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# **Modulation of interactions of *Salmonella* Typhimurium with pigs by stress and T-2 toxin**

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## List of abbreviations

A	<i>Aspergillus</i>
Ach	acetylcholine
ACTH	adrenocorticotropic hormone
ADP	adenosine diphosphate
AFB1	aflatoxin B1
ANOVA	analysis of variance
AP	anterior pituitary
ATP	adenosine triphosphate
ATR	acid tolerance response
BGA	brilliant green agar
BMD	benchmark dose
BPW	buffered peptone water
BGA <sup>NAL</sup>	brilliant green agar with 20 µg/mL nalidixic acid
CAD	caspase-activated Dnase
Caspase	cysteine-dependent aspartate-directed protease
cAMP	cyclic adenosine 5'-monophosphate
CFU	colony forming units
CONTAM	Contaminants in the Food Chain
CRF	corticotropin releasing factor
CSRP	cysteine-rich protein
DAS	diacetoxyscirpenol
DMEM	Dulbecco's modified Eagle's medium
Dnase	deoxyribonuclease
DON	deoxynivalenol
<i>E. coli</i>	<i>Escherichia coli</i>
EEA1	early endosomal antigen 1
EFSA	European Food Safety Authority
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
EST	Expressed Sequence Tags
<i>F</i>	<i>Fusarium</i>
FASFC	Federal Agency for the Safety of the Food Chain
FB1	Fumonisin B1
FCS	fetal calf serum
FOD	Food chain safety and Environment
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate

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h	hour
HBSS	Hank's buffered salt solution
HBSS+	Hank's buffered salt solution with Ca <sup>2+</sup> and Mg <sup>2+</sup>
HIS	histone H3.3
HPRT	hypoxanthine phosphoribosyltransferase
HPA	hypothalamic-pituitary-adrenal axis
IC50	half maximal inhibitory concentration
ICAD	inhibitor of caspase-activated Dnase
IFN	interferon
IgA	immunoglobulin A
IL	interleukin
INS(1,4,5,6)P4	inositol 1,4,5,6-tetrakiphosphate
IPEC	intestinal porcine epithelial cell
iTRAQ	isobaric tags for relative and absolute quantification
ITS	insulin-transferrin-selenium-A supplement
IVET	<i>in vivo</i> expression technology
JNK	c-Jun N-terminal kinase
LB	Luria-Bertani broth
LC-MS	liquid chromatography mass spectrometry
LD50	median lethal dose
lgp	lysosomal membrane glycoproteins
LOAEL	lowest-observed-adverse-effect-level
LPS	lipopolysaccharide
LTB4	leukotriene B <sub>4</sub>
M cells	membranous epithelial cells
MAPk	mitogen activated protein kinase
MCP	monocyte chemotactic protein
mL	milliliter
mM	millimolar
MOI	multiplicity of infection
μg	microgram
μM	micromolar
N	number
NAD	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NE	norepinephrine
NEAA	non essential amino acids
NF-κB	nuclear factor-kappa B
NLR	NOD-like receptor
NOAEL	no-observed-adverse-effect-level

NPF	nucleation-promoting factor
ORF	open reading frame
OTA	ochratoxin A
<i>P</i>	<i>Penicillium</i>
PAM	primary porcine alveolar macrophages
PARP	poly-ADP-ribose polymerase
PCR	polymerase chain reaction
PEEC	pathogen-elicited epithelial chemoattractant
pH	measure of acidity or basicity
pi	post inoculation
PI	protease inhibitor
PMN	polymorphonuclear leucocytes
PMTDI	provisional maximum tolerable daily intake
PPI	phosphatase inhibitor
PtdIns(4,5)P <sub>2</sub>	phosphatidylinositol 4,5-biphosphate
PVN	paraventricular nucleus
RILP	Rab7-interacting lysosomal protein
RNase	ribonuclease
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
<i>Salmonella</i> Enteritidis	<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Enteritidis
<i>Salmonella</i> Typhi	<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Typhi
<i>Salmonella</i> Typhimurium	<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Typhimurium
SAP	<i>Salmonella</i> Action Plan
SCV	<i>Salmonella</i> containing vacuole
SD	standard deviation
Sifs	<i>Salmonella</i> -induced filaments
SNS	sympathetic nervous system
SPI	<i>Salmonella</i> pathogenicity island
T3SS	type III secretion system
TBP	tributylphosphine
TDI	tolerable daily intake
TEER	transepithelial electrical resistance
TEM	transmission electron microscopy
Th	T helper
TNF	tumour necrosis factor
TfnR	transferring receptor
Tris	Tris(hydroxymethyl)aminomethane hydrochloride
vATPase	vacuolar ATPase
WT	wild type

ZEA

zearalenone



# **General Introduction**

**In part adapted from: Veterinary Microbiology (2011) 155: 115-127**



## **1. Pig as a source of human salmonellosis**

Worldwide, *Salmonella* bacteria are one of the most widely distributed foodborne pathogens, and after *Campylobacter* they are the second most common cause of bacterial gastrointestinal illness in humans (European Food Safety Authority, 2011a). The bacterial genus *Salmonella* contains 2 species, namely *Salmonella enterica* and *Salmonella bongori* (Guibourdenche et al., 2010). *Salmonella enterica* is subdivided into six subspecies; *arizonae*, *diarizonae*, *enterica*, *houtenae*, *indica* and *salamae*. Based on flagella (H) and somatic (O) antigens, the genus *Salmonella* can be further classified into serovars. Until now, more than 2,500 serovars of *Salmonella* are recognized (Heyndrickx et al., 2005).

*Salmonella* can cause typhoidal or nontyphoidal human salmonellosis. Typhoidal *Salmonella* serovars such as *Salmonella enterica* subsp. *enterica* serovar Typhi (*Salmonella* Typhi) and *Salmonella* Paratyphi, cause systemic illness which often results into death, with each year an estimated 20 million cases and 200,000 deaths worldwide (Crump et al., 2004; Boyle et al., 2007). Nontyphoidal *Salmonella* serovars such as *Salmonella* Typhimurium, *Salmonella* Enteritidis, *Salmonella* Derby and others, mostly cause self-limiting gastroenteritis in humans (Boyle et al., 2007). Although these serotypes rarely produce systemic infections in healthy adults, it is still a major cause of morbidity and mortality worldwide. It is estimated that nontyphoidal *Salmonella* infections result in 93.8 million illnesses globally each year, of which an estimated 80.3 million are foodborne, and 155,000 result into death (Majowicz et al., 2010). Particularly in parts of Africa, where HIV infection is widespread, nontyphoidal *Salmonella* infections are a major health problem (Gordon et al., 2010).

The main route of human infection is through the consumption of contaminated food. Although more seldom, other routes of infection have been described, such as environmental transmission like fecal contamination of the ground water (O'Reilly et al., 2007) or contact with live animals (Baker et al., 2007). In the European Union, the laying hen reservoir (eggs) and pigs (pork meat) are the most important sources of human salmonellosis. Depending on the region and country, the proportion of disease attributed to layers and pigs is different (Table 1). In Belgium, pigs are the main contributor to human salmonellosis (74.0%), with *Salmonella* Typhimurium being the predominant serovar isolated from slaughter pigs (Table 2; Boyen et al., 2008a; Pires et al., 2011). Since *Salmonella* Typhimurium is a major cause of foodborne salmonellosis in humans, this serotype will be extensively discussed in the next chapters.

**Table 1:** Proportion (%) of *Salmonella* cases attributable to animal sources, outbreaks and travel in the EU, 2007 to 2009. This table was adapted from Pires et al. (2011).

	Broilers	Pigs	Turkeys	Layers	Outbreaks <sup>(a)</sup>	Travel	Unknown	Total
Austria	0.3	13.8	3.6	58.5	3.2	11.6	9.0	8,460
Belgium	2.3	74.0	9.2	2.9	0.5	0.0	11.2	10,917
Cyprus	4.8	51.3	6.3	8.7	0.0	3.8	25.1	461
Czech Republic	0.1	10.9	1.7	84.6	0.2	1.7	0.8	39,032
Germany	0.5	32.7	1.3	51.2	1.6	5.2	7.5	129,704
Denmark	2.8	15.6	15.1	10.5	23.8	18.2	14.1	7,461
Estonia	10.6	24.4	1.8	49.0	4.7	7.1	2.3	1,338
Spain	0.1	31.8	12.4	41.5	3.9	0.0	10.3	12,419
Finland	0.6	4.9	1.7	2.5	2.3	83.2	4.8	8,228
France	12.8	32.5	11.1	6.9	4.8	0.0	32.0	19,849
Greece	0.8	9.5	0.3	78.6	0.0	2.3	8.3	2,154
Hungary	4.2	24.3	4.9	49.7	9.5	0.2	7.3	19,244
Ireland	1.4	26.0	8.4	14.2	5.0	30.3	14.7	1,223
Italy	2.3	73.2	5.3	2.1	0.0	1.3	15.8	11,887
Lithuania	1.6	9.1	0.7	82.8	4.4	0.3	1.2	7,641
Luxembourg	4.3	8.4	6.8	50.0	0.0	9.6	20.9	527
Latvia	3.5	12.2	0.2	69.2	13.2	1.2	0.6	2,664
The Netherlands	3.9	22.9	8.1	27.2	11.3	11.9	14.7	4,077
Poland	21.8	39.8	1.0	23.8	11.3	0.1	2.2	29,268
Portugal	40.2	34.2	0.5	8.1	6.0	0.3	10.7	1,036
Sweden	0.5	4.9	1.7	2.4	2.3	77.7	10.5	11,169
Slovenia	0.3	16.0	3.1	47.3	21.9	0.0	11.4	2,995
Slovakia	0.1	17.5	2.5	75.2	2.3	0.8	1.7	15,879
United Kingdom	0.6	11.7	10.1	35.5	0.0	24.3	17.8	35,972

<sup>(a)</sup> Outbreaks with unknown source. Outbreak cases for which the source was identified were assigned to the correspondent animal sources.

**Table 2:** Within the pig reservoir, the top-5 serovars contributing to human salmonellosis are presented. This table was adapted from Pires et al. (2011).

Serotype	Percentage (%)
<i>Salmonella</i> Typhimurium	63.1
<i>Salmonella</i> Enteritidis	28.3
<i>Salmonella</i> Derby	1.9
<i>Salmonella</i> Infantis	1.5
<i>Salmonella</i> Newport	0.8
Others	4.4
Total cases	113,520

## **2. Pathogenesis of a *Salmonella* Typhimurium infection in pigs**

### **2.1 Intestinal phase of infection: passage through the digestive system**

Airborne *Salmonella* transmission between pigs can occur (Oliveira et al., 2007) but the main route of infection is the fecal-oral route. During ingestion, *Salmonella* is exposed to a number of stressful environments (Rychlik and Barrow, 2005). The tongue and the oral epithelium are the first barriers *Salmonella* encounters. The dorsal tongue expresses porcine epithelial beta-defensin 1 at antimicrobial concentrations (Shi et al., 1999).

Bacteria that survive may enter the soft palate of the tonsils, which are aggregates of lymphoid tissue that act as an immune barrier to invading pathogens (Horter et al., 2003). Since *Salmonella* Typhimurium can persistently colonize the tonsils, they are a reservoir of infection (Wood et al., 1989). *Salmonella* bacteria reside mainly extracellularly in porcine tonsils (Van Parys et al., 2010). Therefore, virulence mechanisms involved in invasion and intracellular survival are of little importance for tonsillar colonization (Boyen et al., 2006; 2008b). Recently, Bearson et al. (2011) identified *poxA*, a member of the family encoding class-II aminoacyl-tRNA synthetases, as a *Salmonella* gene important for the colonization of the tonsils.

In the stomach, *Salmonella* bacteria sense a sudden pH drop to values which may approach 1 to 4.5 (Chesson, 1987). Gastric acidity is a major barrier for *Salmonella* colonization and infection of the gastrointestinal tract. However, these bacteria possess adaptive systems to protect themselves against acid stress. By rapidly modifying transcriptional and translational pathways that are crucial for host colonization, pathogenicity and survival, the bacterium protects itself against the acidity (Bearson et al., 2006). This phenomenon is called the acid tolerance response (ATR) which is associated with the expression of acid shock proteins that play a role in the adaptation of *Salmonella* to acidification (Bearson et al., 2006; Bearson et al., 2011). RpoS and Fur are regulatory proteins essential for the response to low pH induced by organic acids, whereas the 2-component regulatory system PhoP and PhoQ protects the bacterium against inorganic acid stress (Rychlik and Barrow, 2005). These regulatory proteins may play a role in the expression of multiple ATR proteins, such as molecular chaperones, cellular regulatory proteins, transcription and translation factors, envelope proteins and fimbriae (Bearson et al., 1998; 2006; Foley and Lynne, 2008).

*Salmonella* bacteria that survived the acidic environment of the stomach become exposed to new stresses to which they must respond in order to survive. Bile is produced in

the liver and is composed of various bile salts. These bile salts induce DNA damage to *Salmonella* bacteria since they increase the frequency of nucleotide substitutions, frameshifts and chromosomal rearrangements (Prieto et al., 2004; Merritt and Donaldson, 2009). *Salmonella* Typhimurium can be highly resistant against these bile salts (van Velkinburgh and Gunn, 1999). Active efflux pumps such as AcrAB are thought to be a primary defence mechanism of *Salmonella* against bile salts. Furthermore, other genes are known to contribute to bile resistance, including *phoP*, *tolR* and *wec* (Van Velkinburgh and Gunn, 1999; Prouty et al., 2002; Ramos-Morales et al., 2003). Nevertheless, bile salts are known to suppress the invasion machinery of *Salmonella*, resulting in the inhibition of bacterial colonization in the upper parts of the small intestines (Prouty and Gunn, 2000; Boyen et al., 2008a).

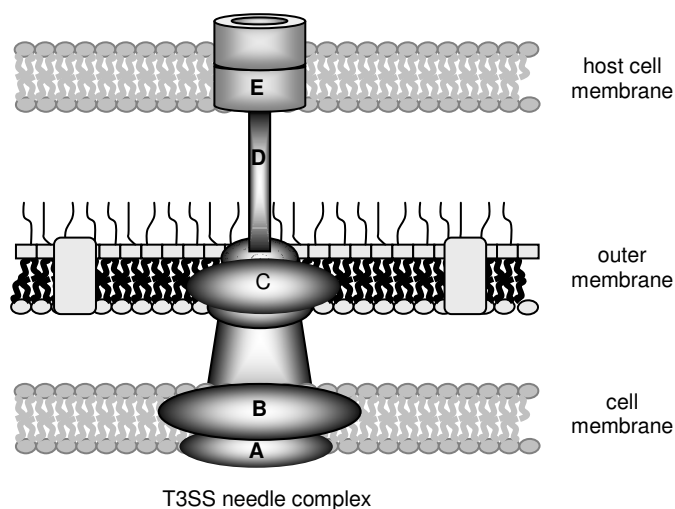
## **2.2 Intestinal phase of infection: invasion of enterocytes**

Adhesion to the intestinal mucosa is considered to be the first step in the intestinal colonization of *Salmonella*. Fimbriae or pili mediate this process and multiple types of fimbriae are known to be expressed by *Salmonella* Typhimurium (Althouse et al., 2003). The bacterium contains a large number of fimbrial operons of which *lpf* encodes for long polar fimbriae that confer attachment to membranous epithelial cells (M cells) (Bäumler et al., 1996; Humphries et al., 2001). M cells are an important cellular component of the follicular-associated epithelium overlaying the Peyer's patches, which are considered as the primary site of *Salmonella* invasion of the murine intestine (Kraehenbuhl and Neutra, 2000; Schauer et al., 2004). By the use of an *ex vivo* porcine ileal loop model, Meyerholz et al. (2002) showed early preferential adherence to M cells within 5 minutes. Besides *lpf*, *Salmonella* Typhimurium contains the fimbrial operon *fim* which encodes type 1 fimbriae (Humphries et al., 2001). These fimbriae have been shown to bind porcine enterocytes, a second major route for intestinal colonization (Althouse et al., 2003; Duncan et al., 2005). Dendritic cells which are present within the follicle-associated epithelium (Iwasaki and Kelsall, 2001) and which can extend their dendrites between the epithelial cells overlaying villi (Niess et al., 2005; Chieppa et al., 2006; Niess and Reinecker, 2006), are also an entry site for *Salmonella* bacteria (Lu and Walker, 2001).

Once *Salmonella* has efficiently migrated through the mucus layer overlaying the intestinal epithelial cells, the bile concentration is expected to decrease, the *Salmonella* Pathogenicity Island (SPI) 1-encoded type III secretion system (T3SS) is activated and the bacterium becomes capable of invading epithelial cells (Prouty and Gunn, 2000). The T3SS is

a complex of proteins that allows the transfer of virulence factors into the host cell through a needle-like structure and SPI's are genetic elements on the chromosome of *Salmonella* encoding many of the virulence factors of the bacterium (Galán and Wolf-Watz, 2006). During its evolution, *Salmonella* acquired many pathogenicity islands of which SPI-1 plays a major role in the intestinal colonization of *Salmonella* Typhimurium in pigs (Marcus et al., 2000; Boyen et al., 2006). However, using signature tagged mutagenesis, Carnell et al. (2007) showed that also other virulence genes, including SPI-2 associated genes, play a role in the short-term colonization of the porcine gut.

The SPI-1 T3SS is composed of an inner ring structure and the cytoplasmic export machinery that span the cell membrane of the bacterium and which are assembled from PrgHK protein subunits, and InvAC and SpaPQRS proteins, respectively. The outer ring structure, composed of InvGH, is assembled in the outer membrane of the bacterium. It connects the inner ring structure and is stabilized by the regulatory protein InvJ. After the base structure, the needle and inner rod structures, which are made up of PrgJ and PrgI subunits, respectively, complete the assembly of the T3SS (Galán and Wolf-Watz, 2006; Foley and Lynne, 2008). Furthermore, the T3SS produces proteins that form a translocation complex for the delivery of additional effector proteins into the cytoplasm of the host cell (Figure 1). The SPI-1 T3SS allows the translocation of at least 20 structural and regulatory proteins from the bacterial cytoplasm to the host cell, resulting in actin cytoskeleton rearrangements, and the induction of diarrhoea and cell death.



**Figure 1:** Schematic representation of the *Salmonella* pathogenicity island 1 associated type III secretion system. (A) the inner ring structure which is composed of PrgHK protein subunits, (B) the cytoplasmic export machinery which is composed of the InvAC and SpaPQRS proteins, (C) the outer ring structure which is connected to the inner ring structure and composed of InvGH, and (D) needle and inner rod structures, made up of PrgJ and PrgI subunits, respectively. The needle complex interacts with the (E) translocase complex to insert bacterial proteins into the host cell. This picture was adapted from Foley and Lynne (2008).

### 2.2.1 Cytoskeletal changes associated with *Salmonella* Typhimurium internalization in porcine enterocytes

SPI-1 T3SS translocates effectors (SipA, SipC, SopB, SopD, SopE and SopE2) into the host cell to cause membrane deformation and rearrangements of the underlying actin cytoskeleton (membrane ruffling), which drives the uptake of the bacterium (Figure 2). SopE and SopE2 mimic cellular guanine exchange factors which catalyse the replacement of guanosine diphosphate (GDP) from Rho-family GTPases by guanosine triphosphate (GTP). This results in the activation of the host Rho-family GTPases Cdc42 and Rac1, which are key regulators of the actin cytoskeleton in eukaryotic cells (Hall, 1998; Hardt et al., 1998a; Stender et al., 2000). *In vitro*, SopE activates Rac1 and Cdc42, whereas SopE2 exhibits specificity for Cdc42 (Patel and Galán, 2005; McGhie et al, 2009). Although SopE and SopE2 are translocated by the SPI-1 T3SS into the enterocyte, they are not encoded within the SPI-1 but by phages (Hardt et al., 1998b; Ehrbar and Hardt, 2005). Indeed, it is known that some SPI-1 effectors are not encoded within SPI-1 itself, but they can be situated on other parts of the bacterial chromosome, in other SPI's or be associated with prophages (Ehrbar and Hardt, 2005).

SopB (SigD) is another effector protein which is injected during *Salmonella* invasion in enterocytes and acts as an inositol phosphatase. It dephosphorylates a range of phosphoinositide phosphate and inositol phosphate substrates (Patel and Galán, 2005). SopB decreases the levels of host phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P<sub>2</sub>) which leads to the rapid fission of the invaginating membranes (Terebiznik et al., 2002), and it increases cellular levels of inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P<sub>4</sub>), leading to Cdc42 activation (Norris et al., 1998; Zhou et al., 2001). This means that *Salmonella* has developed two independent mechanisms to activate Cdc42 and Rac1. The effectors SopE and SopE2 directly activate Cdc42 and Rac1 by mimicking eukaryotic guanine exchange factors, whereas SopB generates PIP fluxes, activating these Rho-family GTPases (McGhie et al., 2009). In addition to this, Patel and Galán indicated that SopB-dependent stimulation of the cellular SH3-containing guanine nucleotide exchange factor, activates the small Rho GTPases RhoG, which contributes to the actin remodelling during invasion of the bacterium (Patel and Galán, 2006; McGhie et al., 2009). RhoG activates Rac1 (Katoh and Negishi, 2003), but it has also been shown that it can act independently of these GTPases (Wennerberg et al., 2002; Hänsich et al., 2010). SopD acts cooperatively with SopB to aid membrane fission and macropinosome formation (Bakowksi et al., 2007).



The bacterium can also modulate the actin dynamics of the host cell in a direct manner through the bacterial effector proteins SipA and SipC (Finlay and Brumell, 2000). SipC is a membrane bound protein of which the N-terminal domain can directly bind to actin and mediate the bundling of actin filaments and of which the C-terminus can mediate nucleation of actin polymers (Hayward and Koronakis, 1999). SipA binds directly to actin, lowers its concentration, stabilizes actin polymers and inhibits depolymerization of actin filaments, resulting in the more outward extension of the *Salmonella*-induced membrane ruffles and consequently facilitating bacterial uptake (Zhou et al., 1999b). SipA also potentiates the actin nucleating and bundling activities of SipC (McGhie et al., 2001) and it enhances the activity of T-plastin, which is a host actin bundling protein (Zhou et al., 1999a; Delanote et al., 2005; McGhie et al., 2009). Furthermore, SipA inhibits the binding of the cellular actin depolymerising proteins ADF/cofilin to F-actin (McGhie et al., 2004; 2009).

