into three subfamilies: extracellular signal-regulated kinase (ERK)/MAPK, p38 and c-Jun-N-terminal kinase (JNK). Yang *et al*. (2008) described that DON induces p21 mRNA stability in terms of interaction with RNA-binding proteins via ERK1/2 activation. P21-activated kinases (PAK) are known to modulate cell morphology, actin/microtubule dynamics and cell motility (Bokoch, 2003) whereas ERK is able to phosphorylate several target proteins in the cytoplasm including cytoskeletal proteins. In contrast to these stimulatory effects, high doses of trichothecenes promote rapid onset of leucocyte apoptosis and this will be manifested as

immunosuppression (Pestka *et al*., 2004).

In European countries, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the predominant serovar isolated from pigs, which most often carry the bacterium without obvious symptoms (Fedorka-Cray *et al*., 2000; Boyen *et al*., 2008). These carrier pigs, however, play an important role as a source of contamination for the environment and for other animals and after slaughter, their carcasses can be a source of contamination for other carcasses in the slaughter facility (Donné *et al*., 2005). Macrophages play an important role in the pathogenesis of *Salmonella* infections in pigs as the bacteria are able to survive and even multiply intracellularly after bacterial entry into the cells. Macrophage invasion coincides with membrane ruffles, bacterium uptake and formation of *Salmonella* containing vacuoles (Finlay *et al*., 1991; Monack *et al*., 1996). Two major virulence determinants of *Salmonella* Typhimurium are the *Salmonella* pathogenicity island (SPI) 1 for invasion and SPI-2 for intracellular proliferation (Marcus *et al*., 2000; Boyen *et al*., 2008). Two major regulators for the SPI-1 or SPI-2 gene expression are respectively HilA and SsrA.

Several studies describe an increased susceptibility to experimental or natural mucosal infections after the ingestion of some mycotoxins (Tai *et al*., 1988; Fukata *et al*., 1996; Stoev *et al*., 2000). Hara-Kudo *et al*. (1996) examined the effects of DON on *Salmonella* Enteritidis infection in mice and suggested that administration of 2 mg/L DON in the drinking water reduced resistance to peroral infection of *Salmonella* Enteritidis presumably by inhibiting the cell-mediated immune function. However, until now there are no data available describing a possible interaction between DON and the pathogenesis of a *Salmonella* Typhimurium infection in pigs. The aim of the present study was to examine the effect of DON on the interaction of *Salmonella* Typhimurium with porcine macrophages, at low DON levels that are not cytotoxic and do not impair *Salmonella* growth and virulence gene expression.

MATERIALS AND METHODS

Chemicals

DON, ERK1/2 inhibitor (U0126 ethanolate) (Favata *et al*., 1998), ERK1/2 activator phorbol myristate acetate (PMA) (Das *et al*., 2000) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DON stock solution of 4 mg/mL was prepared in anhydrous methanol and stored at -20 °C. Serial dilutions of DON were, depending on the experiment, prepared in Luria-Broth (LB, Sigma-Aldrich, Steinheim, Germany) or in the corresponding cell medium allowing the addition of similar volume of vehicle in all experiments. Macrophages seeded onto 24-well or 96-well plates were treated with selected concentrations of DON or equivalent volumes of medium (untreated cells). The ERK1/2 inhibitor U0126 ethanolate was dissolved in DMSO leading to a 3 mM stock solution and stored at -20 °C. For experiments, U0126 ethanolate was diluted in the corresponding cell medium to a final concentration of 10 μM. The PMA stock solution was prepared by dissolving 5 mg of PMA in 1 mL of ethanol and stored in small aliquots at -20 °C. Prior to use, the stock solution was further diluted in the corresponding cell medium to a final concentration of 50 ng/mL.

Bacterial strains and growth conditions

Salmonella enterica subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) strain 112910a was used as the wild type strain (WT) in which all mutant strains were constructed. To obtain highly invasive late logarithmic cultures for invasion assays (Lundberg *et al*., 1999), 2 μL of a stationary phase culture was inoculated in 5 mL LB and grown for 5 h at 37 °C without aeration. The construction and characterization of a deletion mutant in the gene encoding the SPI-1 translocator/effector protein SipB has been described before (Boyen *et al*., 2006). *Salmonella* Typhimurium strains carrying the plasmid containing either the *hilA-luxCDABE* or the *ssrA-luxCDABE* transcriptional fusions were used in the virulence gene expression experiments (Boyen *et al*., 2006). For fluorescence microscopy, the pFPV25.1 plasmid expressing green fluorescent protein under the constitutive promoter of *rpsM* was used (Valdivia and Falkow, 1996; van Immerseel *et al*., 2004; Boyen *et al*., 2008).

Isolation of porcine pulmonary alveolar macrophages

Porcine pulmonary alveolar macrophages (PAM) were isolated by broncho-alveolar washes from lungs of euthanized 3 to 4 week old piglets, obtained from a *Salmonella*-negative farm, as described previously (Dom *et al.*, 1992). The isolated cells were pooled and frozen in liquid nitrogen until further use. Prior to seeding the PAM, frozen aliquots of approximately 10^7 cells/mL, were thawed in phosphate-buffered saline (PBS) with 10% fetal calf serum (FKS, Hyclone, Cramlington, England) at 4 °C. Cells were washed 3 times in PBS and cultured in RPMI (Gibco, Life Technologies, Paisley, Scotland) containing 2 mM L-glutamine (Gibco) and 1 mM sodium pyruvate (Gibco).

Cytotoxicity assays

The Cell Proliferation Reagent WST-1 kit from Roche Applied Science (Bazel, Switzerland) was used to assess the direct cytotoxic effect of DON on PAM. The test was used according to the manufacturer's instructions. The absorbance of DON-treated wells was measured at 450 nm using a microplate ELISA reader (Multiscan MS, Thermo Labsystems, Helsinki, Finland) and compared with a solvent-treated control. The percentages of DON induced cytotoxicity were calculated using the following formula:

$$\% \text{ cytotoxicity} = 100 \times \frac{(c-b)-(a-b)}{(c-b)}$$

In this formula $a = \text{OD}_{450}$ derived from the wells incubated with DON, $b = \text{OD}_{450}$ derived from blank wells, $c = \text{OD}_{450}$ derived from untreated control wells.

In addition to the WST-1 assay, the lactate dehydrogenase cytotoxicity detection kit (LDH, Roche Applied Science) was used to measure cytotoxicity and cell lysis by detecting LDH activity released from damaged cells. The test was used in accordance to the manufacturer's instructions and an ELISA plate reader at 492 nm was used to measure the absorbance.

Effect of DON on the growth and on SPI-1 and SPI-2 virulence gene expression of *Salmonella* Typhimurium

The effect of concentrations of DON from 0.005 to 50 µg/mL on *Salmonella* Typhimurium wild type was examined during 24 h in order to detect a direct toxic effect on the growth of the bacteria. Therefore, *Salmonella* Typhimurium was grown overnight in LB with aeration at 37 °C after which a suspension of the bacteria (Mc Farland 0.5) was added to the different

concentrations of DON in a 96-well plate. The plate was incubated at 37 °C and after 24 h of incubation, the number of colony forming units (CFU) per mL was determined by titration. This step was carried out by making tenfold dilutions of 20 µL of the bacterial suspensions. Then six 20 µL samples of each dilution were inoculated on Brilliant Green agar (BGA) and incubated for 24 h at 37 °C after which the colonies were counted.

A FluoroScan Ascent fluorometer (ThermoLabsystems) was used to quantify SPI-1 and SPI-2 expression of *Salmonella* Typhimurium by measuring the light production (luminescence) of *Salmonella* Typhimurium strains carrying the plasmids containing either the *hilA-lux CDABE* or the *ssrA-lux CDABE* transcriptional fusions. Bacterial cultures were grown in microplates in 200 µL of LB medium supplemented with the different concentrations of DON and in non-supplemented LB medium at 37 °C. Light production was measured every 15 min during 24 h and expressed as the relative gene expression.

Invasion and intracellular survival assays

To examine whether the ability of *Salmonella* Typhimurium and its isogenic *sipB* mutant to invade and proliferate in PAM was altered after pre-exposure of the macrophages to DON, invasion and intracellular survival assays were performed. Therefore, PAM were seeded in 24-well plates at a density of approximately 5×10^5 cells per well and were allowed to attach for at least 2 h. These wells were exposed to different non-cytotoxic concentrations of DON (0.005-0.100 µg/mL). After 24 h, the wells were rinsed and inoculated with *Salmonella* Typhimurium at a multiplicity of infection (moi) of 10:1. To synchronize the infection, the inoculated multiwell plates were centrifuged at 365 g for 10 min. After 30 min incubation at 37 °C under 5% CO_2, the wells were rinsed and fresh medium supplemented with 50 µg/mL gentamicin (Gibco) was added for 1 h. For the invasion assay the PAM were lysed with 1% (v/v) Triton-X (Sigma-Aldrich) for 10 min and 10-fold dilutions were plated out on BGA plates. To assess intracellular growth, the medium containing 50 µg/mL gentamicin was replaced after 1 h incubation with fresh medium supplemented with 10 µg/mL gentamicin and the number of viable bacteria was assessed 6 hours after infection as described above. All measurements were performed in triplicate and the experiment was carried out on 3 independent occasions.

To visualize the effect of DON on the number of cell-associated *Salmonella* bacteria, PAM were seeded in sterile Lab-tek® chambered coverglasses (VWR, Leuven, Belgium) and exposed to 0.025 µg/mL of DON in cell medium or to cell medium only for 24 h at 37 °C. Subsequently the invasion and proliferation assay was performed as described before after

inoculation with green fluorescent protein (gfp)-producing *Salmonella*. Cells were washed three times to remove unbound bacteria. Cell trace® calcein red-orange (Molecular Probes Europe, Leiden, The Netherlands) was added for 30 min at 37°C. Afterwards, cells were washed three times with PBS and fluorescence microscopy was carried out. In 100 macrophages, the number of macrophages containing gfp-*Salmonella* was counted and the average number of cell associated bacteria was calculated.