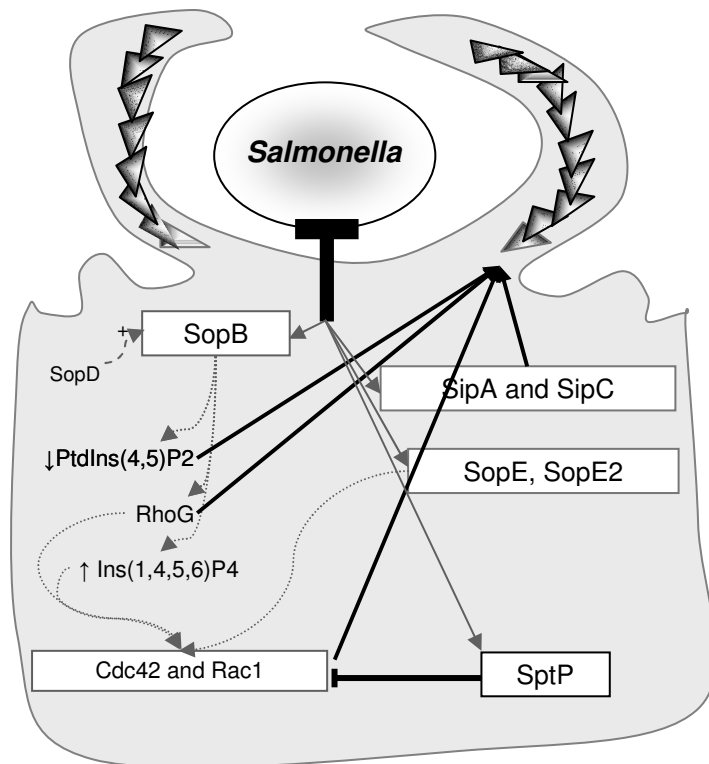
After *Salmonella* internalization has occurred, the bacterium injects the effector protein SptP which promotes the inactivation of Rho family GTPases (Fu and Galán, 1998). This implies that once internalized, the bacterium downregulates the signals that mediate cytoskeletal rearrangements. Consequently, the cytoskeleton of the enterocyte returns to normal (Finlay, 1991; Finlay and Brumell, 2000).

After *Salmonella* is internalized in intestinal epithelial cells, it resides in the membrane bound *Salmonella* containing vacuole (SCV) (Knodler and Steele-Mortimer, 2003). The SCV's are very important for *Salmonella* survival because *Salmonella* can no longer be killed by the normal phago-lysosomal processing pathways (Holden, 2002). Furthermore, the SCV plays a major role in the transport of the bacterium in epithelial cells. During SCV maturation, it migrates from the luminal border of the cell to the basal membrane where the bacterium comes in contact with macrophages associated with Peyer's patches in the submucosal space (Ohl and Miller, 2001). The SCV formation will be more thoroughly discussed in chapter 2.3.1.

## 2.2.2 *Salmonella*-induced diarrhoea

As discussed above, the invasion of *Salmonella* in host cells is accompanied by the translocation of effector proteins into the host cell. SopE, SopE2 and SopB activate Rho GTPases, which stimulate the Mitogen Activated Protein kinase (MAPk) pathway (Brumell et al., 1999). Furthermore, the invasion of *Salmonella* results in an increase in the cytosolic concentration of calcium, which results in the activation of NF- $\kappa$ B (Gewirtz et al., 2000). The

activation of the MAPk pathway and NF- $\kappa$ B results in the secretion of pro-inflammatory cytokines of which interleukin 8 (IL-8) is the most studied one (Eckmann et al., 1993; Cho and Chae, 2003). IL-8 is a chemoattractant for neutrophils and the infiltration of polymorphonuclear leucocytes (PMNs) in the lamina propria is a first line defence of the host against a *Salmonella* infection (Santos et al., 2003). The infiltration of the lamina propria is followed by a massive migration of PMNs through the epithelium into the intestinal lumen (Santos et al., 2003). A large amount of PMNs in the porcine gut could prevent successful salmonellosis (Foster et al., 2003), but the inefficient uptake of the bacterium may however result in the colonization of the porcine gut (Stabel et al., 2002). *In vitro* experiments indicated that IL-8 is secreted at the basolateral side of the epithelial cells. Therefore, the role of IL-8 is recruitment of PMNs to the subepithelial space rather than transepithelial migration into the intestinal lumen (McCormick et al., 1995; Santos et al., 2003).



**Figure 2:** *Salmonella* invasion into enterocytes. SPI-1 T3SS translocates effectors (SipA, SipC, SopB, SopD, SopE and SopE2) into the host cell to cause membrane ruffling, which drives the uptake of the bacterium. SopE and SopE2 directly activate Rac1 and/or Cdc42. SopB acts as an inositol phosphatase that decreases the levels of host phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), which leads to the rapid fission of the invaginating membranes. It increases cellular levels of inositol 1,4,5,6-tetrakisphosphate (Ins(1,4,5,6)P4), leading to Cdc42 activation and it activates the small Rho GTPases RhoG, which contributes to the actin remodelling during invasion of the bacterium through the activation of Rac1 or through a GTPase independent way. SopD acts cooperatively with SopB to aid membrane fission and macropinosome formation. SipA binds directly to actin, it enhances the activity of T-plastin and it potentiates the actin nucleating and bundling activities of SipC. After *Salmonella* internalization has occurred, the bacterium injects the effector protein SptP which promotes the inactivation of Cdc42 and Rac1, returning the cytoskeleton of the porcine enterocyte back to normal. Modified from a model described by Donnenberg (2000).

The transepithelial migration of PMNs is the result of the pathogen-elicited epithelial chemoattractant (PEEC) which is secreted at the apical side of the epithelial cell and which induces direct migration of PMNs across cultured intestinal epithelial cells (McCormick et al., 1998). The activation of PEEC is not NF- $\kappa$ B dependent, but is the result of the SPI-1 T3SS effector protein SipA which is translocated into the cytosol of the host cell (Lee et al., 2000). AvrA, SspH1 and SptP are effector proteins of *Salmonella* which have been described to downregulate NF- $\kappa$ B and the host's inflammatory responses (Collier-Hyams et al., 2002; Haraga and Miller, 2003).

Besides SopE2, SipA and SopB, the effector proteins SopA and SopD participate in the induction of diarrhoea. Individual *Salmonella* mutants of the genes encoding these effectors partially decreased the fluid secretion in a bovine intestinal loop model. The loss of all five genes (*sipAsopABDE2* mutant) resulted in a mild diarrhoea that was more strongly attenuated than the strains having only single mutations and which was characterized by a reduced fluid secretion, PMN influx, and lesions in the bovine intestine (Zhang et al., 2002; Lawhon et al., 2010).

Besides the Rho GTPase activating activity of SopB, it influences the ion balance in host cells (Foley and Lynne, 2008). It is suggested that SopB activity catalyzes the production of an inositol polyphosphate that can act as an antagonist of a negative regulator of calcium-mediated chloride secretion (Galán, 1998). The alteration of the ion balances can lead to fluid secretion into the intestinal tract (Norris et al., 1998; Foley and Lynne, 2008). The damage caused by the massive influx of PMNs and the fluid secretion, can subsequently lead to diarrhoea which is a typical feature of a *Salmonella* infection.

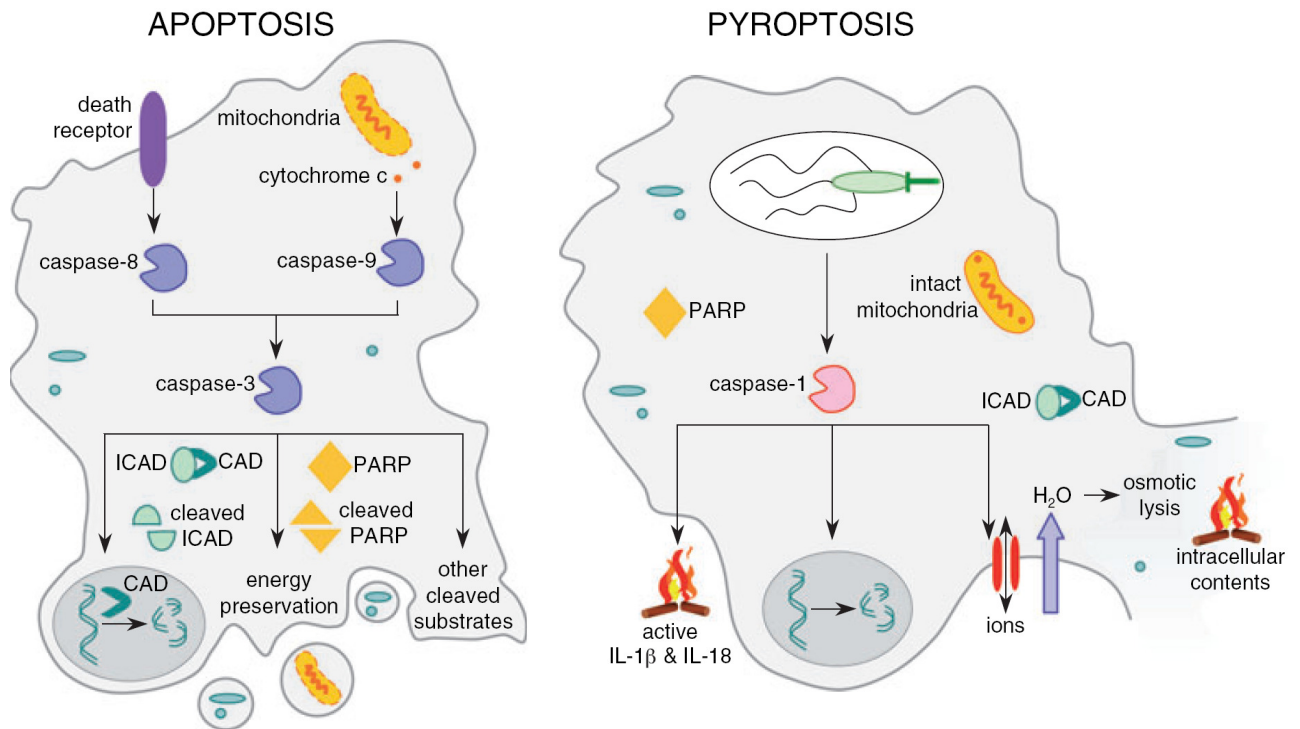
### 2.2.3 *Salmonella*-induced epithelial cell death

In a normal intestinal epithelium, stem cells, located near the crypt base, proliferate and differentiate while migrating towards the top of the villi, with a turnover rate of 3 to 5 days (Potten et al., 1997). During salmonellosis, the turnover rate is disturbed and *Salmonella* induces intestinal epithelial cell death via apoptosis (Schauser et al., 2005). Apoptosis is accompanied by rounding of the cell, reduction of cellular volume, chromatin condensation, nuclear fragmentation, plasma membrane blebbing and engulfment by resident phagocytes (Kroemer et al., 2009). Apoptosis is the result of the activation of cysteine-dependent aspartate-specific proteases (caspases). Ligation of death inducing ligands with cell surface death receptors, including tumour necrosis factor (TNF) receptor and Fas, leads to caspase-8

activation which can promote mitochondrial release of cytochrome c that activates caspase-9. Both caspases activate caspase-3, which cleaves cellular substrates, resulting in cytoplasmic and nuclear condensation, oligonucleosomal DNA cleavage and maintenance of an intact plasma membrane (Green, 2003; Fink and Cookson, 2007). Normally apoptotic cells package their contents into membrane-bound apoptotic bodies and expose surface molecules (e.g. phosphatidylserine) to target phagocytic uptake and removal (Figure 3; Fink and Cookson, 2007).

Shortly after invasion of *Salmonella* Typhimurium in epithelial cells, apoptosis is not detectable because the SPI-1 T3SS effector protein SopB acts as a prosurvival factor (Knodler et al., 2005). SopB suppresses apoptosis in epithelial cells via the sustained phosphorylation and activation of Akt, an important prosurvival kinase, and via inhibition of caspase-3 cleavage (Steele-Mortimer et al., 2000; Knodler et al., 2005).

Twelve to eighteen hours after bacterial entry, *Salmonella* induces apoptosis in human intestinal epithelial cells *in vitro* (Kim et al., 1998). Collier-Hyams et al. (2002) showed that the SPI-1 T3SS effector protein AvrA inhibits the anti-apoptotic NF- $\kappa$ B pathway, resulting in an induced late form of apoptosis in human epithelial cells. *Salmonella*-induced cytotoxicity in epithelial cells is characterized by the activation of caspase-3 and caspase-8, but not of caspase-1 (Paesold et al., 2002; Zeng et al., 2006), and caspase-1 deficient cells respond normally to apoptotic stimuli (Kuida et al., 1995). Using a porcine jejunal loop model, Schauser et al. (2005) showed that a *Salmonella* Typhimurium infection mainly results in the activation of caspase-3 and consequently to apoptosis of epithelial cells, but they also provided evidence for a caspase-3-independent form of programmed cell death. Recently Knodler et al., (2010) observed epithelial pyroptosis in human colonic epithelial cells. This type of cell death will be discussed in chapter 2.3.3.



**Figure 3:** Biochemical mechanism defining apoptosis and pyroptosis. Apoptosis is initiated through the ligation of death inducing ligands with cell surface death receptors, leading to caspase-8 activation, or through the release of cytochrome c, activating caspase-9. Both caspases activate executioner caspases, such as caspase-3 that cleaves the inhibitor of caspase-activated DNase (ICAD), resulting in the activation of caspase-activated DNase (CAD). This nuclease is responsible for DNA fragmentation during apoptosis, which in turn activates the enzyme poly-ADP-ribose polymerase (PARP), which depletes cellular energy stores. However, caspase-3 also cleaves PARP during apoptosis, thereby preserving cellular energy required to carry out apoptosis. Pyroptosis is mediated by the activity of caspase-1 which results in the release of IL-1 $\beta$  and IL-18. Caspase-1 activation also results in nuclease-mediated DNA cleavage and formation of membrane pores, causing loss of ionic equilibrium, water influx, swelling and osmotic lysis with the release of inflammatory intracellular contents. This picture was adapted from Fink and Cookson (2007).

## 2.3 Colonization of macrophages by *Salmonella* Typhimurium and the systemic spread of the bacterium

### 2.3.1 The *Salmonella*-containing vacuole as an intracellular niche for *Salmonella* replication

Internalization of *Salmonella* into macrophages can occur via phagocytic uptake or via the expression of the SPI-1 T3SS (Ibarra and Steele-Mortimer, 2009). The SCV plays a major role in the survival of *Salmonella* in phagocytic macrophages. Once *Salmonella* is inside the host cell SCV, genes located on SPI-2 are expressed, encoding for structural proteins of the T3SS (SsaG through SsaU), effector proteins (SseABCDEF), secretion system chaperones (SscAB) and regulatory proteins (SsrAB) (Hensel, 2000; Foley and Lynne, 2008). This SPI-2 T3SS transfers effector proteins from the bacterium across the SCV membrane into the host

cell in order to interact with targets in these host cells. Its expression is regulated by the SsrA-SsrB 2-component regulatory system, which in turn, is regulated by the OmpR-EnvZ 2-component regulatory system (Lee et al., 2000; Garmendia et al., 2003; Foley and Lynne, 2008).

The intracellular *Salmonella* bacteria induce the formation of an F-actin meshwork around the SCV's. These F-actin rearrangements are independent of the SPI-1 T3SS which induces membrane ruffling during invasion, but it requires a functional SPI-2 T3SS (Méresse et al., 2001). Depolymerization of F-actin with cytochalasin D resulted in an inhibited replication of *Salmonella* Typhimurium and caused the loss of vacuolar membrane around this bacterium (Méresse et al., 2001). Besides the assembly of a meshwork of F-actin, the intracellular bacteria also cause a dramatic accumulation of microtubules around the *Salmonella* Typhimurium microcolonies, unconnected with the SPI-2-dependent actin assembly (Guignot et al., 2004).

When the SCVs form, they acquire the transferring receptor (TfnR), early endosomal antigen 1 (EEA1) and several Rab GTPases (Rab4, Rab5 and Rab11), which are all cellular markers associated with the early endocytic pathway (Figure 4; McGhie et al., 2009). This indicates that shortly after invasion, the SCV interacts with early endosomes. However, within 30 minutes, the SCVs become uncoupled from the endocytic pathway and they do not fuse with lysosomes in order to avoid exposure to the degradative enzymes (Finlay and Brumell, 2000). Although they do not fuse with lysosomes, the early endosomal markers are replaced by late endosome/lysosome markers including Rab7, vacuolar ATPase (vATPase) and lysosomal membrane glycoproteins (lgp) such as LAMP-1 (Steele-Mortimer, 2008). SPI-1 T3SS effector proteins SopB and SopE are required for the SCV recruitment of Rab5 (Mukherjee et al., 2001; Mallo et al., 2008). Rab5 binds the phosphatidylinositol 3-kinase Vps34 which is required for LAMP-1 recruitment and Vps34 in turn generates PI(3)P on the SCV membrane, necessary for the recruitment of EEA1 (Mallo et al., 2008; Steele-Mortimer, 2008, McGhie et al., 2009).

An important step during the maturation of the SCV is the migration towards a predominantly juxtannuclear position near the microtubule organizing centre (Salcedo and Holden, 2003). Due to the close proximity of the SCV to the Golgi apparatus, the SCV may obtain nutrients through the interception of endocytic and exocytic transport vesicles (Ramsden et al., 2007). The SPI-2 T3SS effector proteins SifA, SseF and SseG are required for the redirection of exocytic transport vesicles to the SCV (Kuhle et al., 2006). During the migration of the SCV toward the perinuclear region of the host cell, the SCV transiently

recruits the Rab7-interacting lysosomal protein (RILP). RILP possesses one domain that binds to the GTP-bound form of Rab7 and another domain that recruits the dynein/dynactin complex (Cantalupo et al., 2001; Jordens et al., 2001). The minus end-directed microtubule motor dynein mediates ATP-dependent movements of vesicles and organelles along microtubules toward the cell center (Kuhle et al., 2006). In normal cells, Rab7 controls the fusion of late endosomes with lysosomes and it regulates the fusion of phagosomes with lysosomes (Bucci et al., 2000; Harrison et al., 2003).

After this migration, the *Salmonella* bacteria start to multiply, which is characterized by the formation of *Salmonella*-induced filaments (Sifs). Sifs are LAMP-rich tubulovesicular structures that extend from the original SCV membrane along microtubules (Steele-Mortimer, 2008). Sif tubules extend from the SCV surface and they contain LAMPs, vATPases, and cathepsin D, which suggests that they are derived from late endocytic compartments (Figure 4; Garcia-del et al., 1993; Beuzon et al., 2000; Brumell et al., 2001). Sif formation is driven by the SPI-2 T3SS effector proteins SifA, PipB2, SseF and SseG (Waterman and Holden, 2003; Abrahamns and Hensel, 2006). It has been shown, that SifA mutants display attenuated virulence in the mouse typhoid model and that they fail to replicate in murine macrophages (Stein et al., 1996; Beuzon et al., 2002). These findings implicate that Sif formation is important for bacterial virulence.

Transient overexpression of SifA induces swelling and aggregation of late endosomes and formation of Sif-like tubules in mammalian cells (Steele-Mortimer, 2008). The exact mechanism of how SifA induces the formation of Sifs is unknown, but it has been shown to interact with Rab7 and it is thought that it uncouples Rab7 from the RILP (Kuhle et al., 2006). Harrison and coworkers (2004) showed that, in contrast to the early SCV's, the RILP was not present on Sifs and that the ability of Sifs to extend centrifugally was correlated with a lack of dynein, despite the presence of active Rab7 on their membranes. They also showed that the elongation of Sifs was dependent on kinesin activity. Therefore, it is suggested that the SifA-induced uncoupling of Rab7 from RILP prevents the recruitment of dynein to the Sifs, and as a consequence promotes Sif extension (Harrison et al., 2004; Kuhle et al., 2006).

PipB2 is an effector protein which has been shown to interact directly with the light chain subunit of the plus end-directed microtubule motor kinesin-1 to the SCV (Henry et al., 2006). In contrast to dynein, kinesins mediates ATP-dependent movement of vesicles and organelles along microtubules toward the cell periphery. The interaction with kinesin-1 causes translocation and accumulation of LAMP-1 positive endosomes and/or lysosomes to the cell periphery and it drives the extension of Sif tubules from the juxtannuclear SCV towards the

host cell periphery (Knodler and Steele-Mortimer, 2005; Szeto et al., 2009). *Salmonella* mutants lacking *sseF*, *sseG* or *sopD2* induce fewer Sifs and by contrast, mutants lacking *sseJ* or *spvB* increase numbers of Sifs (Jiang et al., 2004; Ramsden, et al., 2007).

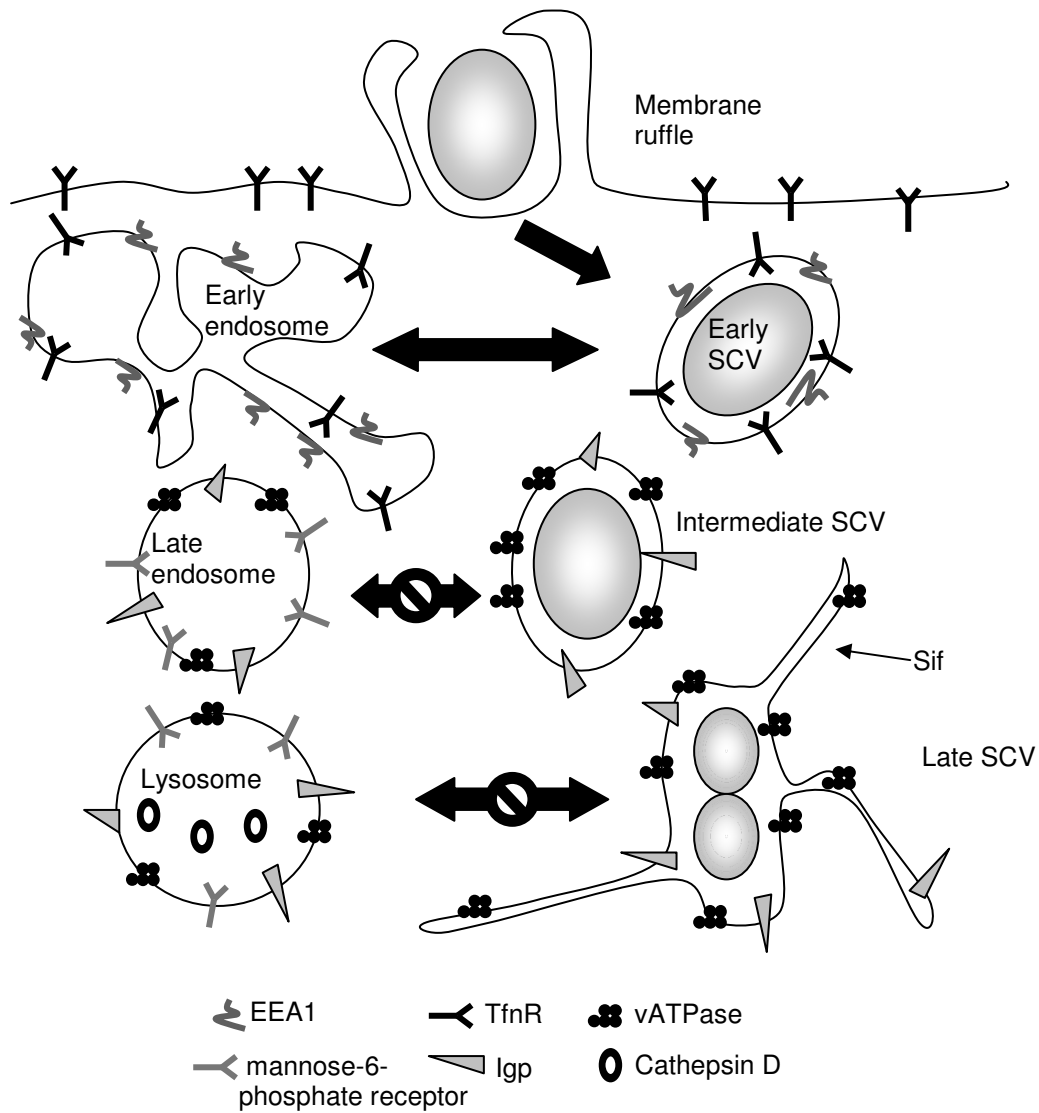
Sif formation was originally attributed to epithelial cells, but Knodler et al. (2003) showed that Sifs are also formed in interferon-gamma (IFN- $\gamma$ ) primed macrophages. Although *Salmonella* is an extensively studied bacterium, still many questions remain about the intracellular environment of *Salmonella* and the SCV formation within different host cells.

### 2.3.2 Systemic spread of *Salmonella* Typhimurium in pigs

Although septicemic episodes of *Salmonella* Typhimurium infections in pigs have been reported, colonization of *Salmonella* Typhimurium in pigs is mostly limited to the gastrointestinal tract (Desrosiers, 1999; Letellier et al., 1999). In mice, *Salmonella* Typhimurium infections result in systemic infections (Stecher et al., 2006). SPI-2 gene products play an important role in the intracellular survival of the bacterium and consequently in the systemic infection (Waterman and Holden, 2003). This implies that the requirement for SPI-2 genes may differ between swine gastrointestinal colonization and the systemic infection in mice (Bearson and Bearson, 2011). The expression of the SPI-2 T3SS is regulated by the SsrA-SsrB 2-component regulatory system, which is required for systemic disease in BALB/c mice (Cirillo et al., 1998; Lober et al., 2006). Boyen et al. (2008b) showed that the oral inoculation of an *ssrAB* mutant into pigs, resulted in an equal colonization of the swine gastrointestinal tract and faecal shedding in comparison to the wild type *Salmonella* Typhimurium strain. However, intravenous inoculation of pigs resulted in a significantly reduced colonization of multiple organs compared to the wild type *Salmonella* Typhimurium strain. These results showed that although the SsrA-SsrB 2-component regulatory system plays a role in the systemic phase of infection, it is not required for the colonization of the bacterium in the gastrointestinal tract in pigs.

A comparison of a *Salmonella* Typhimurium signature-tagged mutant bank screening in calves, chickens and pigs suggested that there may be a core set of genes involved in the colonization of the bacterium in different animal species, but that certain genes may confer host specific colonization mechanisms (Morgan et al., 2004, Carnell et al., 2007, Bearson and Bearson, 2011). These data confirm the hypothesis that there are differences in gene expression of *Salmonella* Typhimurium that contribute to the systemic phase of infection in mice and those that support the gastrointestinal colonization in pigs.





**Figure 4:** Intracellular maturation of the *Salmonella*-containing vacuole (SCV). Once *Salmonella* is internalized in host cells, it resides in the membrane bound *Salmonella* containing vacuole (SCV). Shortly after invasion, the SCV transiently interacts with early endosomes. It acquires the transferring receptor (TfnR), early endosomal antigen 1 (EEA1) and several Rab GTPases (Rab4, Rab5 and Rab11), which are all cellular markers associated with the early endocytic pathway. However, within 30 minutes, the SCVs become uncoupled from the endocytic pathway and they do not fuse with lysosomes. Although, they do not fuse with lysosomes, the early endosomal markers are replaced by late endosome/lysosome markers including Rab7, vacuolar ATPase (vATPase) and lysosomal membrane glycoproteins (Igps) such as LAMP-1. After several hours, intracellular *Salmonella* bacteria begin to replicate and *Salmonella*-induced filaments (Sifs) are formed. Picture adapted from Finlay and Brumell (2000).

### 2.3.3 *Salmonella*-induced cell death of macrophages

During infection of the gastrointestinal mucosa, macrophages infected with *Salmonella* expressing the SPI-1 T3SS rapidly undergo pyroptosis (i.e. within 45 min of infection) (Brennan and Cookson, 2000). This type of cell death is characterized by the activation of caspase-1 (IL-1 $\beta$ -converting enzyme) and not of caspase-3 like during apoptosis (Kroemer et al., 2009). The SPI-1 T3SS effector protein SipB binds and activates caspase-1. Activation of caspase-1 is thought to occur in the inflammasome, a multiprotein complex which contains proteins of the NOD-like receptor (NLR) family and which are thought to be cytosolic pattern-recognition receptors stimulated by exogenous infections agents (Fink and Cookson, 2007; Mariathasan and Monack, 2007). Both the inflammasome adapter protein ASC and the NLR protein Ipaf are required for rapid caspase-1 activation and it is suggested that Ipaf responds to flagellin translocated into the host cytosol by the SPI-1 T3SS (Mariathasan et al., 2004; Franchi et al., 2006; Miao et al., 2006). The activation of caspase-1 results in the release of IL-1 $\beta$  and IL-18 and therefore, pyroptosis may play a relevant role in local and systemic inflammatory reactions (Figure 3; Fink and Cookson, 2007). Non-flagellated *Salmonella* (Franchi et al., 2006; Miao et al., 2006) and *Salmonella* strains harbouring mutations in the genes encoding InvA, InvG, InvJ, PrgH, SipB, SipC, SipD and SpaO are no longer cytotoxic (Chen et al., 1996; Monack et al., 1996; Lundberg et al., 1999; Brennan and Cookson, 2000; Jesenberger et al., 2000; van der Velden et al., 2000). This implies that both the SPI-1 T3SS and flagellin are necessary to induce rapid pyroptosis. Furthermore, bacterial internalization and/or other host processes mediated by host cell actin are also required for rapid pyroptosis, since the inhibition of macrophage actin polymerization with cytochalasin D prevents rapid pyroptosis (Guilloteau et al., 1996; Monack et al., 1996).

During the systemic phase of infection, the expression of SPI-1 T3SS and bacterial flagellin are repressed so that *Salmonella* can reside and multiply in macrophages, without inducing rapid killing. Instead, *Salmonella* activates a delayed form of caspase-1-dependent pyroptosis which requires the SPI-2 T3SS and *spv* genes (Libby et al., 2000; Van der Velden et al., 2000; Monack et al., 2001; Browne et al., 2002). This type of pyroptosis is also associated with the production of IL-1 $\beta$ , DNA cleavage and lysis (Monack et al., 2001; Fink and Cookson, 2007).

Furthermore, although the physiological context remains unknown, a caspase-1 independent macrophage death has been described. Caspase-1-deficient macrophages are resistant to the rapid *Salmonella*-induced SPI-1 and caspase-1-dependent pyroptosis.

However, these cells eventually die after a prolonged infection (Jesenberger et al., 2000; Hernandez et al., 2003). This caspase-1 independent cell death is also dependent on the SPI-1 T3SS SipB effector protein (Hernandez et al., 2003). It is thought that the SipB-mediated macrophage cell death is the result of autophagy by damaging mitochondria or by altering the balance between mitochondria fusion and fission (Hueffer and Galán, 2004). Morphologic features of autophagic cell death are the lack of chromatin condensation, massive vacuolization of the cytoplasm and the accumulation of autophagic vacuoles. In contrast to apoptotic cells, there is little or no uptake by phagocytic cells (Kroemer et al., 2009).

The biological significance of *Salmonella*-induced host cell death remains poorly known. However, it is thought that these intracellular pathogens induce cell death in order to escape and re-infect new host cells and to promote persistence of infection.

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

Pigs infected with *Salmonella* Typhimurium often carry this bacterium asymptotically in their tonsils, gut and gut-associated lymphoid tissue for months resulting in so called *Salmonella* carriers (Wood et al., 1991). Generally, these persistently infected animals intermittently shed low numbers of *Salmonella* bacteria and as a consequence, they are difficult to distinguish from uninfected pigs (Boyen et al., 2008b). However, at slaughter they can be a source of environmental and carcass contamination, leading to higher numbers of foodborne *Salmonella* infections in humans. Until now, the mechanism of prolonged infection in carrier pigs remains poorly known.

In mice, ShdA is important for long-term colonization and persistence of *Salmonella* Typhimurium in the gastrointestinal tract (Bearson and Bearson, 2011). The *shdA* gene is located within the gene cluster of the CS54 and it encodes ShdA which is a fibronectin binding protein (Kingsley et al., 2002). Fibronectin binding proteins are thought to mediate adherence and entry into mammalian cells (Joh et al., 1999; Schwarz-Linek et al., 2004). The disruption of *shdA* does not attenuate the virulence of *Salmonella* Typhimurium in BALB/c mice which were intragastrically inoculated. However, the duration of fecal shedding of a *shdA* mutant in BALB/c and CBA/J mice was significantly decreased in comparison to the parent strain (Kingsley et al., 2000, 2003). In addition to this, the *shdA* mutant showed a reduced persistency in the cecum and Peyer's patches of BALB/c mice (Kingsley et al., 2003). In pigs however, Boyen et al., (2006) showed that a *shdA* deletion mutant was not significantly impaired in persistence and that the fecal shedding of the *shdA* mutant was even

significantly higher at days 1 and 2 post-inoculation. These findings implicate that *shdA* is important for persistent colonization of *Salmonella* Typhimurium in the gastrointestinal tract of mice, but that it is not required for the gastrointestinal colonization and persistence of the bacterium in swine.

In pigs, it has been shown that *Salmonella* Typhimurium downregulates local inflammatory host responses (Wang et al., 2007). Wang et al. (2007) conducted an Affymetrix porcine GeneChip analysis of pig mesenteric lymph nodes to profile the gene expression in these lymph nodes over a time course of infection with *Salmonella* Typhimurium. This study revealed a transcriptional induction of NF- $\kappa$ B target genes at 24 hours post-inoculation and a suppression of the NF- $\kappa$ B pathway from 24 to 48 hours post-inoculation. The authors suggested that the NF- $\kappa$ B suppression in antigen-presenting cells may be the mechanism by which *Salmonella* Typhimurium eludes a strong inflammatory response to establish a carrier status in pigs.

Recently, Van Parys et al. (2011) used *in vivo* expression technology (IVET), to identify *Salmonella* Typhimurium genes that play a role in the persistence of *Salmonella* Typhimurium in pigs. They identified 37 genes that were expressed 3 weeks post oral inoculation in the tonsils, ileum and ileocecal lymph nodes, of which *efp*, encoding the elongation factor P, and *rpoZ*, encoding the RNA polymerase omega subunit, were specifically expressed in the ileocecal lymph nodes. Furthermore, they identified *STM4067* as a factor involved in *Salmonella* persistence in pigs, because the *STM4067 Salmonella* mutant was significantly attenuated in the ileum contents, cecum and cecal contents and faeces of carrier pigs.

#### **4. Stress as a factor influencing the host-pathogen interactions of *Salmonella Typhimurium***

Stress is a concept that has multiple meanings, leading to different definitions (Murray et al., 1996). According to Dhabhar and McEwen (1997), “stress is a constellation of events, consisting of a stimulus (stressor) that precipitates a reaction in the brain (stress perception), which activates physiological fight-or-flight systems in the body (stress response)”. Stress is essential for the survival of an organism as it forms the basis of the innate fight-or-flight response, a fundamental survival mechanism that prepares the body to either challenge or flee from a threat (Dhabhar, 2009; Hughes et al., 2009). The term stress often has a negative connotation since chronic stress suppresses the immune system and increases the susceptibility to infections (Dhabhar and McEwen, 1997). A period of stress results in the release of a variety of neurotransmitters, peptides, cytokines, hormones, and other factors into the circulation or tissues (Freestone et al., 2008; Dhabhar, 2009). The most important mediators of the stress response are the fast-acting catecholamines epinephrine and norepinephrine, which are released by the sympathetic nervous system, and the slow-acting glucocorticoids cortisol and corticosterone, which are secreted by the adrenal gland after activation of the hypothalamic-pituitary-adrenal axis (Dhabhar, 2009).

For a long time, the effects of stress on the course of an infection have been exclusively ascribed to the effect of these stress-related hormones on the immune system. However, during the past decade a new perspective has been introduced which implies that stress-related hormones directly affect the infectious microorganism itself or the host-pathogen interaction (Lyte, 2004). These new insights led to the development of a research area named microbial endocrinology where microbiology and neurophysiology intersect. Recent work from this field shows that bacteria, either from the gastrointestinal tract, the respiratory tract or the skin, can exploit the neuroendocrine alteration due to a stress reaction of the host as a signal for growth and pathogenic processes (Lyte, 2004; Freestone et al., 2008). Both humans and animals are susceptible to the effects of stress on the outcome of an infectious disease. Current animal production practices contain several potentially stressful periods (like inadequate housing conditions, overcrowding, heat, cold, feed deprivation before slaughter and transportation). These stress factors have been linked to increased pathogen carriage, disease susceptibility, carcass contamination and pathogen shedding (Burkholder et al., 2008; Rostagno, 2009).

As some bacteria like *Salmonella* spp. are present in silent carriers, stress induced pathogen shedding could result in an increased transmission of the bacterium and as a consequence interfere with risk assessments. Therefore, it is of great importance to be aware that stress can alter the outcome of an infection in animals

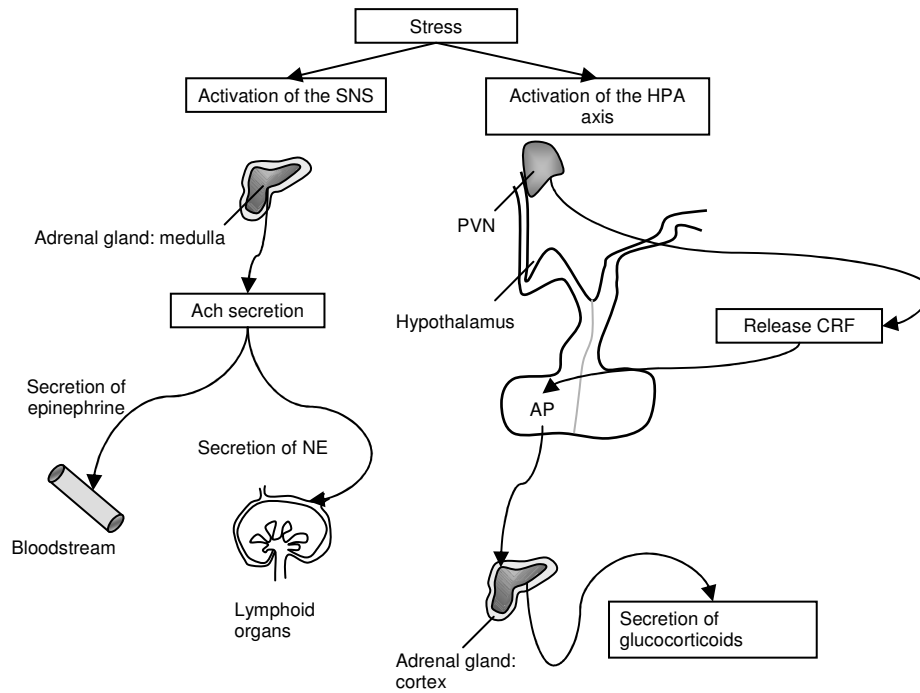
#### **4.1 Stress and stress-related hormones**

The factors causing physical or psychologic stress are different, but they generally result in similar responses, such as the activation of the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis. This results in the rapid and transient release of catecholamines. Subsequently glucocorticoids are released into the circulation, as summarized in Figure 5 (Webster Marketon and Glaser, 2008).

Besides these stress mediators, other neuroendocrine factors can be released following stress, including prolactin, vasoactive intestinal polypeptide, cholecystokinin, growth hormone, nerve growth factor, substance P, neuropeptide Y and serotonin (Joëls and Baram, 2009).

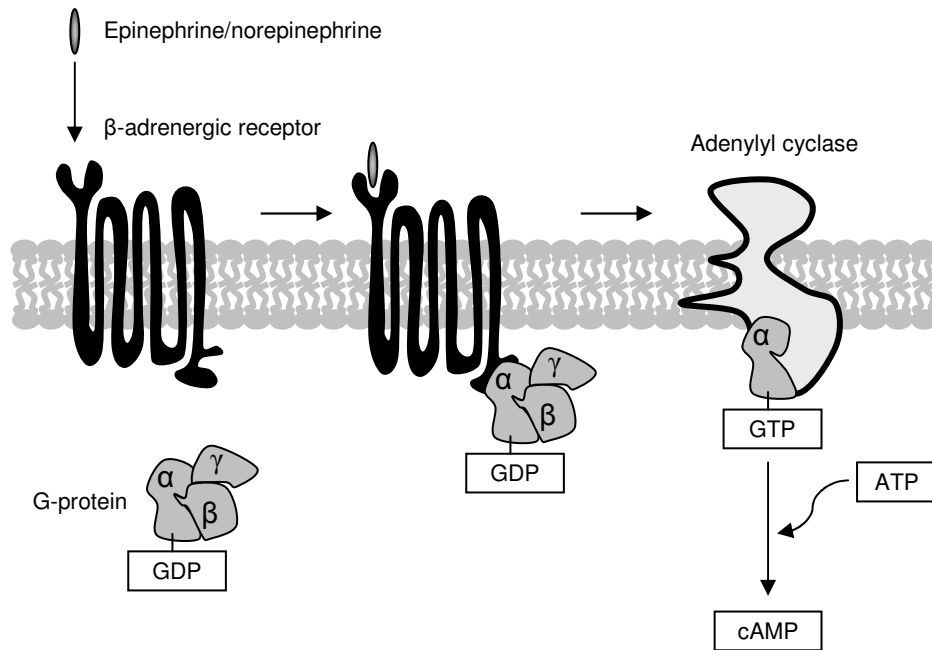
##### **4.1.1 Activation of the sympathetic nervous system: catecholamines**

Stress induces the secretion of acetylcholine from the pre-ganglionic sympathetic fibers in the adrenal medulla via the activation of the sympathetic nervous system. This induces the rapid secretion of epinephrine from the adrenal medulla into the bloodstream and the rapid secretion of norepinephrine from the sympathetic nerve terminals into lymphoid organs, as illustrated in Figure 5. The close association of nerve terminals with immune cells in lymphoid organs facilitates the effects of norepinephrine (Yang and Glaser, 2002).



**Figure 5:** Stress activates the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal axis (HPA), resulting in the release of respectively catecholamines and glucocorticoids. The activation of the sympathetic nervous system induces the secretion of acetylcholine (Ach) from the pre-ganglionic sympathetic fibers in the adrenal medulla. This results in the secretion of epinephrine from the adrenal medulla into the bloodstream and the secretion of norepinephrine (NE) from the sympathetic nerve terminals into lymphoid organs. Besides the activation of the sympathetic nervous system, the hypothalamic-pituitary-adrenal axis becomes activated during a stress response which results in the secretion of corticotropin releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus. Then, corticotropin releasing factor binds to corticotropin releasing factor subtype 1 receptors, located on membranes of anterior pituitary (AP) corticotrope cells, which results in the secretion of the adrenocorticotropic hormone (ACTH) from the anterior pituitary into the systemic circulation. This triggers glucocorticoid secretion from the adrenal glands.

Catecholamines are synthesized from tyrosine and exert their effects by binding to adrenergic receptors. These adrenergic receptors are G-protein coupled receptors which can be subdivided in  $\alpha$ - and  $\beta$ -adrenergic receptors, that comprise  $\alpha_1$  and  $\alpha_2$  subtypes, and  $\beta_1$ ,  $\beta_2$  and  $\beta_3$  subtypes, respectively. Virtually all lymphoid cells express  $\beta$ -adrenergic receptors, with  $\beta_2$ -adrenergic receptors being the most important receptors in terms of the immune system (Elenkov et al., 2000; Webster et al, 2002). When epinephrine and norepinephrine bind the  $\beta_2$ -adrenergic receptors, conformational changes of these receptors take place and G-proteins become activated through the exchange of GDP for GTP. This in turn stimulates enzymes to induce the production of cyclic adenosine 5'-monophosphate (cAMP), which for example can modulate cytokine expression, as illustrated in Figure 6 (Elenkov et al., 2000; Webster et al., 2002). Catecholamines play an important role in many functions in eukaryotic organisms such as energy balance, thermoregulation, cardiovascular function, behaviour and immunity (Thomas and Palmiter, 1997).