Morphological changes in macrophages

Staining techniques were performed to visualize possible morphological changes in macrophages exposed to DON in order to explain the observed difference in uptake of *Salmonella* Typhimurium in macrophages whether or not exposed to DON.

Haemacolor staining

PAM were seeded onto 13-mm-circular glass slides (VWR) in a 24-well plate at a concentration of 1×10^6 cells/mL and were incubated at 37 °C in 5% CO_2 for at least 2 h. Subsequently, either 0.025 µg/mL of DON or cell medium was added. After incubation of 24 h at 37 °C in 5% CO_2, cells were washed three times with PBS containing Ca^{2+} and Mg^{2+} (PBS+) and stained with Haemacolor® stain (Merck, Darmstadt, Germany). Glass slides were mounted with coverslips and observed microscopically in at least three time-independent assays. Attention was paid to cellular and nuclear changes with special focus on changes of the cell membrane, presence of membrane ruffles and apoptotic changes in the nucleus.

Staining of F-actin with phalloidin-Texas Red X®

PAM were seeded onto 13-mm-circular glass slides in a 24-well plate at a concentration of 1×10^6 per mL. After 2 h of incubation, the cells were exposed to cell medium, 0.025 µg/mL of DON, 10 µM of the ERK1/2 inhibitor U0126 ethanolate in combination with 0.025 µg/mL DON or 50 ng/mL of the ERK1/2 activator PMA for 24 h at 37 °C in 5% CO_2. PAM exposed to cell medium served as negative control. Thereafter, they were gently washed twice with 0.5 mL PBS+ at 37 °C, fixed with 0.5 mL 3.0% paraformaldehyde for 10 min at room temperature, gently washed again with 0.5 mL PBS+ and then permeabilized with 0.5 mL 0.1% Triton X-100 in PBS+ for 2 min at room temperature. Following washing with PBS+, 0.25 mL phalloidin-Texas Red X® (Molecular Probes, Eugene, Oregon, USA) (1:100 in PBS+) was added to each well and the 24-well plate was incubated at 37 °C for 1 h. To remove unbound phalloidin, coverslips were gently washed twice with 0.5 mL PBS+ before

visualization of the actin filaments with a fluorescence microscope (Leica Microsystem GmbH, Heidelberg, Germany).

Apoptosis and necrosis staining

A caspase-3 staining in combination with ethidium monoazide (EMA; Sigma-Aldrich) and counterstaining with Hoechst was performed to examine whether the observed cell morphology changes were associated with apoptosis or necrosis. Activation of caspases plays a central role in apoptosis with caspase-3 being one of the key effectors. EMA staining was used for the detection of nonviable cells, which can be either apoptotic or necrotic. The Hoechst staining, used as a nucleus staining technique, helped in the determination of cells as apoptotic or necrotic. Macrophages were described as apoptotic when the caspase-3 staining was positive and the EMA staining positive (late apoptosis) or negative (early apoptosis). Necrotic macrophages were negative for caspase-3 but positive for EMA. Normal living cells were negative for both caspase-3 and EMA.

PAM were seeded in a 24-well plate at 1×10^6 per mL and incubated at 37 °C in 5% CO_2 to allow adhesion. After 2 h, 0.025 µg/mL of DON was added for 24 h. Cell medium served as negative control. Macrophages exposed to 3 µM of staurosporine for 3 h served as a positive control for apoptosis. For the staining protocol, cells were detached from the well through incubation with 0.3 mL trypsine for 10 min and pooled in a falcon tube together with the supernatant. After centrifugation (7 min, 365 g), the cells were resuspended in 0.5 mL of ice-cold PBS. After another centrifugation step, the supernatant was prelevated and the pellet was resuspended in 0.5 mL of ice-cold EMA (1:20 in PBS). After an incubation step in the dark for 20 min, the falcon tubes were put under a light bulb during 10 min after which they were centrifuged (7 min, 365 g). After removal of the supernatant, the cells were fixed by resuspending the pellet in 0.5 mL paraformaldehyde 3% (w/v) in PBS for 10 min at room temperature. After a centrifugation (7 min, 365 g) and washing step with 0.5 mL PBS, the cells were permeabilized by incubating in 0.1 mL Triton-X 0.1% for 2 min followed by the addition of 0.9 mL PBS. From this point on, PBS was supplemented with 20% FCS to prevent the cells from sticking to the tube wall. Again, the cells were centrifuged followed by the addition of 0.1 mL of rabbit anti-active caspase-3 (Sigma-Aldrich) diluted 1:100 in PBS. After 1 h incubation at 37 °C, the cells were centrifuged, washed with PBS and afterwards 0.1 mL of goat anti-rabbit FITC (1:50 in PBS) (Sigma-Aldrich) was added. The cells were incubated at 37 °C, protected from light, for 1 h. After centrifugation and washing in 0.5 mL PBS, the cells were counterstained with Hoechst (1 mg/mL, 1/100 dilution in PBS) for 15 min

protected from light. After a centrifugation step (7 min, 365 g) and a washing step with PBS, the remaining pellet was resuspended in 20-40 µL of PBS and 10 µL was mounted in a small drop of mounting medium (DABCO-glycerine). The cells were observed with a fluorescence microscope.

Statistical Analysis

All experiments were conducted in triplicate with three repeats per experiment, unless otherwise noted. The data were analyzed using ANOVA to address the significance of difference between mean values with significance level set at $p < 0.05$.

RESULTS

Low DON concentrations do not affect macrophage viability

The possible direct cytotoxic effect of DON on PAM at concentrations ranging from 0.005 to 50 µg/mL as determined by the WST-1 assay is shown in Figure 1. DON had no significant effect at concentrations ≤ 0.100 µg/mL. At 0.250 µg/mL, DON had a significant cytotoxic effect of $85 \pm 5\%$ ($p < 0.05$) after 24 h of incubation. Concentrations of DON > 0.250 µg/mL showed a cytotoxicity of more than 97% ($p < 0.05$). The cytotoxicity determined by the LDH test was similar to the previous results (data not shown). Based on these results, only non cytotoxic concentrations of DON (< 0.250 µg/mL) were used in the further experiments.
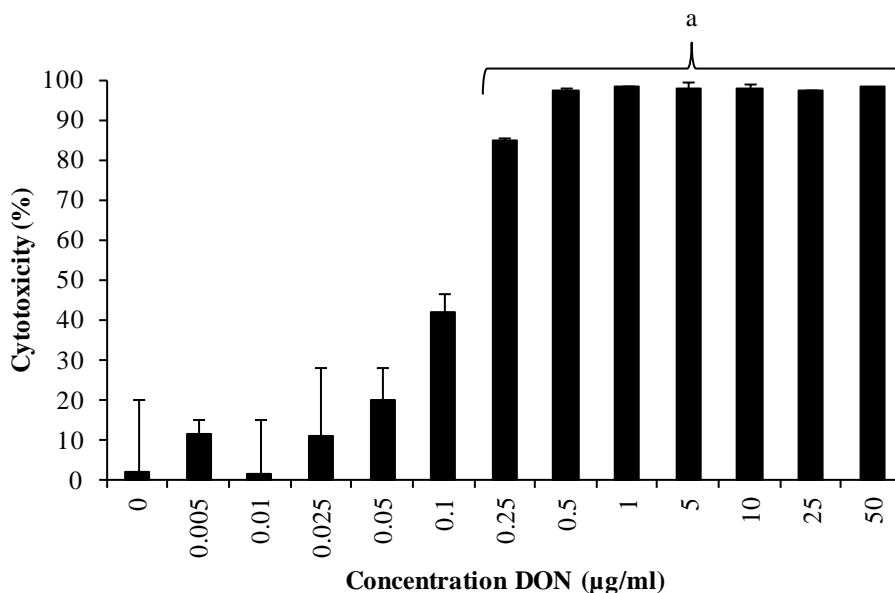
**Figure 1**: Percentage cytotoxicity in porcine macrophages exposed to DON at 0.005 to 50 µg/mL. Twenty-four hours later, the cytotoxic effect was determined by WST-1 assay. Results represent the means of 3 independent experiments conducted in triplicate and their standard deviation. Superscript (a) refers to a significantly higher cytotoxic effect compared to the unexposed control wells ($p < 0.05$).

High concentrations of DON increase SPI-1 and SPI-2 expression of *Salmonella* Typhimurium

Concentrations of DON from 0.005 to 50 µg/mL did not have any effect on the growth of *Salmonella* Typhimurium wild type (data not shown).

High concentrations of DON ($\geq$ 5 µg/mL) in LB medium significantly increased the expression of *hilA* and *ssrA*, which are regulators required for the expression of respectively the SPI-1 invasion genes or SPI-2 genes necessary for the intracellular replication and systemic infection of *Salmonella* Typhimurium ($p < 0.05$). The results are shown in Figure 2.
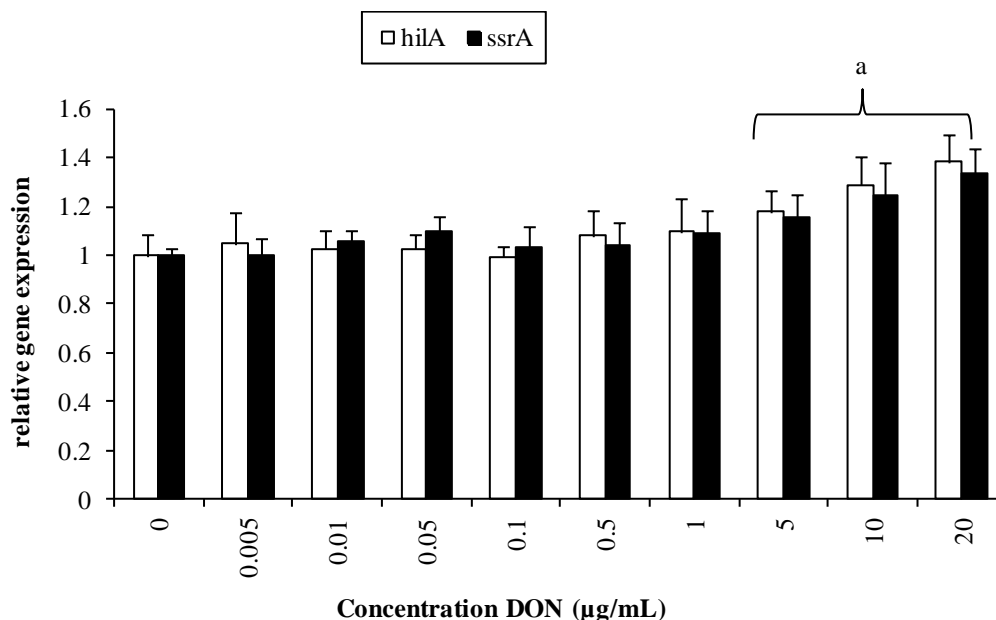
Figure 2: The expression of *hilA* and *ssrA* in *Salmonella* Typhimurium grown in LB medium supplemented with different concentrations of DON. The light production was measured every 15 min during 24 h and is expressed as the relative gene expression. The results represent the means of 3 independent experiments conducted in triplicate and their standard deviation. Superscript (a) refers to a significantly higher expression of *hilA* and *ssrA* compared to the unexposed control wells ($p < 0.05$).

DON promotes the uptake of *Salmonella* Typhimurium in porcine macrophages

The results of the invasion and proliferation test of *Salmonella* Typhimurium WT in PAM with or without prior exposure to DON are summarized in Figure 3A. Uptake was higher in the PAM exposed to 0.025 µg/mL of DON compared to the PAM that were not exposed to DON ($p < 0.05$), with an average increase factor of 1.45. Similar results were obtained using the deletion mutant *sipB* (Fig. 3B). Intracellular replication, represented by log cfu/mL after proliferation minus log cfu/mL after invasion in porcine macrophages, of *Salmonella* Typhimurium WT in porcine macrophages whether or not exposed to 0.025 µg/mL of DON did not differ significantly (0.48 ± 0.339 versus 0.91 ± 0.529 respectively).
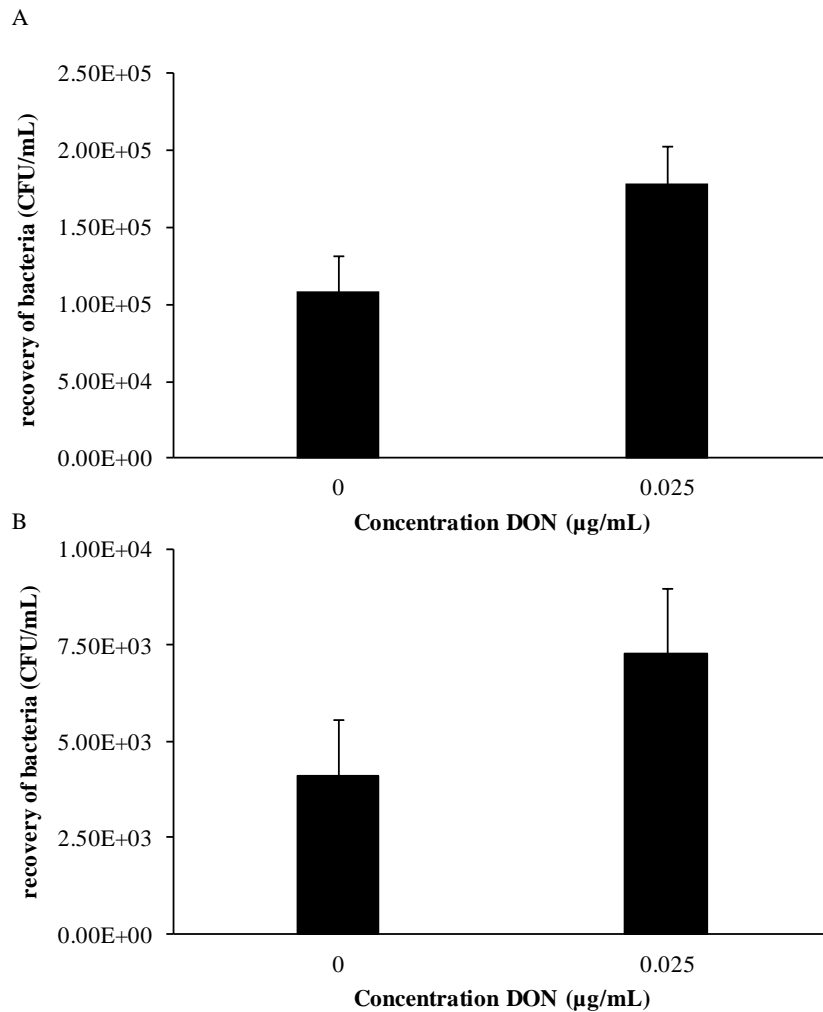
Figure 3: The invasiveness of *Salmonella* Typhimurium WT (A) and its isogenic ΔSipB (B) in porcine pulmonary macrophages whether or not exposed to 0.025 µg/mL of DON is shown. The number of gentamicin-protected bacteria is shown. The results show a representative of three independent experiments conducted in sixfold ± standard deviation for *Salmonella* Typhimurium WT and of two independent experiments conducted in sixfold ± standard deviation for the deletion mutant in *SipB*.

The enhanced uptake of *Salmonella* Typhimurium in PAM exposed to 0.025 µg/mL of DON was confirmed in an invasion and proliferation assay with gfp-*Salmonella*. The results of this experiment are summarized in Table I. Macrophages exposed to 0.025 µg/mL of DON were more frequently infected by *Salmonella* Typhimurium in comparison with the control PAM resulting in a higher bacterial count (average 3.9 versus 2.7 bacteria per macrophage, respectively) and a consequent higher number of bacteria in the PAM after 6 h. The proliferation rate of intracellular bacteria however, did not differ significantly between the control and the DON treated macrophages (2.8 versus 2.6 respectively).

Table I. Results of the invasion and proliferation assay performed with gfp-producing *Salmonella*. The results show a representative of two independent experiments conducted in threefold. The number of cell associated bacteria was counted in 100 macrophages. The results represent the mean bacterial count per macrophages ± standard error of the mean (sem) after invasion (0 h) and after proliferation (6 h), as well as the proliferation rate.

	Mean bacterial count ± sem		**Proliferation rate**
PAM	0 h	6 h	
control	2.7 ± 0.11	7.6 ± 0.35	2.8
0.025 µg/mL DON	3.9 ± 0.13	10.1 ± 0.39	2.6

DON causes morphological alterations and F-actin reorganization in porcine macrophages by activation of ERK1/2

PAM were stained with Haemacolor after exposure to 0.025 µg/mL of DON for an incubation time of 24 h. This staining technique was used to test the hypothesis that DON alters the cell morphology and more specifically induces changes in the cell membranes which might result in an enhanced uptake of *Salmonella* Typhimurium WT. DON caused marked morphological alterations as the majority of the PAM showed ruffling of the cell (Fig. 4A-B). To examine the actin cytoskeleton, phalloidin was used as a specific probe for polymerized (F) actin. Macrophages exposed to 0.025 µg/mL of DON showed some marked morphological changes when compared with the non exposed controls. Macrophages incubated with 0.025 µg/mL of DON for 24 h demonstrated increased formation of ruffling membranes in $78.5 \pm 2.26\%$ of the macrophages in comparison with the control macrophages ($10.3 \pm 3.79\%$) (Fig. 4C-D).

In order to investigate whether the cytoskeletal rearrangements of macrophages after exposure to 0.025 µg/mL of DON, were due to the activation of the MAPK pathway, and more specifically ERK1/2, the actin staining experiment was repeated with on the one hand, DON in combination with the ERK1/2 inhibitor U0126 ethanolate, and on the other hand PMA, which is an ERK1/2 activator.