**Figure 6:** The binding of epinephrine or norepinephrine to a  $\beta$ -adrenergic receptor causes conformational changes of the receptor that allow the association of the trimeric G-protein ( $G_\alpha$ ,  $G_\beta$  and  $G_\gamma$  subunits) with the receptor. The  $G_\alpha$  subunit is bound to guanosine diphosphate (GDP), but the interaction between the G-protein and the  $\beta$ -adrenergic receptor results in the exchange of guanosine diphosphate for guanosine triphosphate (GTP). As a result, the  $G_\alpha$  subunit detaches from the complex and binds to adenylyl cyclase. Consequently adenylyl cyclase becomes activated and catalyzes the formation of the secondary messenger cyclic adenosine 5'-monophosphate (cAMP) from adenosine-5'-triphosphate (ATP).

#### 4.1.2 Activation of the hypothalamic-pituitary-adrenal axis: glucocorticoids

Besides the activation of the sympathetic nervous system, the hypothalamic-pituitary-adrenal axis becomes activated during a stress response, which results in the secretion of corticotropin releasing factor from the paraventricular nucleus of the hypothalamus (Webster Marketon and Glaser, 2008). Next, corticotropin releasing factor binds to corticotropin releasing factor subtype 1 receptors, located on membranes of anterior pituitary corticotrope cells (Taché and Brunnhuber, 2008). This subsequently results in the secretion of the adrenocorticotrophic hormone from the anterior pituitary into the systemic circulation, which then forms the trigger for glucocorticoid secretion from the adrenal glands, as illustrated in Figure 5. Most mammals secrete cortisol as the predominant glucocorticoid, whereas in most rodents and birds, corticosterone is the most important glucocorticoid secreted during a stress reaction. Both are synthesized from cholesterol and besides a minor degree of binding to albumin, in unstressed animals approximately 90% is bound to corticosterone-binding globulin, which is the major transport protein for glucocorticoids in the plasma of mammalian species (Petersen et al., 2006). Only the unbound cortisol or corticosterone can easily cross cell membranes via passive diffusion. Cortisol and corticosterone generally exert their effects



by binding to the glucocorticoid receptor or the mineralocorticoid receptor. In the absence of glucocorticoids these receptors reside in the cytoplasm as a multiprotein complex (Webster et al., 2002). Binding to the mineralocorticoid receptor occurs with a 10-fold higher affinity than to the glucocorticoid receptor. This implies that, under basal resting conditions, the glucocorticoids preferentially bind to the mineralocorticoid receptor and during periods of stress substantial glucocorticoid receptor binding occurs (de Kloet et al., 1993). Upon ligand binding, the glucocorticoid receptor dissociates from its multiprotein complex and is translocated into the nucleus where it acts as a transcription factor via the interaction with genes whose promoter regions contain glucocorticoid response elements (Webster et al., 2002). Furthermore, it can directly interact with transcription factors including NF- $\kappa$ B and AP-1, to inhibit their activation. This results in the inhibition of numerous genes encoding immune effector and pro-inflammatory cytokines (Belgi and Friedmann, 2002). Moreover, there is some evidence that the ligand/receptor complex can interact with protein kinases involved in intracellular signalling, which results in the phosphorylation of various signal transducing kinases and Annexin-1 (Belgi and Friedmann, 2002). In general, glucocorticoids regulate a wide variety of functions, ranging from growth, metabolic functions, cardiovascular functions, to immune modulation (Sapolsky et al., 2000; de Kloet et al., 2008).

## **4.2 The effects of stress-related hormones on the host immune response and the intestinal barrier**

Neuroendocrine stress hormones can modulate various aspects of the immune system. Almost all cells of the immune system have receptors for one or more of the hormones that are released during a stress response. This implies that stress hormones can have direct effects on all cells of the immune system. However, the modulation of the immune response due to a stress reaction can also occur via secondary effects, for example by interfering with cytokine production (Glaser and Kiecolt-Glaser, 2005; Radek, 2010).

### **4.2.1 Effects of stress on the innate and acquired immune system**

Stress hormones regulate a wide variety of functions in cells of the immune system and influence the expression of various cytokines (Webster et al., 2002). Glucocorticoids suppress the production of IL-12 by antigen-presenting cells and downregulate the expression of IL-12 receptors on natural killer and T-cells. As the main inducer of T helper (Th) 1 responses is downregulated, the secretion of IFN- $\gamma$ , which normally further promotes Th1 responses, is inhibited (Elenkov and Chrousos, 1999; Webster Marketon and Glaser, 2008). It

is also known that besides IL-12, glucocorticoids suppress other proinflammatory cytokines and immunoregulatory cytokines including IL-1, IL-2, IL-6, IL-8, IL-11 as well as granulocyte macrophage colony-stimulating factor (Webster et al., 2002). Furthermore, glucocorticoids cause an upregulation of the production of the anti-inflammatory cytokines IL-4 and IL-10 (Webster et al., 2002). Catecholamines enhance these effects by inhibiting and increasing IL-12 and IL-10 production, respectively (Elenkov and Chrousos, 1999). In addition, stress increases the production of IL-10 by Th2 cells (Webster Marketon and Glaser, 2008). Furthermore, stress hormones modulate trafficking, maturation and differentiation of cells of the immune system, the expression of adhesion molecules, chemoattractants and cell migration factors, and the production of inflammatory mediators (Yang and Glaser, 2000, 2002; Webster et al., 2002).

In conclusion, chronic stress stimulates the humoral immunity and inhibits the cellular immunity by altering the cytokine balance from type-1 to type-2 cytokine driven responses, which can influence the course of an infection and/or the susceptibility to a microorganism (Elenkov and Chrousos, 1999).

#### 4.2.2 Effects of stress on the intestinal barrier

The gastrointestinal tract is controlled by the enteric nervous system, that innervates the gut and is bidirectionally linked with the central nervous system by sympathetic and parasympathetic pathways that form the brain-gut axis (Bhatia and Tandon, 2005; Rostagno 2009). The gastrointestinal tract has the challenge of responding to pathogens while at the same time remaining relatively unresponsive to food antigens and the commensal microbiota (Macdonald and Monteleone, 2005). The ability to control the uptake of nutrients across the gut mucosa and to protect the gastrointestinal tract against noxious substances is defined as the intestinal barrier function, which comprises several first line defence mechanisms such as commensal bacteria, the gut mucous lining, the gut epithelium, the lamina propria and the intestinal propulsive motility. Commensal intestinal bacteria can inhibit the colonization of pathogens via the production of antimicrobial substances (bacteriocins), alteration of the pH and competition for binding sites and nutrients required for their growth (Keita and Söderholm, 2010). The mucus layer protects the gut epithelium and serves as a physical barrier to inhibit and entrap invading microorganisms (Moran et al., 2011). The enterocytes of the gut epithelium are connected to each other by junctional complexes, such as tight junctions, which are important for epithelial transport. Under normal conditions, the intestinal

epithelium secretes antimicrobial peptides and constitutes an effective barrier to invading microorganisms (Keita and Söderholm, 2010). Furthermore, the lamina propria includes the enteric nervous system, endocrine system and cells of the innate and acquired immunity and together with the intestinal motility, it can protect the host from invading pathogens (Keita and Söderholm, 2010).

A disruption of the intestinal barrier function can lead to an increased antigen and pathogen passage, and subsequently to altered host-pathogen interactions. The intestinal barrier has many cellular targets for catecholamines and glucocorticoids, including epithelial cells, enteroendocrine cells, leukocytes, mast cells and enteric neurons (Lyte et al., 2011). This implies that stress mediators can alter the mucosa-bacterial interactions and so affect the commensal microbiota and/or the outcome of a bacterial infection (Lyte et al., 2011).

During stress, the release of norepinephrine from sympathetic nerves that innervate the myenteric plexus, the submucosa and mucosa of the intestine, can accelerate intestinal motility, colonic transit and transepithelial ion transport, which can influence the microbial population of the gut (Enck et al., 1989; Mizuta et al., 2006; Freestone et al., 2008). As commensal bacteria inhibit the colonization of pathogens, a stress-induced alteration of the gut microbiota may alter host susceptibility to pathogenic bacteria (Bailey et al., 2004; Keita and Söderholm, 2010). Furthermore, stress can modulate the intestinal permeability and promote the luminal attachment of pathogenic bacteria (Zareie et al., 2006; Lyte et al., 2011).

### **4.3 The effects of stress and stress-related hormones on the course of a *Salmonella* infection**

#### 4.3.1 The effects of stress-related hormones on the course of a *Salmonella* infection

In mammalian hosts, iron availability is very limited because it is bound to proteins such as haemoglobin, ferritin, lactoferrin and transferrin. For many bacteria, including *Salmonella*, the acquisition of iron within the host is essential for their survival. To overcome the low iron availability, *Salmonella* produces the siderophore enterochelin (enterobactin) in order to sequester and transfer iron into the bacterial cell. Due to its hydrophobicity and because it is sequestered by lipocalin 2, a mammalian component of the innate immune system, enterochelin is ineffective as an iron-scavenging agent (Fischbach et al., 2006). However, products of the *iroA* gene cluster in *Salmonella* glucosylate this siderophore in order to produce salmochelins. These modified forms of enterochelin can evade the lipocalin

2 binding and are less hydrophobic than enterochelin, resulting in a restored siderophore iron scavenging ability in mammals (Fischbach et al., 2006).

It has been shown that norepinephrine promotes growth, by providing iron to the bacterial cell via the high affinity siderophore enterochelin, and motility of *Salmonella enterica in vitro* (Bearson and Bearson, 2008; Bearson et al., 2008; Methner et al., 2008). A possible *in vivo* result of this could be the recrudescence of *Salmonella* in carrier animals, increased colonization of the gut due to its increased motility, increased shedding and consequently an increased contamination of the environment and other animals. In fact, Toscano et al. (2007) established that pretreatment of *Salmonella* Typhimurium with norepinephrine *in vitro* is associated with increased replication of this microorganism in various tissues of experimentally infected pigs. However, conflicting results have been obtained by Pullinger et al. (2010) who showed that pre-culture of the bacteria with norepinephrine does not alter the outcome of a *Salmonella* Typhimurium infection in pigs.

Bacterial cells respond to stress hormones via quorum sensing, through the use of small hormone-like molecules (autoinducers) to regulate gene expression within their own species and other bacterial strains in the microenvironment (Boyen et al., 2009; Pacheco and Sperandio, 2009). Bacteria do not express homologues of mammalian adrenergic receptors to respond to catecholamines, but they sense these hormones through histidine sensor kinases. Two histidine sensor kinases characterized in *Salmonella*, QseC and QseE, have been reported to sense epinephrine, norepinephrine and autoinducer 3, and epinephrine, sulphate and phosphate, respectively (Reading et al., 2009). It was shown that a *qseC* mutant reduced the norepinephrine-enhanced motility of *Salmonella* Typhimurium (Bearson and Bearson, 2008). Furthermore, Pullinger et al. (2010) showed that QseC plays a role in the response of *Salmonella* to 6-hydroxydopamine in pigs, but that other factors also may be involved. Six-hydroxydopamine was used to invoke the release of norepinephrine by destruction of noradrenergic nerve terminals in the periphery. The authors showed that the faecal excretion of the *Salmonella* Typhimurium  $\Delta qseC$  mutant could be increased by 6-hydroxydopamine treatment, indicating that QseC is not absolutely essential for the effect. However, the magnitude of the increase in faecal excretion of the  $\Delta qseC$  mutant was lower than compared to the wild type *Salmonella*. These data underscore the important role of QseC in stress-related pathogenesis of *Salmonella* infections in pigs.

Recently, Peterson et al. (2011) showed that physiological concentrations of norepinephrine enhance the horizontal gene transfer efficiencies of a conjugative plasmid encoding multidrug resistance from a clinical strain of *Salmonella* Typhimurium to an

*Escherichia coli* recipient *in vitro*. These results suggest that host stress could also influence the development of bacteria which are multidrug resistant. Furthermore, Karavolos et al. (2008) showed that epinephrine causes an upregulation of genes involved in oxidative stress, which suggests that epinephrine provides an environmental cue to alert the bacterium against the oxidative stress in macrophages. However, it has been shown that epinephrine decreases the resistance to polymyxin B through downregulation of the *pmr* operon, dependent on the 2-component system BasSR (Karavolos et al., 2008). In addition to this, Spencer et al. (2010) showed that epinephrine and norepinephrine decrease the resistance of *Salmonella* Typhimurium to the peptide cathelicidin LL-37, which is a human antimicrobial peptide. Apparently, norepinephrine and/or epinephrine alert the bacterial defences against oxidative stress, but these stress hormones also act in favour of the host by inducing a reduction in bacterial antimicrobial peptide resistance and by downregulation of bacterial virulence (Karavolos et al., 2008; Spencer et al., 2010).

#### 4.3.2 The effects of stress on the outcome of a *Salmonella* infection in pigs

It is thought that stress can increase *Salmonella* shedding in infected pigs and even cause a re-excretion of *Salmonella* in silent carriers (Hurd et al., 2002). This results in an increased cross-contamination during transport and lairage and as a consequence in a higher level of pig carcass contamination (Berends et al., 1996; Hald et al., 2003). An early study by Williams and Newell (1970) pointed out that transportation of pigs may lead to increased shedding of *Salmonella*, however conflicting results have been published (Morrow et al., 2002; Rostagno et al., 2005; Scherer et al., 2008). In more recent studies it was also shown that feed withdrawal and transportation stress are associated with increased shedding of *Salmonella* Typhimurium (Isaacson et al., 1999; Martín-Peláez et al., 2009). In addition, it was established that due to stress, sows become more susceptible to “new” *Salmonella* infections and that carrier sows are more likely to start shedding the pathogen (Nollet et al., 2005).

#### 4.3.3 The effects of stress on the outcome of a *Salmonella* infection in other animal species

Different types of natural stress factors can result in an increased susceptibility of pigs to *Salmonella* infections and a higher shedding of these bacteria. However, only limited data are available in literature with regard to this phenomenon in pigs. Therefore, the effects of

stress on the course of a *Salmonella* infection in mice, poultry and cattle are described in this section of the thesis.

Early studies in mice have established that stress causes an increased frequency and persistency of *Salmonella* infections (Miraglia and Berry, 1962; Previte et al., 1970, 1973; Kuriyama et al., 1996). Pre-treatment of mice with norepinephrine resulted in an enhanced systemic spread of *Salmonella* Typhimurium in mice (Williams et al., 2006).

Environmental stressors such as feed withdrawal and heat cause changes in the normal intestinal microbiota of poultry and the intestinal epithelial structure. This can lead to increased attachment of *Salmonella* Enteritidis (Burkholder et al., 2008). Stressed chickens have higher intestinal and circulating levels of norepinephrine and this stress hormone increases the colonization and systemic spread of *Salmonella* in chicken models (Knowles and Broom, 1993; Cheng et al., 2002; Methner et al., 2008). *In vivo* experiments showed that stress associated with forced molting of egg-laying flocks increases *Salmonella* Enteritidis shedding and that feed withdrawal before slaughter causes an increase of crop contamination by *Salmonella* as well as an enhanced colonization frequency of *Salmonella* in broiler chickens (Holt et al., 1994; Line et al., 1997; Ramirez et al., 1997; Carrier et al., 1999). In contrast, according to Rostagno et al. (2006), preslaughter stress practices (feed withdrawal, catching, loading, transportation and holding) do not significantly alter the prevalence of *Salmonella* in market-age turkeys.

In beef cattle, marketing- and transportation stress induced fecal excretion of *Salmonella* and transportation stress increased the frequency of *Salmonella* infections (Carrier et al., 1990; Barham et al., 2002; Reicks et al., 2007; Dewell et al., 2008). Systemic infections in cattle by *Salmonella* may result in encephalopathy. According to McCuddin et al. (2008), norepinephrine can play a role in these neurological manifestations by *Salmonella* since norepinephrine is needed for *Salmonella* to gain access to the systemic circulation and to induce encephalopathy.

## **5. Mycotoxicosis in pigs with emphasis on T-2 toxin**

Mycotoxins comprise a large group of chemically diverse compounds originating from secondary metabolites produced by certain strains of fungi such as *Aspergillus*, *Penicillium* and *Fusarium* (Hussein and Brasel, 2001; Gutleb et al., 2002). These mycotoxins can end up in the food and feed chain, and due to the diversity of their toxic effects and their synergetic properties, they are considered as risky to the consumers of contaminated foods and feeds (Yiannikouris and Jouany, 2002). Mycotoxins adversely affecting animal health are mainly produced under favourable conditions of temperature and humidity by saprophytic fungi during storage or by endophytic fungi during growth of the crops (Hussein and Brasel, 2001). Pigs are one of the most sensitive species to *Fusarium* mycotoxins and until now, more than 300 different mycotoxins have been identified, but only a few have been shown to affect porcine health and performance (Hussein and Brasel, 2001). These include aflatoxins (aflatoxin B1), ochratoxins (ochratoxin A), ergot alkaloids, zearalenone, fumonisins (fumonisin B1) and the trichothecenes deoxynivalenol (DON) and T-2 toxin. The effects of moderate to high amounts of mycotoxins in pigs have been well characterized. In Table 3 an overview is given of the most important mycotoxins affecting pig health, the most affected crops and the fungal species producing these mycotoxins.

Aflatoxins act as immunosuppressants and reduce overall pig health (Harvey et al., 1990; Lindemann et al., 1993; Rustemeyer et al., 2010). Ochratoxins, especially ochratoxin A, are common contaminants of barley and cause a reduced growth rate, liver and kidney damage and an increased mortality (Yiannikouris and Jouany, 2002). Ergot alkaloids cause a broad range of symptoms, varying from reduced growth and vomiting to reduced lactation and abortion (Diekman et al., 1992). Zearalenone has been shown to reduce fertility and consumption of fumonisins can cause porcine pulmonary oedema, liver damage and heart and respiratory dysfunctions (Diaz and Boermans, 1994). The effects of trichothecenes (DON and T-2 toxin) range from a reduced feed intake and vomiting to complete feed refusal (Wu et al., 2010).

The pattern and amounts of mycotoxins produced by a certain strain vary from year to year and depend on numerous factors such as the crop species, storage conditions and climate (Placinta et al., 1999; Gutleb et al., 2002). In certain geographical areas of the world, some mycotoxins are more easily produced than others (Akande et al., 2006). Aflatoxins are common in hot, humid tropical climate regions like those existing in Asian and African countries and certain parts of Australia (Akande et al., 2006). Ochratoxin A is formed by

*Aspergillus* in tropical and subtropical regions and by *Penicillium* in colder climates (Feier and Tofana, 2009). Wet, rainy weather is particularly favourable to ergot (*Claviceps*) growth (Krska and Crews, 2008). *Fusarium* mycotoxins can be found worldwide and especially in moderate climate regions of North America, Asia and Europe (Placinta et al., 1999).

**Table 3:** Examples of fungal species with their most affected crops and their respective mycotoxins affecting swine health. This table was in part adapted from Hussein and Brasel (2001).