Macrophages incubated with 10 µM U0126 ethanolate in combination with 0.025 µg/mL of DON, for 24 h, showed a normal F-actin distribution. Only $11.0 \pm 2.00\%$ of the macrophages demonstrated ruffling membranes. Macrophages incubated with 50 ng/mL of PMA for 24 h demonstrated increased formation of ruffling membranes in $97.3 \pm 2.52\%$ of the macrophages in comparison with the control macrophages (Fig. 4E-F) and macrophages treated with U0126 ethanolate (data not shown). These results suggest that the increased membrane ruffling, caused by DON, is due to the activation of ERK1/2.
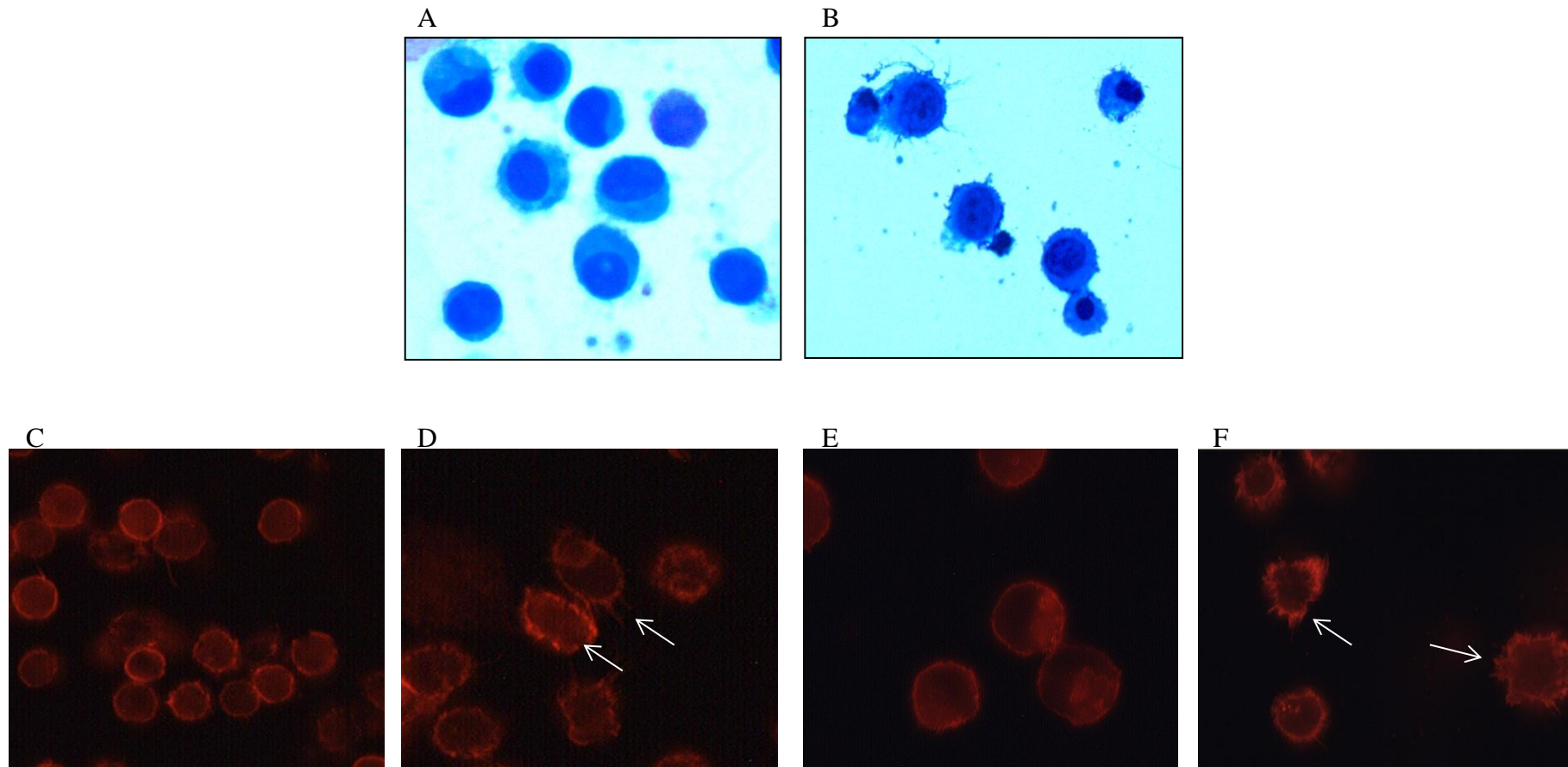
Figure 4: Porcine alveolar macrophages (PAM) whether or not exposed to 0.025 µg/mL of DON, stained with Haemacolor (A-B) or phalloidine-Texas Red® staining technique (C-F). Figure A shows porcine macrophages not exposed to DON, whereas Figure B shows marked membrane ruffling in the DON exposed macrophages. Figures C to F show fluorescence microscopic pictures of actin filament arrangement in PAM either treated with cell medium (C), 0.025 µg/mL of DON (D), 10 µM of the ERK1/2 inhibitor U0126 ethanolate in combination with 0.025 µg/mL of DON(E) or with 50 ng/mL of the ERK1/2 activator PMA (F), for 24 h. Control macrophages (C) and macrophages exposed to U0126 ethanolate in combination with DON (E) demonstrated a normal distribution of F-actin. DON (D) and PMA (F) exposed macrophages demonstrated increased formation of membrane ruffles (indicated by white arrows).

DON induced cell morphology changes are not associated with cellular apoptosis and necrosis

To determine the cellular apoptosis and necrosis of PAM exposed to 0.025 µg/mL of DON during 24 h, the EMA, caspase-3 and Hoechst staining were examined in 100 macrophages on three independent occasions.

No significant difference was seen between the PAM exposed to 0.025 µg/mL of DON and the control PAM which indicates that this concentration does not induce apoptosis nor necrosis. The results of this experiment are summarized in Table II.

Table II. Results of the apoptosis and necrosis staining of porcine alveolar macrophages (PAM) whether or not exposed to 0.025 µg/mL of DON. The results represent the average percentage of cells showing apoptosis or necrosis and their standard deviation. Normal cells are negative for both EMA and caspase-3, necrotic cells are positive for EMA but negative for caspase-3, apoptotic cells are always positive for caspase-3 but can be EMA positive (late apoptosis) or negative (early apoptosis).

	Average percentage (%) of cells ± sd		
PAM	**normal**	**apoptosis**	**necrosis**
control	80.7 ± 6.66	19.3 ± 6.66	0 ± 0
0.025 µg/mL DON	76.3 ± 5.03	21.0 ± 5.00	2.7 ± 2.08

DISCUSSION

DON and salmonellosis are emerging issues causing serious problems in the European pig industry. The toxic effects of the mycotoxin DON have been well documented in several animal species and in diverse cell culture experiments. Several publications indicate that the presence of this *Fusarium* mycotoxin in feed can seriously affect the health status of pigs and other animals (Rotter and Prelusky, 1996; Fink-Gremmels, 1999; Pinton *et al*., 2008). *Salmonella* Typhimurium is the predominant serovar isolated from pigs in Europe and since pigs may be subclinically infected, they are an important threat to both animal and human health. Although DON and *Salmonella* can be commonly encountered in the pig industry, this is the first report describing an interaction between DON and the pathogenesis of a *Salmonella* Typhimurium infection in pigs.

Concentrations of DON higher than 0.1 µg/mL had a significant cytotoxic effect on porcine macrophages after 24 h of exposure. These results correspond with the literature where DON was described to inhibit the proliferation of Caco-2 cells in a dose dependent manner, with a significant effect appearing at 0.2 µg/mL (Sergent *et al*., 2006). Bimczok *et al*. (2007), however, described that DON only had a significant cytotoxic effect on monocyte-derived dendritic cells at 0.8 µg/mL. To eliminate a possible cytotoxic effect of DON on the porcine macrophages, we chose to work with concentrations of DON below 0.1 µg/mL in the further experiments. These low concentrations are relevant in practice since in several publications, depending on the dietary content of DON, serum concentrations of DON varying from 0.013 to 0.026 µg/mL were measured in pigs (Dänicke *et al*., 2006; Goyarts *et al*., 2006).

The growth of *Salmonella* Typhimurium was not affected by concentrations of DON up to 50 µg/mL. Nevertheless, high concentrations of DON ($\geq$ 5 µg/mL) in LB medium, significantly increased the expression of *hilA* and *ssrA*. Although this is an interesting observation, concentrations of 5 µg/mL of DON are exceptional contamination levels under field conditions and hence not relevant for the *in vivo* situation.

Pre-exposure of macrophages to concentrations of DON as low as 0.025 µg/mL during 24 h, enhanced the susceptibility of macrophages for the uptake by *Salmonella* Typhimurium in the macrophages. On average, the colony forming units per mL were 150% higher compared to control macrophages. This was confirmed by microscopic evaluation using gfp-*Salmonella*.

Since 0.025 µg/mL of DON was proven not to affect the expression of SPI-1 and SPI-2 genes of *Salmonella* Typhimurium, the observed enhanced uptake might be caused by changes in the cell morphology. This hypothesis is also supported by the results of the invasion test with the ΔSipB strain which gave similar results as the wild type strain, indicating that the enhanced uptake seen after exposure of the PAM to DON, is not SPI-1 associated.

Salmonella entry in host cells involves a complex series of actin cytoskeletal changes (Guiney and Lesnick, 2005). When we examined the cell morphology focusing on the cell membrane changes, we saw marked changes in DON treated porcine macrophages compared to non treated controls. Macrophages exposed to 0.025 µg/mL of DON demonstrated alterations of the cell membrane on Haemacolor staining. Special attention was paid on the ruffling of the cell membrane as this is a cytoskeletal change that is commonly described with the entry of *Salmonella* Typhimurium in the macrophages. Therefore, staining with phalloidin-Texas Red®, as specific probe for polymerized actin, was performed which clearly indicated a reorganization of F-actin and formation of membrane ruffles.

ERK1/2, a MAPK, is activated both by trichothecenes such as DON via a process termed the 'ribotoxic stress response' and by the invasion of *Salmonella* Typhimurium in macrophages (Procyk *et al*., 1999; Zhou *et al*., 2005). Recently, Yang *et al*. (2008) described that DON induces p21 mRNA stability in human epithelial cells in terms of interactions with RNA binding proteins via ERK 1/2 activation. Since the p21 activated kinases and the Rho-family of small GTP'ases play a central role in both actin dynamics, membrane ruffling and *Salmonella* induced invasion, p21 mRNA stabilization might explain the changes in the cell membrane morphology seen in macrophages exposed to 0.025 µg/mL of DON and might account for the enhanced uptake of *Salmonella* Typhimurium in porcine macrophages pre-exposed to DON.

To test the hypothesis that the modulations of the cytoskeleton in macrophages, caused by DON, are due to the activation of ERK1/2, staining with phalloidin-Texas Red® was performed in macrophages after pre-exposure to an ERK1/2 activator (PMA) or an ERK1/2 inhibitor (U0126 ethanolate) in combination with 0.025 µg/mL of DON.
U0126 ethanolate inhibited the morphological rearrangements in macrophages that were exposed to DON, whereas PMA caused an increased formation of membrane ruffles. Moreover, PMA and DON treated macrophages have a similar morphology. These results

suggest that the increased membrane ruffling, caused by DON, is due to the activation of ERK1/2.

In conclusion, we have shown that low concentrations of DON could modulate the cytoskeleton of macrophages through ERK1/2 F-actin reorganization resulting in an enhanced uptake of *Salmonella* Typhimurium in porcine macrophages.

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GENERAL DISCUSSION

The knowledge that consumption of mycotoxins can seriously affect human and animal health has led to an increasing public awareness on the implications of occurrence of mycotoxins in agricultural commodities. The production of mycotoxins by fungi is not only a climate-, plant- and storage-dependent problem (Paterson and Lima, 2010), also agricultural practices and use of fungicides can affect the mycotoxin contamination level of crops (Simon, 2004). The trichothecene deoxynivalenol (DON), which is mainly produced during the growth of *Fusarium graminearum* on cereals, is one of the most prevalent mycotoxins in temperate climate regions (McMullen *et al.*, 1997). The ubiquitous distribution of DON, together with its resistance to processing, enables the toxin to persist in the human and animal food and feed supply. Whereas the clinical effects of acute exposure to high doses of DON are well documented, the consequences of chronic exposure to low subclinical doses are less specified. It is nevertheless true that the economic impact of decreased productivity, subtle but chronic damage to vital organs and tissues and increased disease incidence due to immune suppression is many times greater than that of acute livestock death (van Egmond and Speijers, 1994). Among farm animals, the pig is the most sensitive animal species to the toxic effects of DON. Being a highly sensitive food-producing animal of economic importance, the pig pre-eminently poses an animal model to study the impact of exposure to DON contaminated feed, not only for animal health but also for human health. Indeed, in addition to the mouse, the pig model has been proven to be a valuable tool for biomedical research due to its anatomic, physiologic and pathologic resemblance to human (Almond, 1996; Meurens *et al.*, 2012).

The molecular mode of action of DON lies in its ability to inhibit protein synthesis and to induce a 'ribotoxic stress response' characterized by the activation of mitogen-activated protein kinases (MAPKs) and their downstream targets (Shifrin and Anderson, 1999; Yang *et al.*, 2000; Pestka, 2008). By this mechanism of toxicity, DON can affect numerous organs and tissues. The continuously proliferating and differentiating intestinal epithelial cells of the gastrointestinal tract, as well as the macrophages and innate immune system in general, appear extremely sensitive to the toxic effects of DON. Because of the important role of these two cell populations in the pathogenesis of a *Salmonella* Typhimurium infection in the pig, the main objective of this thesis was to investigate whether low concentrations of DON could affect the pathogenesis of a *Salmonella* Typhimurium infection in the pig, focusing on the interaction between *Salmonella* Typhimurium and the porcine host. Since DON and salmonellosis are two important issues in pig production, a possible interaction between these

two food-borne agents could have great repercussions not only for animal productivity but also from public health perspective. Although *Salmonella* infections in pigs are typically asymptomatic, carrier pigs can pose a reservoir of infection for humans.

DON and *Salmonella* Typhimurium: working together at the intestinal level

With the gastrointestinal tract being the first organ to be encountered after the intake of DON contaminated food or feed, the intestinal alterations caused by exposure to DON have been addressed in several studies. Of great concern however, is the observation that also low and per definition non-cytotoxic concentrations of DON seem to be able to affect this intestinal barrier function in absence of alterations to the intestinal permeability, leading to the translocation of bacteria such as *Escherichia coli* (Maresca *et al.,* 2008). One could thus expect that this finding might not only be limited to microbiota present in the gut, but might also influence the intestinal phase of the pathogenesis of some adhesive-invasive enteropathogenic bacteria such as *Salmonella* Typhimurium.

The invasion of the bacterium in the intestinal epithelium is an important step in the intestinal phase of a *Salmonella* Typhimurium infection. Factors enhancing this essential step might thus lead towards a higher bacterial load of the gut. Our *in vitro* studies on porcine intestinal epithelial cells showed that pre-exposure to non-cytotoxic concentrations of DON rendered the intestinal monolayer more susceptible to invasion by *Salmonella* Typhimurium, with a subsequent enhanced translocation of the bacterium through an intact intestinal monolayer. The finding that there was an enhanced *Salmonella* invasion in and translocation through the intestinal epithelium at levels of DON plausibly present in the porcine gut lumen after intake of commonly encountered contamination levels in feeding-stuffs, questions the current guidance values (European Commission, 2006). Indeed, whereas the intake of feed according to these guidance levels will probably not lead to acute clinical symptoms in the pig, the effects of subclinical doses have clearly been neglected up till now.

Prudence however is in order when extrapolating *in vitro* results to *in vivo* situations. *In vitro* experiments might overestimate the risk due to mycotoxins as they do not take into account *in vivo* factors able to reduce the final exposure levels, *e.g.* the degrading capacity of bacteria present in the gut lumen. On the other hand, the single mycotoxin exposure mimicked *in vitro* does not totally reflect the *in vivo* situation where multiple contamination poses an important

issue (Grenier and Oswald, 2011) and synergistic actions between different mycotoxins might produce even more detrimental effects in the animal.

**DON and *Salmonella* Typhimurium: united enemies of the immune system?**

The interaction between DON and *Salmonella* Typhimurium on the innate immune system was illustrated in this thesis, not only at the level of the intestinal epithelial cell-derivate immune defence, but also at the level of the macrophage.

The capacity of some mycotoxins, including DON, to alter inflammation, cellular and humoral immune responses was reviewed by Oswald *et al*. (2005). The consequences of the immunomodulation by DON in farm animals comprise both the animal health as public health level as this might decrease resistance to infectious disease, reactivate chronic infection or reduce vaccine and therapeutic efficacy.

Using a porcine ligated intestinal ileal loop model, we were able to demonstrate that DON at low and relevant intestinal concentrations enhanced the intestinal inflammatory response to *Salmonella* Typhimurium. Exposure of the ileal loops to DON alone did not alter the mRNA expression level of any cytokine and chemokines tested. Together with the *in vitro* finding that DON rendered the intestinal epithelium more susceptible to invasion and translocation of *Salmonella* Typhimurium, one could suggest that intake of DON contaminated feed might increase the host's susceptibility to the pathogen.

By promoting inflammation, the innate immune system attempts to defend the host against *e.g*. invading pathogens. The intestinal lumen is colonized by a dense bacterial community called microbiota which protects efficiently against intestinal colonization by most enteric pathogens. Normally an infection with *Salmonella* Typhimurium generates an immune response that clears the infection within less than a week. However, it seems that *Salmonella* Typhimurium is able to trigger intestinal inflammation to create specific growth conditions in the intestinal lumen, which enable it to outcompete the resident microbiota, leading to gut colonization (Stecher *et al.,* 2007; reviewed by Santos *et al*., 2009). The ability of DON to enhance this intestinal inflammatory response when co-exposed to *Salmonella* Typhimurium, together with the fact that intake of DON contaminated feed modifies the dynamics of intestinal bacterial communities (Wache *et al.,* 2009), suggests an additional role of DON in

turning the balance towards *Salmonella* Typhimurium and in enhancing pro-inflammatory stimulation in gut epithelia (Figure 1).
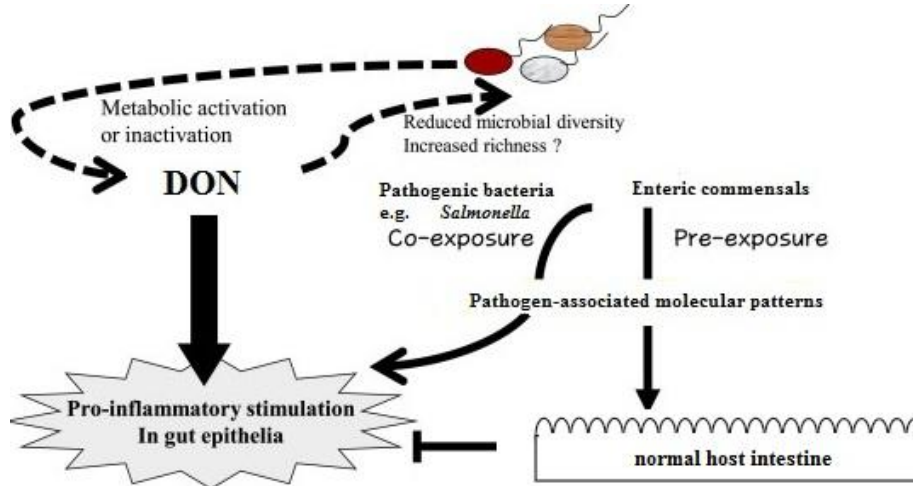
Figure 1: The crosstalk between ribotoxic stress and enteric bacteria. In the normal healthy host intestine beneficial interactions between the intestinal mucosa and the enteric commensals mediate mucosal tolerance and colonization resistance against incoming pathogenic bacteria. Pathogenic bacteria such as *Salmonella* employ specific virulence factors to trigger intestinal inflammation which can alter the luminal conditions and shift the growth competitionwith the commensals in favor of the pathogen. Intake of DON can affect the bacterial diversity and richness in the lumen but can also lead to pro-inflammatory ribotoxic insults. Pathogenic situations are associated with the pro-inflammatory stimulation by co-exposure to mucosal ribotoxic stress and enteric pathogens overwhelming the mucosal tolerance (adapted from Stecher *et al.,* 2007; Moon, 2012).