Mycotoxins	Producing fungi	Most affected crops	Clinical symptoms
Aflatoxins (AFB1)	<i>Aspergillus (A.) flavus</i> , <i>A. nomis</i> and <i>A. parasiticus</i>	Wheat, barley and oats	Reduced growth, liver damage and immunosuppression (Miller et al., 1978; Southern and Clawson, 1979; Miller et al., 1981; Panangala et al., 1986; Harvey et al., 1988; Pang and Pan, 1994; Van Heughten et al., 1994)
Ochratoxins (OTA)	<i>A. ochraceus</i> , <i>Penicillium (P) viridicatum</i> and <i>P. cyclopium</i>	Wheat, barley, oats and maize	Reduced growth, anorexia, faintness, uncoordinated movement, liver and kidney damage, increased water intake, increased urination and increased mortality (Krogh et al., 1974; Huff et al. 1988; Krogh, 1991)
Ergot alkaloids	<i>Acremonium coenophialum</i> and <i>Claviceps purpurea</i>	Rye	Reduced growth, convulsions, respiratory distress, vomiting, reduced lactation in sows and abortion (Burfening, 1973; Diekman and Green, 1992)
Zearalenone (ZEA)	<i>Fusarium (F) culmorum</i> , <i>F. graminearum</i> and <i>F. sporotrichioides</i>	Wheat, barley and maize	Swelling of the vulva, rectal and vaginal prolapse, early embryonic mortality and fertility problems (Chang et al., 1979; Cantley et al., 1982; Etienne and Jemmali, 1982; Long and Diekman, 1984, 1986; Flowers et al., 1987; Diekman and Green, 1992)
Fumonisin (FB1)	<i>F. proliferatum</i> and <i>F. moniliforme</i>	Maize	Reduced feed intake, porcine pulmonary oedema (PPE), liver damage and abortion (Jones et al., 1994; Haschek et al., 2001; Cortyl, 2008)
Trichothecene deoxynivalenol (DON)	<i>F. culmorum</i> , <i>F. graminearum</i> and <i>F. sporotrichioides</i>	Wheat, barley, oats and maize	Immunomodulation, feed refusal, vomiting, weight loss, and reduced growth (Bergsjø et al., 1993; Grosjean et al., 2002; Swamy et al., 2002; Pinton et al., 2004; Etienne et al., 2006; Cortyl, 2008)
Trichothecene T-2 toxin	<i>F. acuminatum</i> , <i>F. equiseti</i> , <i>F. poae</i> and <i>F. sporotrichioides</i>	Wheat, barley, oats and maize	Immunomodulation, feed refusal, vomiting, weight loss, reduced growth and skin lesions (Weaver et al., 1987; Rafai et al., 1995b; Meissonier et al., 2008; Schuhmacher-Wolz, 2010)

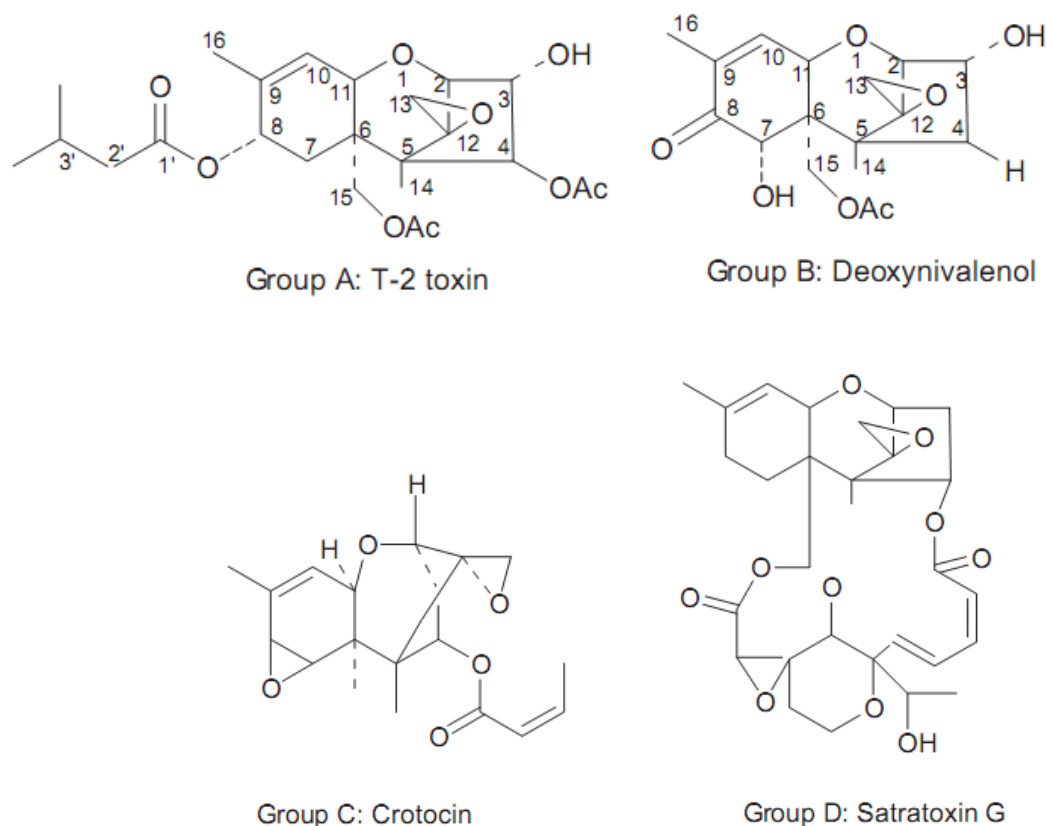
## 5.1 Trichothecenes

The trichothecenes are a large group of structurally related mycotoxins that comprise the largest group of *Fusarium* mycotoxins found in Europe (Binder et al., 2007). Other fungi species such as *Cephalosporium*, *Myrothecium*, *Stachybotrys* and *Trichothecium* are also able to produce trichothecenes, although to a lesser extent (Wu et al., 2010).

Trichothecenes are non-volatile, low-molecular-weight tricyclic sesquiterpenes that are characterized by the presence of a double bond at C-9,10 and an epoxy-ring at C-12,13. Generally, they are classed as 12,13-epoxy-trichothecenes (Gutleb et al., 2002). Depending on their functional groups, the trichothecenes are subdivided in 4 classes, illustrated in Figure 7. Type A trichothecenes have a functional group other than a ketone at C-8, whereas type B trichothecenes have a ketone at C-8. Examples of group A trichothecenes are represented by



T-2 toxin, HT-2 toxin and diacetoxyscirpenol (DAS), and DON is known as an important member of the type B trichothecenes. Group C members such as crotoxin, have another epoxy group between C-7 and C-8 or C-8 and C-9 positions. Type D trichothecenes like satratoxin contain a macrocyclic ring between C-4 and C-5 (Wu et al., 2010; Gutleb et al., 2002).



**Figure 7:** The chemical structures of trichothecenes: examples of groups A-D (Wu et al., 2010).

The skeleton of trichothecenes is chemically stable and the 12,13-epoxide ring is essential for their toxicity (Rocha et al., 2005). These trichothecenes are resistant to heat and autoclaving (Wannemacher et al., 2000) and they are not degraded during normal food processing conditions (Eriksen, 2003). Furthermore, trichothecenes are stable at neutral and acidic pH (Ueno et al., 1987), which implies that they are not hydrolysed in the stomach (Eriksen, 2003).

Up to now, already 150 trichothecenes and trichothecene derivatives have been isolated and characterized (Gutleb et al., 2002). However, data concerning their natural occurrence in foods and feeds are mostly limited to nivalenol, DAS, DON and T-2 toxin, since these are the most prevalent in the field (Smith et al., 1994; Wu et al., 2010). DON is the most widely occurring trichothecene in nature. However, contamination of cereals with T-2 toxin is an emerging issue (van der Fels-Klerx, 2010) and T-2 toxin is considered being the

most acutely toxic trichothecene (Gutleb et al., 2002). Monbaliu et al. (2010) analyzed 82 feed samples including sow feed, wheat and maize with a multimycotoxin LC-MS/MS method for the presence of mycotoxins. The samples were collected in the Czech Republic, Spain and Portugal and at least 67 samples were contaminated with at least one mycotoxin. Table 4 shows that seven samples were positive for T-2 toxin with detected minimum and maximum concentrations of 10 and 112  $\mu\text{g}$  T-2 toxin per kg feed, respectively.

Recently, the European Food Safety Authority (2011b) established a tolerable daily intake (TDI) value for the sum of T-2 toxin and HT-2 toxin of 100 ng/kg body weight. Nevertheless, control of exposure is limited since no maximum guidance limits for T-2 toxin in food and feedstuff are yet established by the European Union. In the next chapters, its mechanism of action, its effect on pig health and its interference with host-pathogen interactions will be discussed.

**Table 4:** Summary of the results of a multimycotoxin LC-MS/MS analysis of 67 contaminated feed samples for the presence of mycotoxins. This table was adapted from Monbaliu et al. (2010).

Mycotoxins	no. of contaminated samples	mean $\pm$ SD, $\mu\text{g}/\text{kg}$	minimum, $\mu\text{g}/\text{kg}$	maximum, $\mu\text{g}/\text{kg}$
Alternariol methyl ether	1		19	19
Ochratoxin A	2	27.5 $\pm$ 7.8	22	33
Diacetoxyscirpenol	3	5.1 $\pm$ 1.5	3,5	6,3
Alternariol	3	20.3 $\pm$ 4.2	17	25
Roquefortine-C	4	4.6 $\pm$ 6.1	1,3	14
T-2 toxin	7	28.9 $\pm$ 37.1	10	112
HT-2 toxin	7	47.0 $\pm$ 32.6	22	116
Nivalenol	9	416.2 $\pm$ 807.3	70	2547
Zearalenone	12	157.2 $\pm$ 117.5	58	387
Fumonisin B3	23	95.8 $\pm$ 55.2	25	246
Fumonisin B2	29	292.5 $\pm$ 305.5	28	1527
15-acetyldeoxynivalenol	31	118.3 $\pm$ 187.9	9.9	1047
3-acetyldeoxynivalenol	35	35.8 $\pm$ 56.3	6	339
Fumonisin B1	36	913.6 $\pm$ 1125	36	5114
Deoxynivalenol	52	948.6 $\pm$ 1772	74	9528

## 5.2 T-2 toxin: mechanism of action

The biochemical basis of the toxicity of T-2 toxin is a non-competitive inhibition of the protein synthesis (Cole and Cox, 1981). T-2 toxin binds to the 60S subunit in the ribosomes of eukaryotic cells, and thereby inhibits the peptidyl transferase activity at the transcription site (Cundliffe et al., 1974; Hobden and Cundliffe, 1980; Yagen and Bialer, 1993). Trichothecene-producing fungi contain an altered ribosomal protein L3, which is a component of the 60S ribosomal subunit. Therefore, these fungi are protected against the primary effect of T-2 toxin on the protein synthesis (Fried and Warner, 1981).

Inhibition of the protein synthesis by T-2 toxin leads to a ribotoxic stress response that activates c-Jun N-terminal kinase (JNK)/p38 mitogen-activated protein kinases (MAPKs), and as a consequence modulates numerous physiological processes including cellular homeostasis, cell growth, differentiation and apoptosis (Shifrin and Anderson, 1999).

Secondary to the inhibition of protein synthesis, T-2 toxin also inhibits the synthesis of DNA and RNA (Rocha et al., 2005). Both in *ex vivo* cell cultures (bone marrow, spleen and thymus of mice, after one, three or seven daily doses of 0.75 mg T-2 toxin/kg body weight) as in *in vitro* cell lines, the synthesis of DNA and RNA was inhibited by T-2 toxin (Scientific Committee on Food, 2001; World Health Organization, 2001; Schuhmacher-Wolz, 2010).

Furthermore, T-2 toxin interferes with cell membrane functions. According to Bunner and Morris (1988), T-2 toxin at a concentration of 0.4 pg/ml already affected the permeability of cell membranes of L-6 myoblasts within 10 minutes, *in vitro*. Once T-2 toxin has crossed the plasma membrane barrier, it can interact with mitochondria (Pace et al., 1988). It has been shown that T-2 toxin inhibits mitochondrial functions *in vivo* and *in vitro* (Holt et al., 1988; Pace et al., 1988; Rocha et al., 2005). Generally, cells depending on high protein synthesis, such as epithelial cells and lymphocytes, are considered to be the most sensitive to T-2 toxin (Eriksen, 2003).

## 5.3 T-2 toxin toxicokinetics in pigs

T-2 toxin is toxic to several animal species, but cattle are less sensitive than most monogastric species (Eriksen, 2003). The reason for this is that trichothecenes are largely de-epoxidised in the rumen of cattle before absorption to the blood (Cote et al., 1986; Swanson and Corley, 1989). Since the 12,13-epoxy group is essential for the toxicity of T-2 toxin, such a de-epoxidation is considered as a detoxification of the mycotoxin. Reduction of this epoxide

is catalyzed by anaerobic microbiota present in the gastrointestinal tract and ruminal fluids (Yagen and Bialer, 1993). Of all monogastric species, pigs are one of the most sensitive ones to T-2 toxin (Hussein and Brasel, 2001).

After ingestion, T-2 toxin is rapidly and efficiently absorbed in the gastrointestinal tract which results in the passage of T-2 toxin to the blood and distribution to other organs such as the liver for which T-2 toxin has a high affinity (Yagen and Bialer, 1993). T-2 toxin is also rapidly absorbed via the inhalation route, whereas dermal absorption is reported to be slow (Schuhmacher-Wolz et al., 2010). Eriksen and Pettersson (2004) showed that T-2 toxin was already detected in pig blood before 30 min after their ingestion. Its plasmatic half-life is less than 20 min and T-2 toxin is more rapidly absorbed than DON after ingestion by most species (Beasley et al., 1986; Larsen et al., 2004; Cavret and Lecoecur, 2006).

After its absorption, T-2 toxin is rapidly biotransformed into its metabolites (Cavret and Lecoecur, 2006). The major metabolic fates of T-2 toxin in animals are hydroxylation, hydrolysis, conjugate formation, and de-epoxidation (Bauer 1995; Yagen and Bialer, 1993; Wu et al., 2010). T-2 toxin is rapidly metabolized to HT-2 toxin which is detected in the blood just after the ingestion of T-2 toxin. This suggests that intestinal cells can metabolize T-2 toxin by hydrolysis at the C-4 position (JECFA, 2002). After being transformed to HT-2, it undergoes further hydroxylation (Wu et al., 2010). According to Ge et al. (2010), cytochrome P450 (CYP3A22) is responsible for the 3'-hydroxylation of T-2 toxin and HT-2 toxin into their less toxic metabolites, 3'-hydroxy-T-2 and 3'-hydroxy-HT-2, respectively. In addition to this, Wu et al. (2011) showed that CYP3A29 could catalyze the hydroxylation of T-2 toxin, indicating that the CYP3A gene subfamily is important for the transformation of T-2 toxin into its metabolites in pigs. Metabolite profiling of T-2 toxin in the bile and urine of swine pointed out that glucuronide-conjugated products were found to be the main metabolites in the urine of swine (Corley et al., 1985; Wu et al., 2010). According to Corley et al. (1986), who studied the T-2 toxin metabolism profiles in plasma and tissues of swine, deepoxy metabolites (deepoxy-HT-2, deepoxy-T-2 triol, and deepoxy-T-2 tetraol) were also found in swine. A proposed metabolic pathway of T-2 toxin in animals was adapted from Wu et al. (2010) and is represented in Figure 8.

T-2 toxin and its metabolites are rapidly eliminated in pigs via urine and faeces, which are the main routes of excretion (Beasley et al., 1986; JECFA, 2002). Due to the rapid metabolism of T-2 toxin, it is unlikely that T-2 toxin will be found in edible tissues (Bauer, 1995). However, Robison et al. (1979) intubated pigs with [<sup>3</sup>H]-T-2 toxin to determine the tissue distribution and the excretion pattern of T-2 toxin and/or its metabolites. Eighteen hours

after administration of 0.1 mg [<sup>3</sup>H]-T-2 toxin/kg body weight, the distribution pattern was 0.7% (muscle), 0.43% (liver), 0.08% (kidney), 0.06% (bile), 21.6% (urine) and 25.0% (faeces). The distribution pattern after administration of 0.4 mg [3H]-T-2 toxin/kg body weight was 0.7% (muscle), 0.29% (liver), 0.08% (kidney), 0.14% (bile), 17.6% (urine) and 0.84% (faeces). In this experiment, the distribution pattern of [<sup>3</sup>H]-T-2 toxin was investigated in only one animal per concentration. Therefore, differences in percentage present in the faeces may be attributed to differences within individual animals. Possibly T-2 toxin and its metabolites undergo enterohepatic circulation, which may differ between animals. Yoshizawa et al. (1981) showed that T-2 toxin and its metabolites can also be secreted in cow milk.

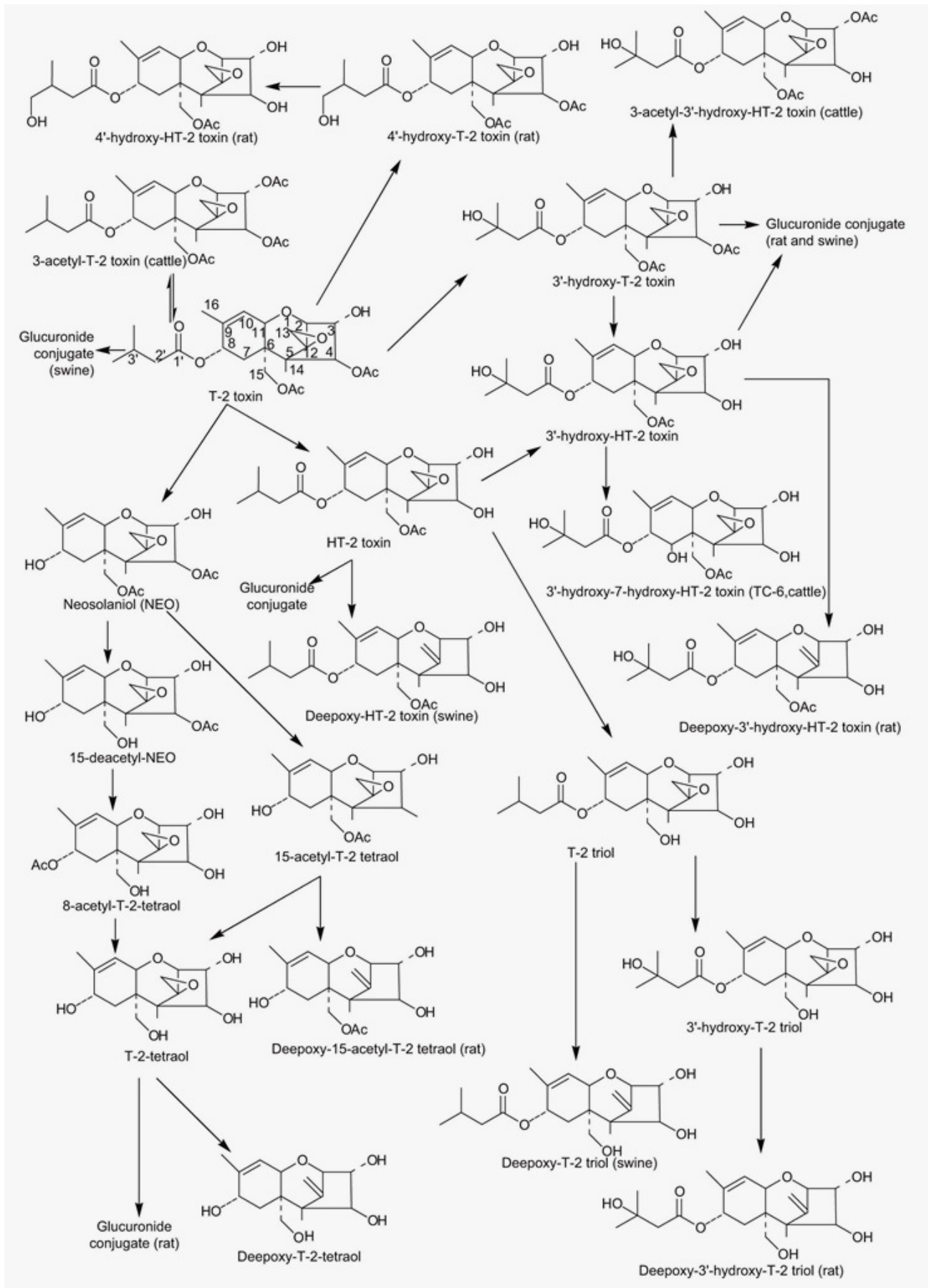


Figure 8: Proposed metabolic pathways of T-2 toxin in animals (Wu et al. 2010).

## 5.4 Effects of T-2 toxin on pig health

### 5.4.1 Acute toxicity

Effects observed in various species after acute oral T-2 toxin exposure (ranging from 0.06 to 10 mg/kg body weight) include non-specific symptoms such as weight loss, feed refusal, dermatitis, vomiting, diarrhoea, haemorrhages and necrosis of bone marrow, spleen, testis, ovary and the epithelium of the stomach and intestine (Shuhmacher-Wolz, 2010). Studies with pigs revealed an oral median lethal dose (LD50) of 5 mg/kg body weight and an intravenous LD50 of 1.2 mg/kg body weight (Shuhmacher-Wolz, 2010). Furthermore, acute effects in pigs include disturbance of the circulatory system such as hypotension and arrhythmia. This could be due to an effect on blood pressure and catecholamine elevation (Shuhmacher-Wolz, 2010). Dong et al. (2008) showed that a single subcutaneous administration of T-2 toxin of 0.3 mg T-2 toxin/kg body weight results in the modulation of Phase I and Phase II drug metabolizing enzymes. Protein levels of CYP1A2, 2E1, 3A4, glutathione S-transferase alpha and glutathione S-transferase M1-1 were increased at 24 hours after application (Dong et al., 2008).

### 5.4.2 Repeated dose toxicity

Effects observed in various species after repeated exposure to T-2 toxin include signs such as poor weight gain, weight loss, bloody diarrhoea, dermal necrosis, beak and mouth lesions, haemorrhage and decreased production of milk and eggs and immunological effects (Figure 9; Schuhmacher-Wolz, 2010). Pigs (seven-week-old) that were fed a prestarter containing T-2 toxin at concentrations ranging from 0.5 to 15.0 mg/kg feed, for three weeks, showed a reduced feed intake (10%) and a lowered glucose, inorganic phosphorus and magnesium levels (Rafai et al., 1995a). A reduced body weight gain and reduced haemoglobin and serum alkaline phosphatase values were seen in pigs that received 8.0 mg T-2 toxin/kg feed during 30 days (Harvey et al., 1994). Ad libitum treatment of pigs with 10 mg T-2 toxin/kg feed during 28 days, induced necrotizing contact dermatitis on the snout, buccal commissures and prepuce and it resulted in increased serum triglyceride and decreased serum iron concentrations (Harvey et al., 1990).

Besides oral exposure, T-2 toxin is also rapidly absorbed via the inhalation route. Pang et al. (1987, 1988) showed that T-2 toxin aerosol (equivalent to 8 mg/kg body weight) treatment resulted in vomiting, cyanosis, anorexia, lethargy, lateral recumbency, slightly

elevated rectal temperature and a depressed body weight. Most of this research is however limited to the clinical effects of high and sometimes irrelevant concentrations of T-2 toxin. The influence of low concentrations of T-2 toxin remains largely unknown.



**Figure 9:** Skin lesions caused by T-2 toxin exposure. This picture was adapted from <http://www.knowmycotoxins.com/pig.htm#8>.

#### 5.4.3 Immunity

The immune system is one of the main targets of mycotoxins and they can affect both the humoral and cellular immune response. *In vitro* studies showed that immunosuppression caused by aflatoxins occurs mainly at cellular and not humoral level (van Heugten et al., 1994). This was confirmed *in vivo* by Miller et al. (1981, 1987). A decreased lymphocyte blastogenic response to mitogens and a reduced macrophage migration was noticed when pig received 0.4 to 0.8 mg aflatoxins per kg feed for 10 weeks (Miller et al., 1987). On the other hand, no effect on swine humoral immune response was noticed at levels ranging from 0.4 to 0.8 mg aflatoxin per kg of feed (Miller et al., 1981) and even at a high acute concentration of 500 mg/kg of feed (Panangala et al., 1986).

Ingestion of FB1 (0.5 mg/kg body weight during 7 days) decreased the expression of IL-8 mRNA in the ileum of piglets (Bouhet et al., 2006). This decrease in IL-8 may lead to a reduced recruitment of inflammatory cells in the intestine and may participate in the increased susceptibility to intestinal infections (Oswald et al., 2003). In line with these results, Devriendt et al. (2009) showed that fumonisin B1 (1 mg/kg body weight during 10 days) reduces the intestinal expression of IL-12/IL-23p40. They also showed that the function of intestinal antigen presenting cells is impaired, with a decreased upregulation of major histocompatibility complex class II molecules and a reduced T cell stimulatory capacity upon



stimulation. According to these authors, fumonisin B1 reduces *in vivo* antigen presenting cell maturation, resulting in a prolonged F4<sup>+</sup> enterotoxigenic *Escherichia coli* infection.