Although the importance of the systemic phase of a *Salmonella* Typhimurium infection in pigs is still questioned, macrophages appear to play an important role in the long term persistence of the bacterium in the gut and gut associated lymphoid tissues (Boyen *et al.,* 2006, 2009). Pre-exposure of porcine macrophages to DON resulted in a *Salmonella* Pathogenicity Island-1 (SPI-1) independent increased uptake of *Salmonella* Typhimurium, as proven by using a non-invasive strain ($\Delta sipB$). Again, this observation occurred after exposure of macrophages to a concentration of DON that is fairly low yet measurable in both serum and gastrointestinal tissues after consumption of DON contaminated feed.

As illustrated in this thesis with *Salmonella* Typhimurium, DON might possibly increase the susceptibility of pigs to infectious disease with subsequent reduced productivity. From public health point of view, an increased animal colonization with *Salmonella* Typhimurium may result in increased animal-to-human transmission.

DON and *Salmonella* Typhimurium: close collaboration in enhancing bacterial engulfment

The induction of dramatic cytoskeletal changes on the membrane surface of epithelial cells and macrophages is a hallmark of invasive *Salmonella* Typhimurium, in order to establish its entry into the cells. After binding to the surface of a macrophage, *Salmonella* Typhimurium induces generalized membrane ruffling that results in the internalization of the bacterium into the spacious phagosome (Aderem *et al.,* 1999).

When exposing macrophages to a concentration of DON that led to an enhanced uptake of *Salmonella* Typhimurium, this coincided with a membrane ruffling formation analogous to those seen at bacterial engulfment induced by SPI-1 encoded Type 3 Secretion System (T3SS) of *Salmonella* Typhimurium. This finding suggests a synergistic action between the mycotoxin and *Salmonella* Typhimurium in the bacterial invasion. By activating the mitogen-activated protein kinase (MAPK) cascade, in particular extracellular signal-regulated kinase (ERK) 1/2, DON appears to be able to enhance the uptake of *Salmonella* Typhimurium through membrane ruffling formation. As this concentration of DON did not induce apoptosis nor necrosis of the macrophage, this might suggest that DON could enhance the long term persistence of *Salmonella* Typhimurium in macrophages, although the role of macrophages in persistent colonization of pigs is still under debate.

Clearly, it cannot be denied that low non-cytotoxic concentrations of DON can affect the interaction of *Salmonella* Typhimurium with both the intestinal epithelial cell and the macrophage, in terms of an increased host cell susceptibility to the pathogen. However, one could assume that the toxin's effect might not only be limited to the eukaryotic cells but could also comprise the bacterium. Although exposure to even high concentrations of DON did not seem to affect the growth of *Salmonella* Typhimurium in itself, microarray analysis on the effect of DON on the gene expression of *Salmonella* Typhimurium revealed a general downregulation of the bacterium's metabolism and an increased expression of multidrug efflux systems. The effect of DON on the expression of genes involved in virulence and motility however, was limited. It appears thus that in the interaction with the pathogenesis of *Salmonella* Typhimurium infections, the effect of DON is more oriented towards the host cells than towards the bacterium. As knowledge on the effects of trichothecenes, or mycotoxins in general, on prokaryotic cells is scarce, not to say unexisting, this possible bacterial intoxication should be kept in mind when studying the interaction between other,

127

more potent toxins and pathogens. Indeed, research performed at our department on the interaction between T-2 toxin and the pathogenesis of a *Salmonella* Typhimurium infection in pigs showed that the effect of T-2 toxin was more pronounced on the level of the bacterium than at the host cell (Verbrugghe *et al.,* 2012) and this was seen at T-2 toxin contamination levels (0.01 and 0.1 mg/kg) measured in swine feed in field situations (Monbaliu *et al.,* 2010).

DON and *Salmonella* Typhimurium: could they call the tune in Inflammatory Bowel Disease?

Many gastrointestinal disorders including inflammatory bowel diseases (IBD) have been associated with mucosal intolerance derived from disruption of the epithelial barrier (Park *et al.,* 2010). A recent systematic review illustrated that the incidence and prevalence of IBD are increasing with time and in different regions around the world, indicating its emergence as global disease (Molodecky *et al.*, 2011). Genetic factors play an important role in the disease development although it appears that environmental factors might play an important etiological role. There is mechanistic evidence that the ribotoxic stress-induced cellular responses, as can be seen after intake of DON contaminated food, could be involved as critical etiological factors in mucosa-associated diseases, in particular epithelial inflammatory diseases (Maresca and Fantini, 2010; Moon, 2012). Moreover, enteric infections with *e.g. Salmonella* have also been suggested to play an etiological role in the development of IBD, triggering an already altered immune response or a defect in downregulation of the immune response, hence leading to the typical chronic intestinal inflammation seen with IBD (Helms *et al*., 2006; Gradel *et al*., 2009; Jess *et al.,* 2011).

Considering these facts above, the interaction between DON and *Salmonella* Typhimurium seen at both the intestinal epithelial cell level as macrophage level, might be rather alarming as this inflammatory crosstalk between enteric bacteria and ribotoxic stress can enhance pro-inflammatory responses and subsequent disorders.

There's no end to the mycotoxin's menace

Regulations relating to mycotoxins levels in food and feed have been established in many countries in order to ensure the health of both consumers and animals. However, the shortcoming of these regulations is that they are primarily based on known and particularly acute toxic effects of a single mycotoxin. Step by step it becomes clear that the subtle toxicity

of chronic low doses could be more pronounced than initially thought. These effects might even be more detrimental for pronounced cytotoxic trichothecenes such as T-2 toxin, for which, apart from provisional maximum tolerable daily intake (PMTDI) levels, no maximal doses in food or feed have been established yet (Maresca and Fantini, 2010). The fact that mycotoxins seldom occur as single contaminant also questions the applicability of the proposed safety levels.

Several pitfalls might be encountered when studying mycotoxicoses, starting at the level of food and feed analysis. Factors as inadequate sampling and the presence of masked or conjugated forms of mycotoxins that might escape the routine analysis, but could still present a risk after being enzymatically hydrolyzed in the gut, might also lead to an underestimation of correct values. Further research on the effects of these mycotoxins for animal and human health is thus required.

When assessing the risk associated with mycotoxin exposure, one should be aware that although the hazard is more or less the same all over the world, exposure varies due to different levels of contamination and dietary habits in the various parts of the world (van Egmond *et al.,* 2007). Indeed, the presence of mycotoxins in food is often overlooked in developing countries due to public ignorance about their existence, lack of regulatory mechanisms, dumping of food products and the introduction of contaminated commodities into the human food chain during chronic food shortage due to various factors (Wagacha *et al.,* 2008). This puts these low-income countries at high risk to the toxic effects of various mycotoxins with besides the direct health risk, enormous economic losses.

In this context, the development of validated biomarkers for mycotoxin exposure can be very useful. Up till now only few biomarkers have been validated for some specific mycotoxins, whereas more than 25 mycotoxins are frequently reported to contaminate foodstuff. Biomarkers of exposure for DON include the parent compound or glucuronide conjugate (Meky *et al*., 2003; Turner *et al*., 2008) whereas the clinical relevant growth related proteins insulin-like growth factor acid labile subunit (IGFALS) and the insulin-like growth factor 1 (IGF1) are potential biomarkers of effect (Riley *et al*., 2011). These detection tools for mycotoxin exposure can play a critical role in the effort to reduce the existing uncertainty in risk assessment of the most prevalent mycotoxins in humans but also in developing a better understanding of the impact of mycotoxin exposure on farm animal productivity (Baldwin *et al.*, 2011; Riley *et al.,* 2011).

Future research perspectives

Implications of the interaction between DON and Salmonella *Typhimurium for public health*

The results of the studies described in this thesis indicate that, when exposed to low and subclinical concentrations of DON, the porcine host becomes more susceptible to *Salmonella* Typhimurium infections. This enhanced susceptibility could lead to a higher infection level in the herd, posing a higher public health risk for salmonellosis through the consumption of contaminated pork meat. Moreover, since the intake of DON contaminated feed is known to cause in the pig an abdominal distress syndrome associated with a reduced feed intake, one could assume that this period of stress could induce re-excretion of *Salmonella* in asymptomatic carrier pigs. Indeed, it has been described that a natural stress stimulus like feed withdrawal causes recrudescence of a *Salmonella* Typhimurium infection in carrier pigs (Verbrugghe *et al*., 2011).

Porcine *in vivo* trials combining a chronic exposure to DON-contaminated feed with an experimental infection of *Salmonella* Typhimurium are necessary to investigate these assumptions.

Involvement of DON in human IBD

In order to determine the direct role of DON and trichothecene mycotoxins in general as mechanistic mediators in human IBD, toxicological risk assessments are needed. Therefore, DON or DON-glucuronide as biomarkers of exposure could be assessed in the human body and in specimens (urine) of normal and clinically diseased groups.

Another interesting aspect in the development of IBD is the involvement of the ATP-binding cassette protein (ABC) transporter P-glycoprotein (P-gp). Using knockout mice, a relationship between the downregulation of P-gp and the development of IBD has been demonstrated (Panwala *et al.,* 1998) and this reduced expression of P-gp was also observed in intestinal biopsies obtained from patients with gastrointestinal disorders (Blokzijl *et al.,* 2007). Although some ABC transporters including P-gp are used by intestinal epithelial cells to export ribotoxic trichothecenes (Tep *et al*., 2007; Videmann *et al*., 2007), no information is available on whether the intake of mycotoxin contaminated food or feed could modulate the expression of these ABC transporters. Recent studies described a role of P-gp in bacterial attachment to human gastrointestinal cells (Crowe *et al.,* 2011) as well as the capacity of *Salmonella* Typhimurium to functionally downregulate the efflux capabilities of P-gp

(Siccardi *et al.,* 2008). Interestingly, the results of our micro-array analysis showed that exposure of *Salmonella* Typhimurium to DON led to an increased expression of *emrAB* multidrug efflux systems in the bacterium, indicating, at least on prokaryotic level, the potential of DON to affect the expression of efflux systems. In this context, investigating the effect of both DON and co-exposure of DON and *Salmonella* Typhimurium on the expression and function of P-gp could offer valuable information, to gain not only better insights in the pathogenesis but also in the treatment and prevention of environmentally-linked human IBD.

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SUMMARY

The intake of deoxynivalenol (DON) contaminated food and feed as well as salmonellosis are ever-growing problems, not only in pig production because of their impact on animal health and productivity but also from public health perspective. The high sensitivity to the toxic effects of DON together with the high exposure level resulting from their cereal rich diet, sets the pig in an extremely vulnerable position toward the intake of DON through the feed. The European Union suggested a concentration of 0.9 mg/kg DON as a guidance value in complete feed and feedingstuffs for pigs (2006/576/EG). Salmonellosis remains one of the most important bacterial zoonoses worldwide and the importance of human *Salmonella* Typhimurium infections through the consumption of contaminated pork meat and derived products is still increasing. Although *Salmonella* Typhimurium infections in pigs can lead to a self-limiting enteritis, septicemia and death, most of the infections in pigs are subclinical. The latter can be persistently infected, thus posing an important source of contamination for the human food chain.

The inhibition of the protein synthesis and the activation of mitogen-activated protein kinases (MAPKs) in a process called the 'ribotoxic stress response', is the underlying mechanism for the toxicity of DON. Although these molecular mechanisms of toxicity can affect numerous organs and tissues, the rapidly dividing and differentiating intestinal epithelial cells and the macrophages, both being important components of the innate immune response, are extremely sensitive to the effects of DON. Because of the importance of these cells in the pathogenesis of a *Salmonella* Typhimurium infection in the pig, the overall aim of this thesis was to investigate whether co-occurrence of both DON and *Salmonella* Typhimurium may result in altered interactions between *Salmonella* Typhimurium and the porcine host.

In chapter one, the effects of low concentrations of DON on the intestinal phase of a *Salmonella* Typhimurium infection in the pig were examined.
In a first series of experiments, a polarized porcine intestinal epithelial cell model was used to examine whether the ability of *Salmonella* Typhimurium to invade in and translocate through the intestinal monolayer was affected after pre-exposure to DON. A distinction was made between actively dividing undifferentiated and fully differentiated intestinal epithelial cells, as these both cell populations are present in the gut. Concentrations of DON higher than 0.1 µg/mL had a significant cytotoxic effect on actively dividing cells, whereas concentrations of DON up to 10 µg/mL did not affect the viability of the fully differentiated cells. Pre-exposure to non-cytotoxic concentrations of DON not only promoted the invasion of *Salmonella*

Typhimurium in the intestinal epithelial cells but also led to an increased translocation of *Salmonella* through the intestinal epithelial monolayer without affecting the integrity of the cell monolayer.

Secondly, a porcine intestinal ileal loop model was used to study the effect of DON on the intestinal inflammatory response induced by *Salmonella* Typhimurium by analyzing the intestinal cytokine response. A real-time quantitative PCR was developed to quantify a series of 8 cytokines and chemokines (IL-1β, IL-6, IL-8, IL-12, IL-18, TNFα, IFNγ and MCP-1), all of them involved in the early intestinal immune response. Co-exposure of the ileal loops for 6 hours to 1 μg/mL of DON and *Salmonella* Typhimurium resulted in a potentiated intestinal immune response in comparison to loops incubated with *Salmonella* Typhimurium only. This was shown by the significant higher gene expression of TNFα and IL-12 and an increase in IL-1β, IL-6, IL-8 and MCP-1. Exposure to 1 μg/mL of DON without *Salmonella* Typhimurium did, however not affect the expression level of any cytokine and chemokine tested.

In a third experiment, the effect of DON on the gene expression of *Salmonella* Typhimurium was performed using a Salsa *Salmonella* serovar microarray, covering 5080 genes. This revealed that exposure of the logarithmic phase cultures of *Salmonella* Typhimurium to 0.250 μg/mL of DON, caused a differential expression of 159 genes. These included, apart from a general reduction in the expression of genes involved in energy production, also an increased expression of *emrAB* multidrug efflux systems. These findings could indicate a possible toxic effect of DON on the bacterium, but also an attempt of the bacterium to enhance the elimination of the toxin. The expression level of only six genes of possible importance in the pathogenesis of a *Salmonella* infection was affected by exposure to DON, including three genes (*marT*, *fidL* and *cigR*) with no described significant role for the virulence of *Salmonella* Typhimurium. Three other downregulated genes were involved either in the flagellar biosynthesis (*fliD* and *flgL*) or in the invasion of *Salmonella* Typhimurium in epithelial cells (*sipA*). No changes in gene expression were seen when exposing the stationary phase cultures of *Salmonella* Typhimurium to DON.

Taking together these results indicate that for the intestinal phase of a *Salmonella* Typhimurium infection, the action of DON is more likely to occur on the intestinal epithelial cell level than on *Salmonella*.

In the second chapter, the effect of exposure to DON on the systemic phase of infection was mimicked, by studying the uptake and intracellular proliferation of *Salmonella* Typhimurium in DON treated porcine pulmonary alveolar macrophages. Experiments showed that concentrations of DON, even up to 50 µg/mL did not affect the growth of *Salmonella* Typhimurium and that *Salmonella* Pathogenicity Island (SPI)-1 and SPI-2 virulence gene expression was only increased after exposure to concentrations of DON higher than 5 µg/mL. To exclude a direct cytotoxic effect of DON on the macrophages when studying the host-pathogen interactions, a non-cytotoxic concentration of DON (0.025 µg/mL) was selected. Even though this concentration is low, it is still relevant in practical situations. Pre-exposure of macrophages to 0.025 µg/ml of DON enhanced the uptake of *Salmonella* Typhimurium, which was confirmed by microscopic evaluation using a green fluorescent protein expressing strain of *Salmonella*. Similar results were obtained using the ΔSipB strain, indicating that this enhanced uptake seen after exposure of macrophages to DON, was not SPI-1dependent. The proliferation rate of *Salmonella* Typhimurium in macrophages was however not affected by pre-exposure to DON.

Morphological examination of macrophages treated with 0.025 µg/mL of DON demonstrated marked alterations of the cell membrane on Haemacolor staining when compared to untreated control macrophages. Additional fluorescence staining with phalloidin-Texas Red®, a specific probe for polymerized actin, showed an increased ruffling of the macrophage cell membrane by reorganization of F-actin after exposure to DON, and this morphological change was not associated with apoptosis nor necrosis. By using a specific extracellular signal-regulated kinase (ERK)1/2 activator and inhibitor, we demonstrated that this modulation of the cytoskeleton of porcine macrophages by DON was due to the activation of this specific MAPK family member, which is also described to play a role in the invasion of *Salmonella* Typhimurium in macrophages. In conclusion, these results propose a specific mode of action of DON to alter the morphology of macrophages, enhancing the uptake of *Salmonella* Typhimurium in this cell population.

To our knowledge, this is the first study describing an interaction between the mycotoxin DON and the pathogenesis of a *Salmonella* Typhimurium infection in the pig.

The results of this thesis clearly demonstrate that exposure to DON affects the interaction of the bacterium with both the intestinal epithelial cell and the macrophage, and through this renders the porcine host more susceptible to a *Salmonella* Typhimurium infection. The fact that these host-pathogen interactions occur at low and non-cytotoxic concentrations of DON,

stresses the need for further research on the toxicity of long term exposure to low doses of DON on both animal and human health.

Although this being a theoretical statement, the finding that exposure to DON leads to an enhanced invasion and translocation of *Salmonella* Typhimurium through an intact intestinal epithelium could offer perspectives in the research on the development of chronic intestinal inflammatory disease or IBD, in which both the intake of DON contaminated food and infections with *Salmonella* are put forward as potential etiological factors.

SAMENVATTING

Zowel de opname van voeder gecontamineerd met deoxynivalenol (DON) als salmonellose zijn in belang toenemende problemen, niet alleen in de varkensindustrie omwille van hun impact op de diergezondheid en productiviteit, maar ook omwille van hun risico voor de volksgezondheid. De hoge gevoeligheid voor de toxische effecten van DON, samen met het hoge blootstellingsrisico dat voorvloeit uit hun graanrijke dieet, maakt het varken tot één van de gevoeligste diersoorten voor de opname van DON via het voeder. De Europese Unie legde 0.9 mg/kg DON vast als richtwaarde voor aanvullende en volledige dierenvoeders voor varkens (2006/576/EG). Salmonellose blijft wereldwijd één van de belangrijkste bacteriële zoönosen en het belang van *Salmonella* Typhimurium infecties als gevolg van de consumptie van besmet varkensvlees en afgeleide producten neemt alleen maar toe. In een aantal zeldzame gevallen kunnen infecties met *Salmonella* Typhimurium leiden tot een zelflimiterende enteritis, septicemie en sterfte, de meeste infecties bij het varken blijven echter subklinisch. Nochtans kunnen deze varkens blijvend geïnfecteerd zijn en bijgevolg een belangrijke besmettingsbron vormen voor de menselijke voedselketen.

Het werkingsmechanisme van DON berust niet enkel op een inhibitie van de eiwitsynthese, het toxine is eveneens in staat om mitogen-activated protein kinases (MAPKs) te activeren via een mechanisme dat gekend is als 'ribotoxic stress reponse'. Hoewel het toxine op deze manier de functie van diverse organen en weefsels kan beïnvloeden, blijken zowel de sneldelende intestinale epitheliale cellen als de macrofagen, een belangrijk celtype in het aangeboren immuunsysteem, uitermate gevoelig voor de schadelijke effecten van DON. Deze cellen spelen bovendien een belangrijke rol in de pathogenese van *Salmonella* Typhimurium infecties bij het varken. Daarom was het hoofddoel van dit onderzoek om na te gaan of gelijktijdig voorkomen van zowel DON als *Salmonella* Typhimurium een invloed zou kunnen hebben op de interactie tussen *Salmonella* Typhimurium en het varken.