DON can either be immunostimulatory or immunosuppressive, depending on the dose and the exposure frequency (Pestka and Smolinski, 2005). It is stated that low doses of DON act immunostimulating by increasing production and secretion of pro-inflammatory cytokines. DON affects the humoral immune response by increasing IgA in the serum of pigs, as well as the levels of expression of several cytokines such as IL-6, IL-10, IFN- $\gamma$  and TNF- $\alpha$  (Bergsjö et al., 1993; Grosjean et al., 2002; Swamy et al., 2002; Pinton et al., 2004). Vandembroucke et al. (2011) showed that co-exposure of pig loops to 1 mg/mL of DON and *Salmonella* Typhimurium compared to loops exposed to *Salmonella* Typhimurium alone, causes an increased expression of IL-12/IL-23p40 and TNF- $\alpha$ . High doses of DON act rather immunosuppressive by causing apoptosis of leucocytes (Yang et al., 2000).

Rafai et al. (1995b) investigated the effects of various levels of T-2 toxin on the immune system of growing pigs. The animals received feed contaminated with T-2 toxin at concentrations ranging from 0.5-3.0 mg/kg feed, for three weeks. On the first and the fourth day of the treatment, the animals were immunised with horse globulin. Blood samples were collected before the immunization and at day 7, 14 and 21 and they showed that the synthesis of antibodies towards horse globulin, was reduced at all dose groups of T-2 toxin and at all time points. Depletion of lymphoid elements in the thymus and spleen was noticed and the leukocyte counts and the portion of T lymphocytes were decreased in all exposure groups (Rafai et al., 1995b, Schuhmacher-Wolz et al., 2010). The effect of T-2 toxin on the acquired immune response of a vaccine antigen was confirmed by Meissonier et al. (2008) who showed that pigs fed 1324 or 2102  $\mu$ g T-2 toxin/kg feed exhibited reduced anti-ovalbumin antibody production. However, in contrast to Rafai et al. (1995b), Meissonier et al. (2008) did not observe effects on the leukocyte proliferation and the spleen histopathology. *In vitro*, numerous experiments have been performed to investigate the effect of T-2 toxin on the immune system. Summarized, severe damage to actively dividing cells in the lymph nodes, spleen, thymus, bone marrow and intestinal mucosa has been observed (Schuhmacher-Wolz et al., 2010).

Generally, the effects of moderate to high amounts of mycotoxins on the immune system have been well characterized, but less attention has been focused on their effects on the local intestinal immune response (Bouhet and Oswald, 2005). Nevertheless, the intestine and the intestinal epithelial cell layer are exposed to these mycotoxins following ingestion of contaminated food or feed. Several mycotoxins cause a decrease of the transepithelial

electrical resistance (TEER) of several cell types (Mahfoud et al., 2002; Maresca et al., 2001, 2002; Bouhet et al., 2004). The TEER is a good indicator of the epithelial integrity and tight junction organization (Hashimoto and Shimizu, 1993). This implies that mycotoxins can alter the intestinal barrier function. T-2 toxin even induces necrosis of epithelial and crypt cell of the jejunum and ileum in pigs, chickens and mice (Hoerr et al., 1981; Li et al., 1997; Williams, 1989). Besides the effect on the epithelial barrier and its inter-cellular junctions, trichothecenes have been shown to modulate the immunoglobulin pathway. T-2 toxin suppresses membrane immunoglobulin A (IgA)-bearing cells in mouse Peyer's patches (Nagata et al., 2001). Recently, Kruber et al. (2011) established that T-2 toxin strongly induces IL-8 production in a Caco-2 intestinal epithelial cell line. This implies that T-2 toxin also affects the cytokine production in the intestine. These cytokines are important mediators in the regulation of the immune and inflammatory responses (Bouhet and Oswald, 2005). These data indicate that T-2 toxin is able to disrupt the intrinsic barrier function of intestinal epithelial cells and that it affects the release of protective molecules (Bouhet and Oswald, 2005).

### **5.5 Effects of T-2 toxin on bacterial infections**

Several articles pointed out that repetitive exposure to T-2 toxin increases the susceptibility to a diverse array of pathogens. Mice that were challenged with T-2 toxin at various stages of infection, showed an increased susceptibility to *Mycobacterium* (Kanai and Kondo, 1984). The immunosuppressive effect of the mycotoxin was also seen in mice exposed to T-2 toxin after *Listeria monocytogenes* infection. T-2 toxin induced a rapid growth of the bacterium and significantly increased the mortality due to listeriosis (Corrier and Ziprin, 1986a). The trichothecene caused a depletion of T lymphocytes and failure of surviving immunologically committed T cells and T-cell dependent immune-activated macrophages to clear the host of pathogens (Corrier and Ziprin, 1986a). Tai and Pestka (1988a) showed that co-challenge of *Salmonella* Typhimurium and T-2 toxin in mice results in a five log reduction in LD50 of *Salmonella* Typhimurium. When coadministered with DON, an increased susceptibility to lipopolysaccharide (LPS) was observed in mice. These results suggest that bacterial LPS and trichothecenes interact synergistically (Tai and Pestka 1988b; Zhou et al., 1999c). Other mycotoxins such as DAS have also been shown to influence the susceptibility to pathogens. Immunosuppression by repeated injections of DAS increased the sensitivity to *Cryptococcus neoformans* (Fromentin et al., 1981). Besides repetitive

exposure, a single injection of DAS into mice challenged by *Candida albicans* significantly increased the development of experimental candidiasis (Salazar et al., 1980).

Depending on the dose and timing of exposure, T-2 toxin and trichothecenes in general can be either immunosuppressive or immunostimulatory (Bondy and Pestka, 2000). This partly explains why mycotoxin-enhanced resistance to several pathogens has also been described. Corrier and Ziprin showed that short-term preinoculation with T-2 toxin enhances the resistance to *Listeria monocytogenes*, whereas the authors showed that postexposure to this mycotoxin result in immunosuppression (Corrier and Ziprin, 1986a, 1986b; Corrier et al., 1987a, 1987b). It has been suggested that this enhanced resistance is associated with an increased migration of macrophages and an elevated phagocytic activity which may have been mediated by altered T-regulatory cell activity (Corrier 1991; Bondy and Pestka, 2000). In addition to this, Taylor et al. (1989, 1991) showed that pretreatment of mice with a single dose of T-2 toxin significantly reduced the virulence of *Escherichia coli* and *Staphylococcus aureus* in mice. Successive treatment with T-2 toxin for 14 days prior to inoculation also slightly lowered the virulence in T-2 toxin treated mice.

Tai and Pestka (1988a) showed that co-challenge with T-2 toxin and *Salmonella* Typhimurium led to an impaired murine resistance to the bacterium. Increased mortality in response to the bacterium was dependent on the mycotoxin dose. T-2 toxin did not significantly affect the intestinal infection, but it increased splenic counts in *Salmonella* infected mice. If the T-2 toxin treatment was started 1 day prior to the infection or at 5 or 9 days after infection, the mortality rate was equal to the co-challenge as described above. However, when the T-2 toxin administration was started at 13 or 23 days after infection, a time-related decreased mortality was observed. These data support the hypothesis that depending on the challenge dose and exposure regimen, T-2 toxin can either increase or decrease the susceptibility to an infection.

Probably, other factors also influence the effects of mycotoxins on the outcome of an infection. Ziprin and McMurray (1988) investigated the effect of pretreatment of mice with a single dose of T-2 toxin (gastric gavage; 4 mg/kg body weight) on the course of a *Listeria monocytogenes*, *Salmonella* Typhimurium or *Mycobacterium bovis* infection. Seven days after toxin administration, the mice were either infected using intraperitoneal inoculation, or by inhalation of respirable droplet nuclei containing the bacteria. T-2 toxin had no effect on resistance to infection initiated by inhalation and on the course of salmonellosis induced after intraperitoneal inoculation. However, the toxin increased the resistance to infection with *Listeria monocytogenes* and reduced the resistance to *Mycobacterium bovis* infection after

intraperitoneal inoculation. These data indicate that the effect of T-2 toxin on the course of a bacterial infection also depends on the nature of the infective agent and on the route of inoculation (Ziprin and McMurray, 1988).

Most of the research concerning the influence of mycotoxins on the susceptibility to infections has been conducted in mice and only little information is available of these effects in pigs. Tenk et al. (1982) showed that feeding pigs with 5 mg T-2 toxin per kg feed, resulted in a substantial increase of aerobic bacterial counts in the intestine. Oswald et al. (2003) showed that FB1 (0.5 mg/kg body weight daily for 6 days) increased the intestinal colonization by pathogenic *Escherichia coli* in pigs. However, Tanguy et al. (2006) stated that feeding pigs with FB1 did not induce modifications in the number of *Salmonella* bacteria in the ileum, cecum and colon of asymptomatic carrier pigs.

## **5.6 Mycotoxin reduction**

### **5.6.1 Prevention of intoxication by mycotoxins**

Mycotoxin contamination may occur either before harvest of a crop (field stage) or during storage. Some moulds such as *Fusarium* spp. are most frequently encountered in the field. These fungi can grow on grains and produce mycotoxins before harvest. However, although *Fusarium* spp. mostly infect cereals, they can also be found after harvest (Yainnikouris and Jouany, 2002). Other moulds such as *Aspergillus* spp. usually do not invade the crop prior to harvest. These moulds are called “storage fungi” and they grow during storage of the crops (Yainnikouris and Jouany, 2002).

A first step to prevent field mycotoxin formation is to minimize plant contamination. Several agricultural practices such as a proper irrigation and fertilization of the crops, and choosing varieties that are adapted to the growing area, have resistance to fungal disease and have resistance to insect damage, could reduce the mycotoxin formation in the field (Whitlow, accessed on 06/01/2012). Moreover, several fungicides such as triazoles and strobilurins, are valuable tools against several species of the *Fusarium* complex (Audenaert et al., 2011). However, even the best management of agricultural strategies cannot completely eradicate mycotoxin contamination (Jouany, 2007), and the use of fungicides can even increase T-2 toxin levels. Gaurilcikiene et al. (2011) demonstrated increased T-2 toxin levels in azoxystrobin applied plots and Audenaert et al. (2011) showed that sublethal prothioconazole and fluoxastrobin concentrations can cause fungicide stress, leading to an increased T-2 toxin production of *Fusarium poae*.

A second step in the prevention is to reduce mycotoxin formation during harvest by avoiding lodged or fallen material, because contact with soil can increase mycotoxin formation and damaged or broken kernels support more mould growth. Cleaning and maintaining harvest equipment in good condition can greatly reduce mycotoxin concentrations in the feedstuff (Whitlow, accessed on 06/01/2012).

The storage conditions are critical in the prevention of storage mould growth and storage mycotoxin production. Good storage conditions include rapid filling of the silo, sufficient packing of the silage, appropriate covering to eliminate air and water, proper moisture contents, cooling operations and routinely cleaning of the storage facilities (Jouany, 2007; Whitlow, accessed on 06/01/2012).

### 5.6.2 Mycotoxin binders

Detoxification methods for mycotoxins are limited. Removal or dilution of the contaminated feed is a common practice, however mixing batches with the aim of decreasing the level of contamination is not authorized within the European Union (Kolosova and Stroka, 2011). One of the most promising methods for mycotoxin decontamination is the use of mycotoxin-detoxifying agents. A first strategy for reducing the exposure to mycotoxins is the use of mycotoxin-adsorbing agents in the feed. Theoretically, these adsorbing agents adsorb the toxin in the gut, resulting in the excretion of the toxin in the faeces. A second strategy is the use of biotransforming agents that modify the toxin into non-toxic metabolites (European Food Safety Authority, 2009). The extensive use of these agents resulted in the introduction of a wide range of new products and in the introduction of a new functional group of feed additives by the European Commission in 2009. This group was entitled “substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action”. Since the mycotoxin-detoxifying agents have little or no effect in or on the feed itself, but act after ingestion by the animal, *in vitro* testing is not sufficient to examine the safety and efficacy of these mycotoxin-detoxifying agents. Therefore the European Food Safety Authority recently implemented that *in vivo* trials should be performed to test the efficacy and safety of these mycotoxin-adsorbing agents (European Food Safety Authority, 2010).

### 5.6.2.1 Mycotoxin-adsorbing agents

Mycotoxin-adsorbing agents are compounds with a high molecular weight, which are able to bind mycotoxins after ingestion. Ideally, this complex does not dissociate in the gastrointestinal tract of the animal, resulting in an efficient elimination via faeces and hereby preventing or minimizing exposure of animals to mycotoxins (European Food Safety Authority, 2009b). These mycotoxin-adsorbing agents include aluminosilicates (bentonite, montmorillonite, zeolite, phyllosilicates), activated carbon, complex indigestible carbohydrates (cellulose, polysaccharides from the cell walls of yeast and bacteria such as glucomannans and peptidoglycans) and synthetic polymers (cholestyramine and polyvinylpyrrolidone) (European Food Safety Authority, 2009b).

Based on the literature, the protective role of some of these mycotoxin-adsorbing agents against T-2 toxin has been demonstrated. Aravind et al. (2003) showed that the addition of esterified glucomannan to a naturally contaminated diet (including T-2 toxin), was effective in counteracting the toxic effects, such as growth depression of broilers. Raju and Devegowda (2000) showed that the addition of an esterified glucomannan in the diet of broiler chickens minimized the adverse effects of aflatoxin B1, ochratoxin A and T-2 toxin. The protective role of the modified glucomannan binder Mycosorb<sup>TM</sup> against the detrimental effects of T-2 toxin on growing chickens has also been demonstrated (Dvorska et al., 2007). Furthermore, the use of a yeast-derived glucomannan demonstrated protective effects against T-2 toxin immunotoxicity during a vaccinal protocol in pigs (Meissonier et al., 2009). These organic binders are biodegradable and they do not accumulate in the environment after excretion by animals. Another advantage of these yeast cell wall based mycotoxin binders is that they are more adapted to multi-contaminated feeds because they are more efficient against a larger range of mycotoxins than inorganic binders (Kolossova et al., 2009).

Several inorganic binders such as bentonites, zeolites, and aluminosilicates are the most common feed additives against aflatoxins (Kolossova et al., 2009). However, they seem to be less effective against T-2 toxin contaminations. Curtui (2000) showed that the inclusion of 0.5% zeolite did not diminish the adverse effects of trichothecenes in broiler chickens. Edrington et al. (1997) investigated the effectiveness of a super activated charcoal in alleviating mycotoxicosis in broiler chicks which were fed diets containing aflatoxin or T-2 toxin. The addition of super activated charcoal was of little benefit when T-2 toxin was fed to growing broiler chickens. Furthermore, if the concentration of essential nutrients in the animal

feed is much higher compared to those of a mycotoxin, then super activated charcoal also adsorbes these nutrients (Huwig et al., 2001).

#### 5.6.2.2. Biotransforming agents

Biotransforming agents such as bacteria, yeasts, fungi and enzymes, are able to degrade mycotoxins (European Food Safety Authority, 2009b). Since the 12,13-epoxide ring is responsible for the toxicity of trichothecenes, de-epoxidation of these mycotoxins results in a significant loss of toxicity. Ruminal and intestinal microbiota have been shown to de-epoxidate trichothecenes (Yoshizawa et al., 1983; He et al., 1992; Kollarczik et al., 1994). In addition to this, an *Eubacterium* strain isolated from bovine rumen contents and referred as BSSH 797, has been shown to transform DON into its non-toxic deepoxide metabolite DOM-1 (Binder et al., 2000; Fuchs et al., 2002). This bacterial strain also caused a simultaneous deacetylation and de-epoxidation of A-trichothecenes and actually, BSSH 797 is used in a feed additive proposed to counteract the deleterious effects of trichothecenes (Fuchs et al., 2002; Jouany et al., 2007).

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# **Scientific Aims**





Salmonellosis is one of the most important zoonotic bacterial diseases and pigs are considered as one of the main sources of human salmonellosis. Worldwide, *Salmonella* Typhimurium is the predominant serotype isolated from slaughter pigs. Often, these pigs are persistently infected with *Salmonella* Typhimurium, which means that they carry the bacterium asymptotically in their tonsils, gut and gut-associated lymphoid tissue for months resulting in so called *Salmonella* carriers. Interactions of the bacterium with the porcine host are very complex and may be affected by external factors such as host stress and exposure to feed contaminants like mycotoxins.

Periods of stress like transport to the slaughterhouse, induce increased fecal shedding of *Salmonella*. This could lead to increased cross-contamination during transport and lairage and to a higher degree of carcass contamination, thus affecting human health. Stress results in the release of catecholamines and glucocorticoids and pigs secrete cortisol as the predominant glucocorticoid. We hypothesized that glucocorticoids may play an important role in the stress related recrudescence of the bacterium. However, when we started our research, little was known about the role of glucocorticoids. Therefore, it was the **first aim** of this thesis to determine the role of cortisol in the stress related recrudescence of *Salmonella* Typhimurium by pigs and to elucidate if it alters bacterium-host cell interactions.

Besides stress, *Salmonella* infected pigs can also be exposed to T-2 toxin. This mycotoxin is produced by various *Fusarium* species which are common contaminants of cereals. Since T-2 toxin is very stable under normal food processing conditions, it can end up in the food and feed chain and cause numerous toxic effects, especially to pigs which are one of the most sensitive species. T-2 toxin mostly affects rapidly dividing cells, such as intestinal epithelial cells, and cells of the immune system, which both play an important role in the pathogenesis of a *Salmonella* infection. As a result, we hypothesized that T-2 toxin may interfere with the pathogenesis of a *Salmonella* Typhimurium infection in pigs. A widespread strategy for reducing the exposure to T-2 toxin is the use of mycotoxin-adsorbing agents in the feed. Some mycotoxin-adsorbing agents, like glucomannans, are derived from yeast cell walls that contain  $\alpha$ -D-mannans and  $\beta$ -D-glucans. Since both polysaccharides have been described to interfere with the pathogenesis of a *Salmonella* infection, we assumed that some mycotoxin-adsorbing agents could influence the course of the infection. For that reason, it was the **second aim** to investigate whether and how T-2 toxin and a commercially available modified glucomannan feed additive affect the pathogenesis of a *Salmonella* Typhimurium infection in pigs.



# **Experimental Studies**

- 1) **Stress induced *Salmonella* Typhimurium recrudescence in pigs coincides with cortisol induced increased intracellular proliferation in macrophages**
- 2) **Cortisol modifies protein expression of *Salmonella* Typhimurium infected porcine macrophages, associated with *scsA* driven intracellular proliferation**
- 3) **T-2 toxin induced *Salmonella* Typhimurium intoxication results in decreased *Salmonella* numbers in the cecal contents of pigs, despite marked effects on *Salmonella*-host cell interactions**
- 4) **A modified glucomannan feed additive counteracts the reduced weight gain and diminishes cecal colonization of *Salmonella* Typhimurium in T-2 toxin exposed pigs.**



## CHAPTER 1:

### **Stress induced *Salmonella* Typhimurium recrudescence in pigs coincides with cortisol induced increased intracellular proliferation in macrophages**

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## Abstract

*Salmonella* Typhimurium infections in pigs often result in the development of carriers that intermittently excrete *Salmonella* in very low numbers. During periods of stress, for example transport to the slaughterhouse, recrudescence of *Salmonella* may occur, but the mechanism of this stress related recrudescence is poorly understood. Therefore, the aim of the present study was to determine the role of the stress hormone cortisol in *Salmonella* recrudescence by pigs. We showed that a 24 h feed withdrawal increases the intestinal *Salmonella* Typhimurium load in pigs, which is correlated with increased serum cortisol levels. A second *in vivo* trial demonstrated that stress related recrudescence of *Salmonella* Typhimurium in pigs can be induced by intramuscular injection of dexamethasone. Furthermore, we found that cortisol, but not epinephrine, norepinephrine and dopamine, promotes intracellular proliferation of *Salmonella* Typhimurium in primary porcine alveolar macrophages, but not in intestinal epithelial cells and a transformed cell line of porcine alveolar macrophages. A microarray based transcriptomic analysis revealed that cortisol did not directly affect the growth or the gene expression of *Salmonella* Typhimurium in a rich medium, which implies that the enhanced intracellular proliferation of the bacterium is probably caused by an indirect effect through the cell. These results highlight the role of cortisol in the recrudescence of *Salmonella* Typhimurium by pigs and they provide new evidence for the role of microbial endocrinology in host-pathogen interactions.

## Introduction

For a long time it has been known that stress may cause recrudescence of some bacterial infections in food-producing animals, such as poultry and pigs (Burkholder et al., 2008; Rostagno, 2009). Salmonellosis is one of the most important zoonotic bacterial diseases and pigs are considered as one of the main sources of human salmonellosis (Berends et al., 1996, 1998; Alban et al., 2002; Snary et al., 2010). Worldwide, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the predominant serovar isolated from slaughter pigs (Boyen et al., 2008a). Pigs infected with *Salmonella* Typhimurium can carry this bacterium asymptotically in their tonsils, gut and gut-associated lymphoid tissue for months resulting in so called *Salmonella* carriers. Generally, these persistently infected animals intermittently shed low numbers of *Salmonella* bacteria. However, during periods of stress, like transport to the slaughterhouse, recrudescence of *Salmonella* may occur. This results in increased cross-contamination during transport and lairage and to a higher degree of carcass contamination, which could lead to higher numbers of foodborne *Salmonella* infections in humans (Berend et al., 1998; Wong et al., 2002). Until now, the mechanism of stress related recrudescence of *Salmonella* is not well understood and this study aimed at elucidating this phenomenon.