In hoofdstuk 1 werd het effect van lage concentraties DON op de intestinale fase van een *Salmonella* Typhimurium infectie bij het varken bestudeerd.

In een eerste reeks experimenten werd een gepolariseerde intestinale varkensepitheelcellijn gebruikt om na te gaan of het vermogen van *Salmonella* Typhimurium om te invaderen in en te transloceren doorheen een intestinale cellulaire monolaag, beïnvloed werd door voorbehandeling met DON. Hierin werd een onderscheid gemaakt tussen ongedifferentieerde actief delende cellen en volledig gedifferentieerde intestinale epitheelcellen, gezien deze celpopulaties aanwezig zijn in de darm. Er werd aangetoond dat DON concentraties hoger dan 0.1 µg/mL een significant cytotoxisch effect hadden op de actief delende cellen, terwijl DON

concentraties tot 10 µg/mL geen invloed hadden op de leefbaarheid van volledig gedifferentieerde cellen. Voorbehandeling met niet-cytotoxische concentraties DON zorgde niet alleen voor een verhoogde invasie van *Salmonella* Typhimurium in de intestinale epitheelcellen, het leidde eveneens tot een verhoogde passage van *Salmonella* doorheen de epitheliale celmonolaag zonder aantasting van de integriteit van deze monolaag.

Vervolgens werd een varkensdarmlusmodel gebruikt om, via analyse van de intestinale cytokine respons, de invloed van DON na te gaan op de intestinale ontstekingsreactie geïnduceerd door *Salmonella* Typhimurium. Hiertoe werd een kwantitatieve PCR methode ontwikkeld om een reeks van 8 cytokines en chemokines (IL-1β, IL-6, IL-8, IL-12, IL-18, TNFα, IFNγ en MCP-1), elk betrokken bij de vroege intestinale immuunreactie, te kwantificeren. Gelijktijdige blootstelling van ileale darmlussen gedurende 6 uur aan 1 µg/mL DON en *Salmonella* Typhimurium resulteerde in een versterkte intestinale immuunreactie in vergelijking met loops die enkel waren geïncubeerd met *Salmonella* Typhimurium. Dit werd aangetoond door de significant hogere genexpressie van TNFα en IL-12, en een verhoging van IL-1β, IL-6, IL-8 en MCP-1. Het expressiegehalte van geen enkel getest cytokine en chemokine werd echter beïnvloed door blootstelling aan 1 µg/mL DON alleen.

In een derde experiment werd het effect van DON op de genexpressie van *Salmonella* Typhimurium zelf nagegaan, door middel van een Salsa *Salmonella* serovar microarray, die 5080 genen omvat. Blootstelling van logaritmische fase culturen van *Salmonella* Typhimurium aan 0.250 µg/mL DON veroorzaakte een veranderd expressiepatroon van 159 genen. Deze omvatten, naast een algemene reductie van de expressie van genen betrokken in de energieproductie, ook een verhoogde expressie van *emrAB* multidrug efflux systemen. Deze bevindingen kunnen wijzen op een mogelijk toxisch effect van DON op de kiem, maar eveneens op een poging van de kiem om het toxine te elimineren. Het expressie patroon van slecht zes genen van mogelijk belang in de pathogenese van een *Salmonella* infectie werd beïnvloed door blootstelling aan DON, waaronder drie genen (*marT*, *fidL* en *cigR*) met een beperkte rol in de virulentie van *Salmonella* Typhimurium. Drie andere onderdrukte genen waren ofwel betrokken in de flagellaire biosynthese (*fliD* en *flgL*) ofwel in de invasie van *Salmonella* Typhimurium in epitheelcellen (*sipA*). Er werden geen veranderingen gezien in het genexpressie patroon bij blootstelling van stationaire fase culturen van *Salmonella* Typhimurium aan DON.

Samengevat tonen deze resultaten aan dat voor de intestinale fase van een *Salmonella* Typhimurium infectie, het effect van DON wellicht meer uitgesproken is op het niveau van de epitheelcellen dan op *Salmonella*.

In het tweede hoofdstuk werd het effect van DON op de macrofaag nagegaan, door de opname en intracellulaire vermeerdering van *Salmonella* Typhimurium te bestuderen in varkensmacrofagen die werden voorbehandeld met DON. Experimenten toonden aan dat hoge concentraties DON tot 50 µg/mL geen effect hadden op de groei van *Salmonella* Typhimurium en dat de expressie van virulentiegenen gelegen op de *Salmonella* Pathogeniciteitseilanden SPI-1 en SPI-2 (*Salmonella* Pathogenicity Islands; SPI) enkel werd verhoogd door concentraties DON hoger dan 5 µg/mL. Om een direct cytotoxisch effect van DON op de macrofagen uit te sluiten bij het bestuderen van de kiem-gastheer interacties, werd een niet-cytotoxische concentratie van DON (0.025 µg/mL) geselecteerd met behulp van een cytotoxiciteitstest. Hoewel deze concentratie laag is, blijkt ze toch nog van belang in praktijksituaties. Voorafgaandelijke blootstelling van macrofagen aan 0.025 µg/mL DON bevorderde de opname van *Salmonella* Typhimurium bacteriën, wat microscopisch werd bevestigd met behulp van een groen fluorescente *Salmonella* stam. Gelijkaardige resultaten werden bekomen met de deletiemutant in het *sipB* gen, hetgeen aantoonde dat de verhoogde opname na blootstelling van de macrofagen aan DON, SPI-1 onafhankelijk was. De vermeerderingsratio van *Salmonella* Typhimurium in macrofagen werd echter niet beïnvloed door voorafgaandelijke blootstelling aan DON.

Morfologisch onderzoek na Haemacolor kleuring van DON behandelde macrofagen toonde opmerkelijke veranderingen aan de celmembraan wanneer deze werden vergeleken met onbehandelde controle macrofagen. Bijkomende fluorescente kleuringen met phalloidine-Texas Red®, een specifieke probe voor gepolymeriseerd actine, toonde een verhoogde rafeling van de celmembraan van de macrofagen ten gevolge van een reorganisatie van F-actine na blootstelling aan DON. Deze morfologische verandering bleek niet geassocieerd te zijn met apoptose of necrose. Door gebruik te maken van een specifeke extracellular signal-regulated kinase (ERK)1/2 activator en inhibitor werd aangetoond dat deze cytoskeletale verandering van de varkensmacrofaag door DON te wijten was aan de activatie van dit specifiek eiwit van de MAPK familie. Dit eiwit speelt bovendien een rol in de opname van *Salmonella* Typhimurium in de macrofagen. Samengevat suggereren deze resultaten een specifiek werkingsmechanisme van DON om de morfologie van macrofagen te veranderen, hetgeen kan leiden tot een verhoogde opname van *Salmonella* Typhimurium in deze celpopulatie.

Voor zover bekend, is dit de eerste studie die een interactie beschrijft tussen het mycotoxine DON en de pathogenese van een *Salmonella* Typhimurium infectie bij het varken.

De resultaten van dit proefschrift tonen duidelijk aan dat blootstelling aan DON de interactie van de kiem met zowel de intestinale epitheliale cel als met de macrofaag beïnvloedt en het varken hierdoor meer gevoelig wordt voor een infectie met *Salmonella* Typhimurium. Het feit dat deze kiem-gastheer interactie voorkomt bij lage en niet-cytotoxische concentraties DON benadrukt de nood aan verder onderzoek naar de schadelijke effecten van chronische blootstelling aan lage concentraties DON voor de gezondheid van mens en dier.

Hoewel speculatief, zou de bevinding dat blootstelling aan DON leidt tot een verhoogde invasie en passage van *Salmonella* Typhimurium doorheen een intact intestinaal epitheel, perspectieven kunnen bieden in het onderzoek naar de ontwikkeling van chronische darmontstekingen zoals IBD, daar zowel de opname van DON gecontamineerd voedsel als infecties met *Salmonella* voorgesteld worden als mogelijke etiologische factoren voor dit ziektebeeld.

CURRICULUM VITAE

Virginie Vandenbroucke werd geboren op 3 mei 1979 te Kortrijk.

Na het beëindigen van haar middelbare studies Latijn-Wiskunde in 't Fort te Kortrijk, begon ze in 1997 aan de studie geaggregeerde in de Medische Laboratoriumtechnologie aan de Katholieke Hogeschool Brugge-Oostende. Na het behalen van haar diploma, startte ze in 2000 met de studies Diergeneeskunde aan de Universiteit Gent waar ze in 2006 afstudeerde als dierenarts (optie varken-pluimvee-konijn) met grote onderscheiding.

In augustus 2006 vatte ze bij de Vakgroep Farmacologie, Toxicologie en Biochemie haar doctoraatsonderzoek aan, waarin ze de mogelijke interactie tussen het mycotoxine deoxynivalenol en de pathogenese van *Salmonella* Typhimurium infecties bij het varken bestudeerde. Dit onderzoek gebeurde in nauwe samenwerking met de dienst Bacteriologie van de Vakgroep Pathologie, Bacteriologie en Pluimveeziekten. Als assistent Toxicologie stond ze eveneens in voor de toxicologische dienstverlening op de vakgroep.

In het kader van haar onderzoek is ze auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en gaf ze presentaties op nationale en internationale congressen.

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"The clock is running. Make the most of today. Time waits for no man. Yesterday is history. Tomorrow is a mystery. Today is a gift. That's why it is called the present."

(Alice Morse Earle)