Although stress is hard to define and the factors causing stress can be very different, they generally result in similar physiological responses. A period of stress results in the release of a variety of neurotransmitters, peptides, cytokines, hormones, and other factors into the circulation or tissues of the stressed organism (Freestone and Lyte, 2008; Merlot et al., 2011; Muráni et al., 2011). Besides the fast-acting catecholamines, which are released by the sympathetic nervous system, the hypothalamic-pituitary-adrenal axis becomes activated, resulting in the release of the slow-acting glucocorticoids by the adrenal gland (Dhabhar, 2009). These stress hormones can not only affect the host immune response via the modulation of various aspects of the immune system, but they also can have a direct effect on the bacteria and may influence their interactions with the host cells (Verbrugghe et al., 2011). Indeed, several bacterial species can exploit the neuroendocrine alteration of a host stress reaction as a signal for growth and pathogenic processes (Lyte, 2004; Freestone et al., 2008; Dhabhar, 2009).

Pigs secrete cortisol as the predominant glucocorticoid (Worsaae and Schmidt, 1980). Therefore, it was the aim of the present study to determine the role of this hormone in the

stress related recrudescence of *Salmonella* Typhimurium by pigs and to elucidate if it alters bacterium-host cell interactions.

## Materials and Methods

### Chemicals

Cortisol and dexamethasone (Sigma-Aldrich, Steinheim, Germany) stock solutions of 10 mM were prepared in water and stored at  $-20\text{ }^{\circ}\text{C}$ . Serial dilutions of cortisol were, depending on the experiment, prepared in Luria-Bertani broth (LB, Sigma-Aldrich NV/SA) or in the corresponding cell culture medium.

### Bacterial strains and growth conditions

*Salmonella* Typhimurium strain 112910a, isolated from a pig stool sample and characterized previously by Boyen et al. (2008b), was used as the wild type strain in which the spontaneous nalidixic acid resistant derivative strain (WT<sub>nal</sub>) was constructed. For fluorescence microscopy, *Salmonella* Typhimurium strain 112910a carrying the pFPV25.1 plasmid expressing green fluorescent protein (GFP) under the constitutive promoter of *rpsM* was used (Van Immerseel et al., 2004; Boyen et al., 2008b).

Unless otherwise stated, the bacteria were generally grown overnight (16 to 20 h) as a stationary phase culture with aeration at  $37\text{ }^{\circ}\text{C}$  in 5 mL of LB broth. To obtain highly invasive late logarithmic cultures for invasion assays, 2  $\mu\text{L}$  of a stationary phase culture were inoculated in 5 mL LB broth and grown for 5 h at  $37\text{ }^{\circ}\text{C}$  without aeration (Lundberg et al., 1999).

For the oral inoculation of pigs, the WT<sub>nal</sub> was used to minimize irrelevant bacterial growth when plating tonsillar, lymphoid, intestinal and faecal samples. The bacteria were grown for 16 h at  $37\text{ }^{\circ}\text{C}$  in 5 mL LB broth on a shaker, washed twice in Hank's buffered salt solution (HBSS, Gibco, Life Technologies, Paisley, Scotland) by centrifugation at  $2\,300 \times g$  for 10 min at  $4\text{ }^{\circ}\text{C}$  and finally diluted in HBSS to the appropriate concentration of  $10^7$  colony forming units (CFU) per mL. The number of viable *Salmonella* bacteria per mL inoculum was determined by plating 10-fold dilutions on Brilliant Green agar (BGA, international medical products, Brussels, Belgium) supplemented with 20  $\mu\text{g}/\text{mL}$  nalidixic acid (BGA<sup>NAL</sup>, Sigma-Aldrich) for selective growth of the mutant strains.



## Cell cultures

Primary porcine 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 cells, frozen aliquots of approximately  $10^8$  cells/mL were thawed and washed 3 times in Hank's buffered salt solution with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (HBSS+, Gibco) with 10% (v/v) fetal calf serum (FCS, Hyclone, Cramlington, England) at 4 °C. Finally, these cells were cultured in Roswell Park Memorial Institute medium (RPMI, Gibco) containing 10% (v/v) FCS, 2 mM L-glutamine (Gibco), 1 mM sodium pyruvate (Gibco), 1% (v/v) non essential amino acids (NEAA, Gibco), 100 units penicillin per mL and 100 µg streptomycin per mL (penicillin-streptomycin, Gibco). The porcine macrophage cell line (3D4/31) is derived from PAM and was obtained from Weingartl et al. (2002). These cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 1% (v/v) NEAA and 10% (v/v) FCS.

The polarized intestinal porcine epithelial (IPEC-J2) cell line is derived from jejunal epithelia isolated from a neonatal piglet and was grown in DMEM supplemented with 47% (v/v) Ham's F12 medium (Gibco), 5% (v/v) FCS, 1% insulin-transferrin-selenium-A supplement (ITS, Gibco), and antibiotics as described above (Rhoads et al., 1994; Schierack et al., 2006).

## *In vivo* trials

All animal experiments were carried out in strict accordance with the recommendations in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The protocols were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2007/101 and EC 2010/108).

## Experimental inoculation of piglets

A standardized infection model was used to create *Salmonella* carrier pigs (Boyen et al., 2009). For this purpose, four-week-old piglets (commercial closed line based on Landrace) were obtained from a serologically negative breeding herd (according to the Belgian *Salmonella* monitoring program). The *Salmonella*-free status of the piglets was verified serologically using a commercially available *Salmonella* antibody test kit (IDEXX,

Hoofddorp, The Netherlands), and bacteriologically via repeated faecal sampling. The piglets were housed in pairs in separate isolation units at 25 °C under natural day-night rhythm with *ad libitum* access to feed and water. Seven days after they arrived, the piglets were orally inoculated with 2 mL HBSS containing  $10^7$  CFU of WT<sub>nal</sub> per mL.

In a first *in vivo* trial (EC 2007/101), we investigated the effect of different types of stress on the recrudescence of *Salmonella* Typhimurium by pigs. In a second *in vivo* trial, (EC 2010/108), we injected pigs intramuscularly with 2 mg dexamethasone per kg body weight to test our hypothesis that corticosteroids induce the recrudescence of *Salmonella* Typhimurium in pigs.

#### Effect of different types of stress on the *Salmonella* Typhimurium load in carrier pigs

At day 23 post inoculation (pi), pigs were submitted to either social stress ( $n = 12$ ) or feed withdrawal stress ( $n = 6$ ), mimicking the transport and starvation period before slaughter, respectively. The remaining six pigs were not stressed and served as a negative control group. To induce social stress, the piglets were mixed for 24 h. One piglet was removed from its pen and transferred to another pen, which already contained 2 piglets. This was done in triplicate, so finally there were three groups of 3 piglets per pen (overcrowding) and three groups of 1 piglet per pen (isolation). To mimic feed withdrawal stress, three groups of 2 piglets per pen were not fed for 24 h. After the stress period, the animals were euthanized. Blood samples were taken of all pigs at the same time and the serum cortisol concentrations were determined in twofold via a commercially available enzyme-linked immunosorbent assay (ELISA, Neogen, Lansing, USA), according to the manufacturer's instructions. Furthermore, samples of tonsils, ileocaecal lymph nodes, ileum, cecum, colon and contents of ileum, cecum and colon were collected for bacteriological analysis to determine the number of *Salmonella* bacteria, with a detection limit of 50 CFU per gram tissue or contents.

#### Effect of dexamethasone on the *Salmonella* Typhimurium load in carrier pigs

The animals ( $n = 18$ ) were housed and inoculated as described above to create *Salmonella* carrier pigs (Boyen et al., 2008b). At day 42 pi, pigs were intramuscularly injected with either dexamethasone (Kela laboratoria, Hoogstraten, Belgium) ( $n = 9$ ) or HBSS ( $n = 9$ ). Since cortisol has a short half-life of 1 to 2 h (Perogamvros et al., 2011), we used dexamethasone, which is a long-acting glucocorticoid with a half-life of 36 to 72 h (Shefrin and Goldman, 2009), at a concentration of 2 mg dexamethasone per kg body weight. Pigs are remarkably resistant to dexamethasone-mediated immunosuppression at the dose used

(Flamin et al., 1994). At 24 h after dexamethasone injection, the animals were euthanized and samples of tonsils, ileocaecal lymph nodes, ileum, cecum, colon and contents of ileum, cecum and colon were collected for bacteriological analysis, with a detection limit of 83 CFU per gram tissue or contents.

### Bacteriological analysis

All tissues and samples were weighed and 10% (w/v) suspensions were prepared in buffered peptone water (BPW, Oxoid, Basingstoke, United Kingdom). The samples were homogenized with a Colworth stomacher 400 (Seward and House, London, United Kingdom) and the number of *Salmonella* bacteria was determined by plating 10-fold dilutions on BGA<sup>NAL</sup> plates. These were incubated for 16 h at 37 °C. The samples were pre-enriched for 16 h in BPW at 37 °C and, if negative at direct plating, enriched for 16 h at 37 °C in tetrathionate broth (Merck KGaA, Darmstadt, Germany) and plated again on BGA<sup>NAL</sup>. Samples that were negative after direct plating but positive after enrichment were presumed to contain 50 or 83 CFU per gram tissue or contents (detection limit for direct plating). Samples that remained negative after enrichment were presumed to contain less than 50 or 83 CFU per gram tissue or contents and were assigned value “1” prior to log transformation. Subsequently, the number of CFU for all samples derived from all piglets was converted logarithmically prior to calculation of the average differences between the log<sub>10</sub> values of the different groups and prior to statistical analysis.

### **The effects of cortisol and dexamethasone on host-pathogen interactions of *Salmonella* Typhimurium with porcine host cells**

To examine whether the ability of *Salmonella* Typhimurium to invade and proliferate in primary porcine alveolar macrophages (PAM) and IPEC-J2 cells was altered after exposure of these cells to cortisol, invasion and intracellular survival assays were performed. For the invasion assays, PAM and IPEC-J2 cells were seeded in 24-well plates at a density of approximately  $5 \times 10^5$  and  $10^5$  cells per well, respectively. PAM were allowed to attach for 2 h and IPEC-J2 cells were allowed to grow for 24 h. Subsequently, the cells were exposed to different concentrations of cortisol ranging from 0.001 to 100  $\mu$ M. After 24 h, the invasion assay was performed as described by Boyen et al. (2009). Briefly, *Salmonella* was inoculated into the wells at a multiplicity of infection (MOI) of 10:1. To synchronize the infection, the inoculated multiwell plates were centrifuged at  $365 \times g$  for 10 min and incubated for 30 min

at 37 °C under 5% CO<sub>2</sub>. Subsequently, the cells were washed 3 times with HBSS+ and fresh medium supplemented with 100 µg/mL gentamicin (Gibco) was added. After 1 h incubation, the PAM and IPEC-J2 cells were washed 3 times and lysed for 10 min with 1% (v/v) Triton X-100 (Sigma-Aldrich) or 0.2% (w/v) sodium deoxycholate (Sigma-Aldrich), respectively, and 10-fold dilutions were plated on BGA plates.

To assess intracellular proliferation, cells were seeded and inoculated with *Salmonella* Typhimurium, but the medium containing 100 µg/mL gentamicin was replaced after 1 h incubation with fresh medium containing 20 µg/mL gentamicin, with or without cortisol or dexamethasone ranging from 0.001 to 100 µM. The number of viable bacteria was assessed 24 h after infection. To examine whether cortisol could also increase the intracellular proliferation of *Salmonella* Typhimurium in a macrophage cell line, the intracellular proliferation assay was repeated in the 3D14/31 cell line.

To determine whether the observed effect was cortisol specific, invasion and proliferation assays were also performed after exposure of PAM to epinephrine, norepinephrine or dopamine at concentrations ranging from 5 to 50 µM to reflect experiments previously performed by others (Bearson et al., 2008).

To visualize the effect of cortisol on the intracellular proliferation of *Salmonella* bacteria, PAM were seeded in sterile Lab-tek<sup>®</sup> chambered coverglasses (VWR, Leuven, Belgium), inoculated with GFP-producing *Salmonella* at a multiplicity of infection of 2:1, as described by Boyen et al. (2009), and exposed to cortisol at a high physiological stress concentration of 1 µM (Wei et al., 2010) in cell medium or to cell medium only. After 24 h at 37 °C, cells were washed three times to remove unbound bacteria and cellTrace<sup>™</sup> calcein red-orange (Molecular Probes Europe, Leiden, The Netherlands) was added for 30 min at 37 °C. Afterwards, cells were washed three times and fluorescence microscopy was carried out. Per experiment the number of cell associated bacteria was determined in 100 macrophages and the average number of cell associated bacteria was calculated from four independent experiments.

### **The effect of cortisol on the viability of porcine host cells**

It is possible that cortisol affects the toxicity of *Salmonella* Typhimurium for host cells, resulting in an increased or reduced cell death. Therefore, the cytotoxic effect of cortisol on uninfected and infected PAM and IPEC-J2 cells was determined using the neutral red uptake assay. For this purpose, PAM were seeded in a 96-well microplate at a density of

approximately  $2 \times 10^5$  cells per well and were allowed to attach for 2 h. The IPEC-J2 cells were seeded and allowed to grow for 24 h in a 96-well microplate at a density of approximately  $2 \times 10^4$  cells per well. As earlier, uninfected and infected cells with *Salmonella* Typhimurium were treated with medium whether or not supplemented with cortisol concentrations ranging from 0.001 to 100  $\mu\text{M}$  for 24 h. To assess cytotoxicity, 150  $\mu\text{L}$  of freshly prepared neutral red solution (33  $\mu\text{g}/\text{mL}$  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 h. The cells were then washed two times with HBSS+ and 150  $\mu\text{L}$  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:

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

In this formula  $a = \text{OD}_{540}$  derived from the wells incubated with cortisol,  $b = \text{OD}_{540}$  derived from blank wells,  $c = \text{OD}_{540}$  derived from untreated control wells.

## **Effect of cortisol on the growth and gene expression of *Salmonella* Typhimurium**

### Effect of cortisol on the growth of *Salmonella* Typhimurium

It is possible that cortisol directly increases the growth of *Salmonella* Typhimurium. Therefore, we examined the effect of cortisol concentrations (1 nM, 100 nM, 1  $\mu\text{M}$  and 100  $\mu\text{M}$ ) on the growth of *Salmonella* Typhimurium, during 24 h. For this purpose, *Salmonella* Typhimurium was grown in LB broth or DMEM medium with or without cortisol. The number of CFU per mL was determined at different time points ( $t = 0, 2.5, 5, 7.5$  and 24 h) by titration of 10-fold dilutions of the bacterial suspensions on BGA. After incubation for 24 h at 37 °C, the number of colonies was determined.

### Effect of cortisol on the gene expression of *Salmonella* Typhimurium

A direct effect of cortisol on the pathogenicity of *Salmonella* Typhimurium could explain the increased intracellular proliferation in macrophages. Therefore, a microarray analysis was conducted to investigate the effect of cortisol on the gene expression of the bacterium.

RNA was isolated from *Salmonella* Typhimurium at logarithmic and stationary growth phase (2.00  $\text{OD}_{600 \text{ nm}}$  units) in the presence or absence of 1  $\mu\text{M}$  cortisol, for 5 and 16 h

respectively (Lundberg et al., 1999). This was done according to procedures described on the IFR microarray web site ([www.ifr.ac.uk/Safety/microarrays/](http://www.ifr.ac.uk/Safety/microarrays/)). The quantity and purity of the isolated RNA was determined using a Nanodrop spectrophotometer and Experion RNA StdSens Analysis kit (Biorad).

Triple biological replicates were performed for each experiment, RNA labeled and hybridized to *Salmonella* Typhimurium SALSA2 microarrays, consisting of 5080 ORFs, according to protocols described on the IFR microarray web site ([www.ifr.ac.uk/Safety/microarrays/](http://www.ifr.ac.uk/Safety/microarrays/)). Following washing and scanning of the hybridized microarrays, the expression data was processed and statistically filtered. All transcriptomic data were normalized to that of the wild-type strain. A Benjamini and Hochberg multiple testing correction was then applied to adjust individual P-values so that only data with a false discovery rate of 0.05 and a  $\geq 1.5$ -fold change in the expression level was retained.

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 GSE30923 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30923>).

### **Statistical analysis**

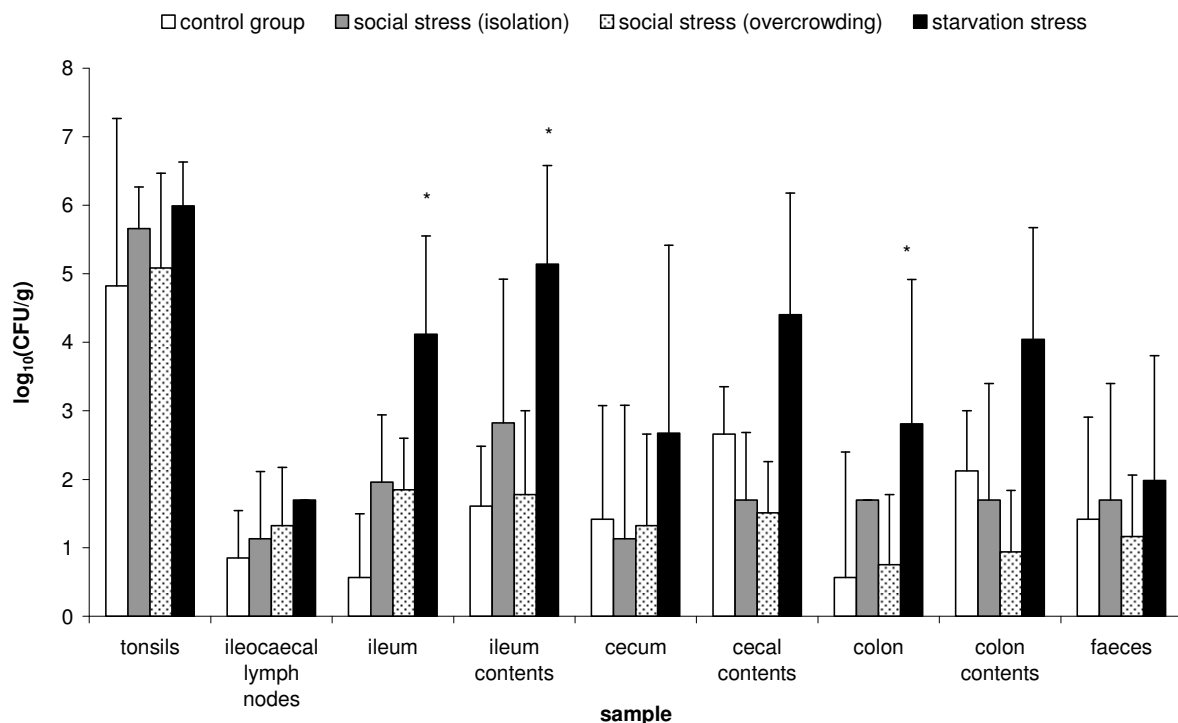
All *in vitro* experiments were conducted in triplicate with 3 repeats per experiment, unless otherwise stated. All statistical analyses were performed using SPSS version 17 (SPSS Inc., Chicago, IL, USA). Normally distributed data were analyzed using unpaired Student's t-test or one-way ANOVA to address the significance of difference between mean values with significance set at  $p \leq 0.05$ . Bonferroni as post hoc test was used when equal variances were assessed. If equal variances were not assessed, the data were analyzed using Dunnett's T3 test. Not normally distributed data were analyzed using the non parametric Kruskal-Wallis analysis, followed by Mann-Whitney U test.

## Results

### Feed withdrawal results in increased numbers of *Salmonella* Typhimurium bacteria in the gut of pigs and elevated blood cortisol levels

Carrier pigs subjected to feed withdrawal, 24 h before euthanasia, showed elevated numbers of *Salmonella* Typhimurium per gram in their bowel contents and organs in comparison to the control group (Figure 1). This increase was significant in the ileum ( $p \leq 0.001$ ), ileum contents ( $p = 0.022$ ) and colon ( $p = 0.014$ ). The social stress groups (overcrowding and isolation) showed no significant differences in comparison to the control group.

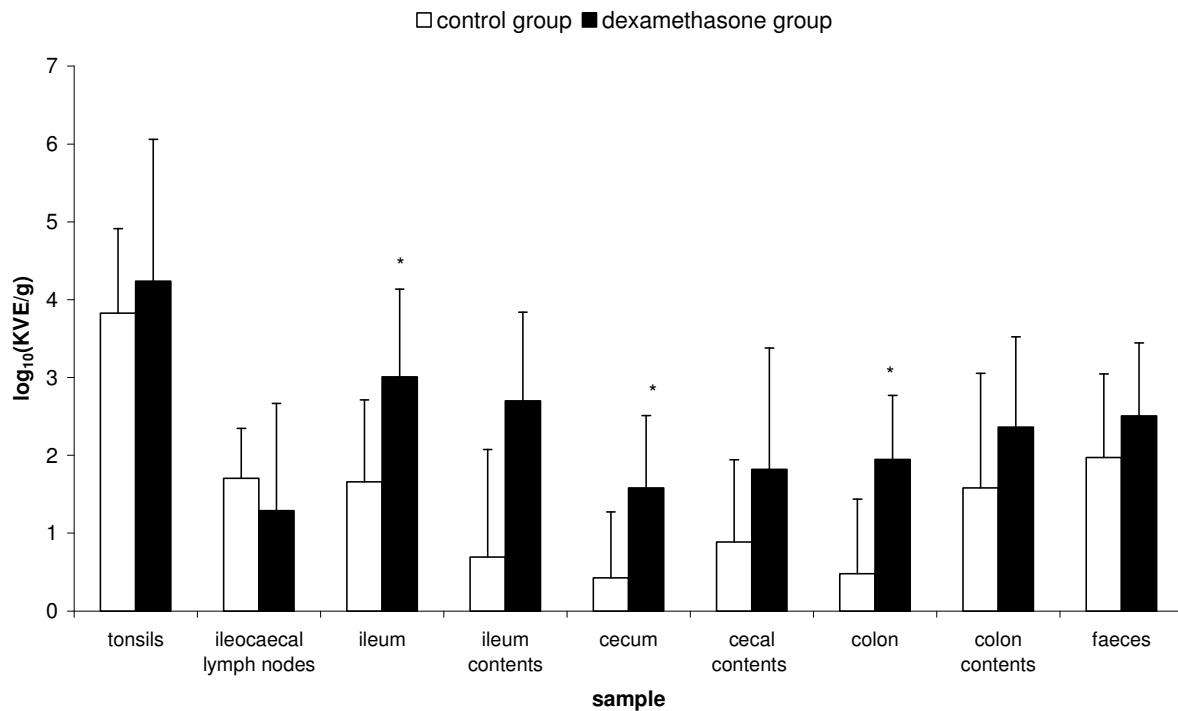
Pigs that were subjected to feed withdrawal ( $p = 0.004$ ) and overcrowding ( $p = 0.001$ ) showed significantly elevated serum cortisol levels compared to the control group that had a mean cortisol concentration  $\pm$  standard deviation of  $48.65 \pm 4.67$  nM. Pigs that were starved 24 hours before euthanasia had the highest mean serum cortisol level  $\pm$  standard deviation of  $66.88 \pm 6.72$  nM. Pigs that were housed per 3 (overcrowding) and housed separately (isolation) at 24 h before euthanasia, had a mean cortisol concentration  $\pm$  standard deviation of  $59.26 \pm 3.47$  nM and  $53.66 \pm 2.06$  nM, respectively.



**Figure 1: Effect of different types of stress on the *Salmonella* load in carrier pigs.** Recovery of *Salmonella* Typhimurium bacteria from various organs and gut contents of carrier pigs that were subjected to either feed withdrawal ( $n = 6$ ) or social stress, isolation ( $n = 3$ ) and overcrowding ( $n = 9$ ), 24 h before euthanasia. Six pigs were not stressed and served as a control group. The  $\log_{10}$  value of the ratio of CFU/gram sample is given as the mean + standard deviation. Superscript (\*) refers to a significant difference compared to the control group ( $p \leq 0.05$ ).

## Dexamethasone increases the number of *Salmonella* Typhimurium bacteria in the gut of carrier pigs

Carrier pigs that were intramuscularly injected with 2 mg dexamethasone per kg body weight, 24 h before euthanasia, showed elevated numbers of *Salmonella* Typhimurium in their gut tissues and contents in comparison to the control group that was intramuscularly injected with HBSS (Figure 2). This increase was significant in the ileum ( $p = 0.018$ ), cecum ( $p = 0.014$ ) and colon ( $p = 0.003$ ).



**Figure 2: Effect of dexamethasone on the *Salmonella* Typhimurium load in carrier pigs.** Recovery of *Salmonella* Typhimurium bacteria from various organs and gut contents of carrier pigs that were injected with either HBSS (control group,  $n = 9$ ) or 2 mg dexamethasone per kg body weight (dexamethasone group,  $n = 9$ ), 24 h before euthanasia. The  $\log_{10}$  value of the ratio of CFU/gram sample is given as the mean + standard deviation. Superscript (\*) refers to a significant difference compared to the control group ( $p \leq 0.05$ ).



### **Cortisol and dexamethasone, but not catecholamines, promote the intracellular proliferation of *Salmonella* Typhimurium in primary porcine macrophages but not in 3D4/31 and IPEC-J2 cells**

The results of the intracellular survival assay of *Salmonella* Typhimurium in PAM with or without exposure to cortisol or dexamethasone are summarized in Figure 3. Exposure to concentrations ( $\geq 100$  nM) of cortisol or dexamethasone for 24 h led to a significant dose-dependent increase of the number of intracellular *Salmonella* Typhimurium bacteria compared to non-treated PAM.

Cortisol concentrations ranging from 0.001 to 100  $\mu$ M did neither affect the intracellular proliferation of *Salmonella* Typhimurium in IPEC-J2 and 3D4/31 cells (Figure 4), nor the invasion in PAM and IPEC-J2 cells (Figure 5).

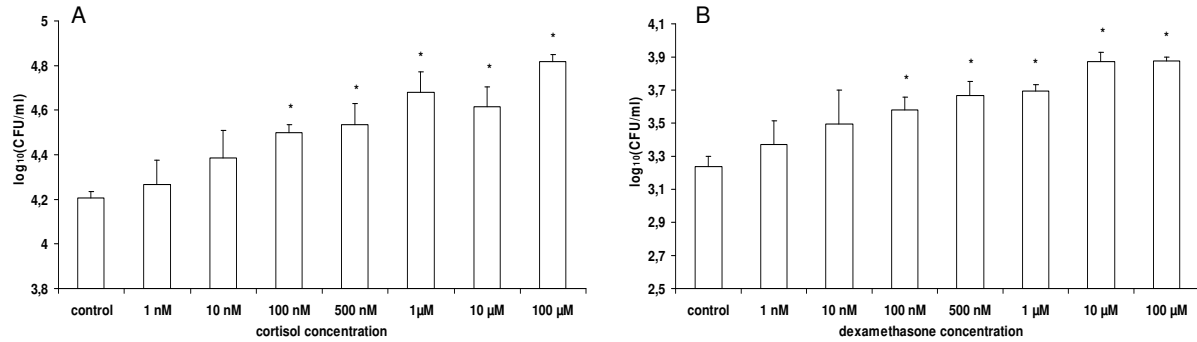
The enhanced intracellular proliferation of *Salmonella* Typhimurium in PAM exposed to a high physiological stress concentration of 1  $\mu$ M cortisol (Wei et al., 2010) was confirmed in a proliferation assay with GFP-*Salmonella*. No difference was seen in the mean number of macrophages containing GFP-*Salmonella*  $\pm$  standard error of the mean, after exposure to 1  $\mu$ M cortisol for 24 h in comparison to untreated PAM ( $41.0 \pm 0.53$  versus  $40.5 \pm 0.59$  percentage *Salmonella* positive macrophages, respectively). However, the proliferation rate of intracellular bacteria that were exposed to 1  $\mu$ M cortisol for 24 h was significantly ( $p = 0.001$ ) increased in comparison with the control PAM, resulting in a higher mean bacterial count  $\pm$  standard error of the mean ( $3.1 \pm 0.14$  versus  $2.0 \pm 0.07$  bacteria per macrophage, respectively).

Epinephrine, norepinephrine and dopamine at a concentration of 1  $\mu$ M did neither affect the invasion nor the intracellular proliferation of *Salmonella* Typhimurium in PAM (Figure 6) and IPEC-J2 cells (Figure 7).

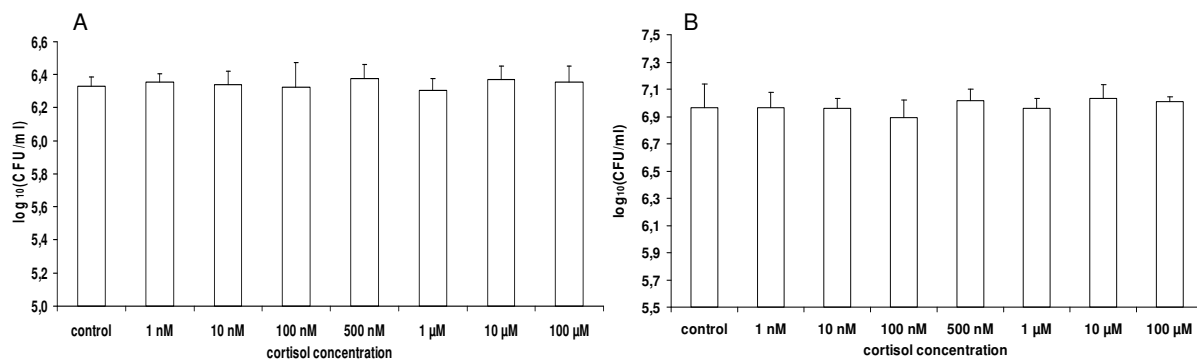
### **Cortisol does not affect the viability of primary porcine macrophages and intestinal epithelial cells and it does not directly affect *Salmonella* cytotoxicity, growth and gene expression**

Cortisol concentrations ranging from 0.001 to 100  $\mu$ M did neither affect the viability of PAM and IPEC-J2 cells, nor the cytotoxicity of *Salmonella* Typhimurium for these cells (Figure 8). Furthermore, we showed that cortisol did not affect the growth of *Salmonella* Typhimurium in LB and DMEM medium (Figure 9) and transcriptomic analysis revealed that exposure of stationary and logarithmic phase cultures of *Salmonella* Typhimurium to cortisol

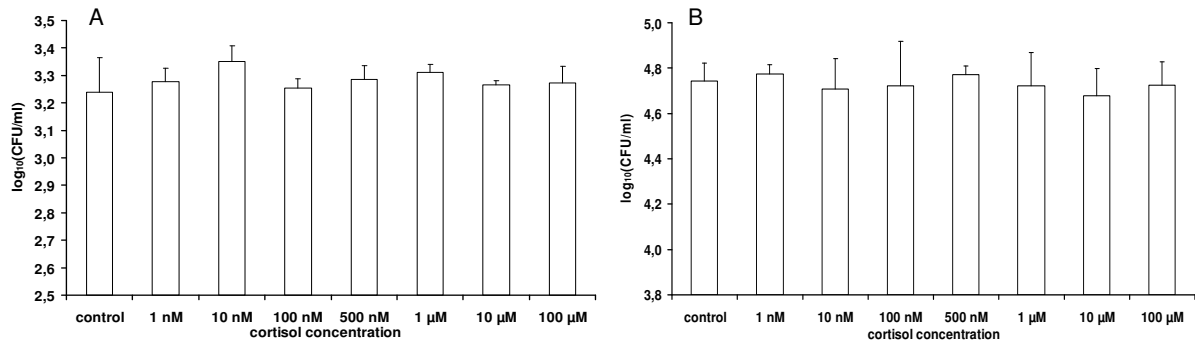
at a high physiological stress concentration of 1  $\mu\text{M}$  (Wei et al., 2010), did not significantly affect gene expression levels compared to the untreated strain in LB medium ([http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE30923](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30923)).



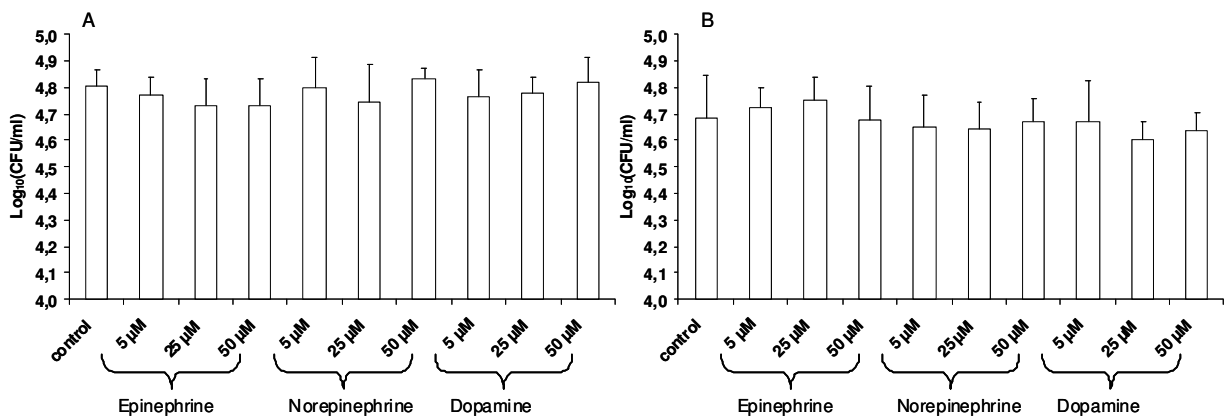
**Figure 3: Effect of cortisol and dexamethasone on the intracellular proliferation of *Salmonella Typhimurium* in macrophages.** Number of intracellular *Salmonella Typhimurium* bacteria in PAM that were treated with control medium or different concentrations of A) cortisol or B) dexamethasone, for 24 h after invasion. The  $\log_{10}$  values of the number of gentamicin protected bacteria + standard deviation are shown. Results are presented as a representative experiment conducted in triplicate. Superscript (\*) refers to a significant difference compared to the control ( $p \leq 0.05$ ).



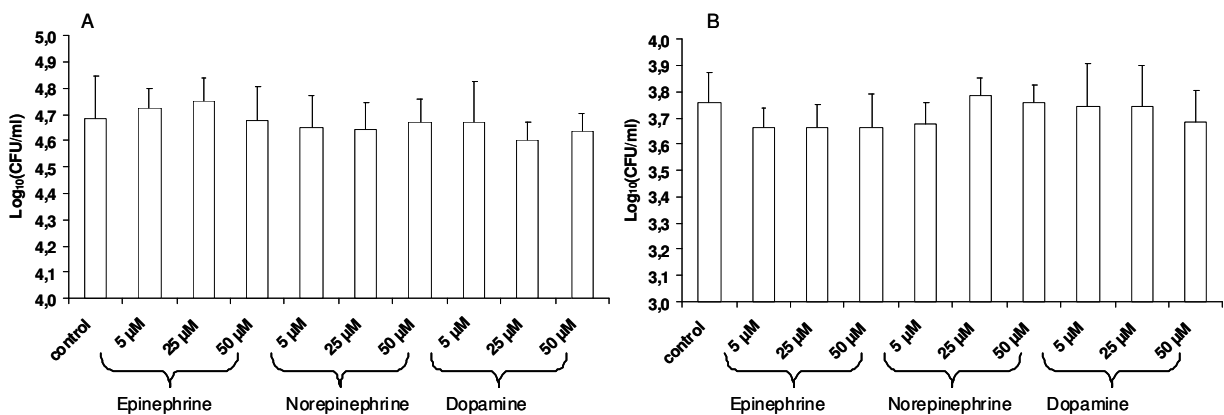
**Figure 4: Effect of cortisol on the intracellular proliferation of *Salmonella Typhimurium* in IPEC-J2 and 3D4/31 cells.** Number of intracellular *Salmonella Typhimurium* bacteria in (A) IPEC-J2 cells and (B) 3D4/31 cells, that were treated with control medium or cortisol (0.001  $\mu\text{M}$ -100  $\mu\text{M}$ ), for 24 h after invasion. The  $\log_{10}$  values of the number of gentamicin protected bacteria + standard deviation are given. Results are presented as a representative experiment conducted in triplicate.



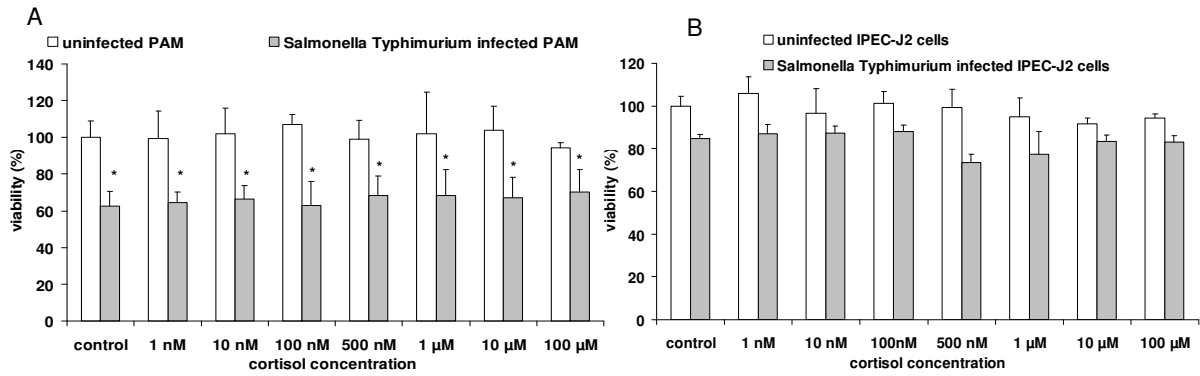
**Figure 5: Effect of cortisol on the invasion of *Salmonella Typhimurium* in PAM and IPEC-J2 cells.** The invasiveness of *Salmonella Typhimurium* in (A) PAM and (B) IPEC-J2 cells, whether or not exposed to cortisol (0.001-100  $\mu\text{M}$ ) is shown. The  $\log_{10}$  values of the number of gentamicin protected bacteria + standard deviation are given. Results are presented as a representative experiment conducted in triplicate.



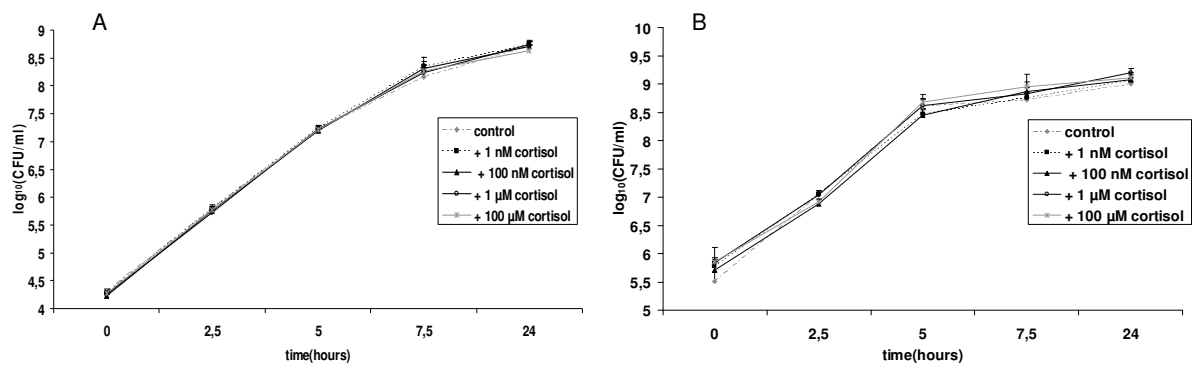
**Figure 6: Effects of catecholamines on the invasion and intracellular proliferation of *Salmonella Typhimurium* in macrophages.** The invasiveness (A) and the survival (B), 24 h after invasion, of *Salmonella Typhimurium* in PAM whether or not exposed to epinephrine, norepinephrine or dopamine (5-50  $\mu\text{M}$ ) are shown. The  $\log_{10}$  values of the number of gentamicin protected bacteria + standard deviation are given. Results are presented as a representative experiment conducted in sixfold.



**Figure 7: Effect of catecholamines on the invasion and intracellular proliferation of *Salmonella Typhimurium* in IPEC-J2 cells.** The invasiveness (A) and the survival (B), 24 h after invasion, of *Salmonella Typhimurium* in IPEC-J2 cells whether or not exposed to epinephrine, norepinephrine or dopamine (5-50  $\mu\text{M}$ ) is shown. The  $\log_{10}$  values of the number of gentamicin protected bacteria + standard deviation are given. Results are presented as a representative experiment conducted in sixfold.



**Figure 8: Effect of cortisol on the viability of *Salmonella* infected and uninfected macrophages and IPEC-J2 cells.** Percentage viability (%) of *Salmonella Typhimurium* infected and uninfected (A) PAM and (B) IPEC-J2 cells, exposed to different concentrations of cortisol (0.001-100 μM). Twenty-four hours after incubation with cortisol, the cytotoxic effect was determined by neutral red assay. Results represent the means of three independent experiments conducted in triplicate and their standard deviation. Superscript (\*) refers to a significant difference compared to control uninfected cells ( $p \leq 0.05$ ).



**Figure 9: Effect of cortisol on the growth of *Salmonella Typhimurium*.** The log<sub>10</sub> values of the CFU/mL + standard deviation are given at different time points ( $t = 0, 2.5, 5, 7.5, 24$  h). *Salmonella Typhimurium* growth was examined in (A) LB and (B) DMEM medium, with or without cortisol (0.001-100 μM). Results are presented as a representative experiment conducted in triplicate.

## Discussion

Conflicting results have been published concerning the effect of different stressors on the shedding of *Salmonella* Typhimurium in pigs (Williams and Newell, 1970; Isaacson et al., 1999; Nollet et al., 2005; Rostagno et al., 2005; Scherer et al., 2008; Martín-Peláez et al., 2009). However, our findings elucidate that a natural stress stimulus like feed withdrawal causes recrudescence of a *Salmonella* Typhimurium infection in carrier pigs (Figure 1). Feed withdrawal before transport to the slaughterhouse is a common practice to reduce the risk of carcass and environmental contamination because a decrease of the gastrointestinal tract weight results in a lower risk of lacerations during evisceration (Miller et al., 1997). However, we showed that feed withdrawal practices could result in an increased risk of contamination. Martín-Peláez et al. (2009) hypothesized that the increased faecal excretion of *Salmonella* Typhimurium after feed withdrawal could be the result of a change in short-chain fatty acids concentration, an altered pH and/or a change in the number of lactic acid bacteria such as lactobacilli.

Until now, the mechanism of stress related recrudescence of *Salmonella* is not well understood and the investigation of this phenomenon is hindered by the lack of appropriate animal models (Stabel and Fedorka-Cray, 2004; Griffin et al., 2011). The higher *Salmonella* Typhimurium numbers in pigs subjected to feed withdrawal stress, suggest that this model is a valuable tool for the study of stress related *Salmonella* recrudescence. We hypothesized that cortisol plays a role in the stress related recrudescence of *Salmonella* Typhimurium by pigs. During a stress reaction, the sympathetic nervous system and hypothalamic-pituitary-adrenal axis become activated, resulting in the release of catecholamines and glucocorticoids, respectively (Freestone et al., 2008). These stress hormones can affect the host immune response, but the pathogenesis of an infection can also be altered by direct effects of these stress mediators on the bacteria (Verbrugge et al., 2011).

We showed that social stress and starvation result in elevated serum cortisol levels. Starvation can result in hypoglycaemia, which causes an increased secretion of cortisol to stimulate the gluconeogenesis (Guettier and Jorden, 2006). Müller et al. (1982) showed that a starvation period up to 5 days in miniature pigs, results in a slight, but insignificant elevation of plasma cortisol levels. The elevated serum cortisol levels, seen in the carrier pigs that were subjected to feed withdrawal, are probably the result of both hypoglycaemia and stress caused by starvation.

We revealed that a short-term treatment of carrier pigs with a high dose of dexamethasone results in the recrudescence of *Salmonella* Typhimurium. This confirms that the release of corticosteroids in the bloodstream itself could alter the outcome of a *Salmonella* Typhimurium infection in pigs, resulting in recrudescence of the infection. Smyth et al. (2008) showed that long-term treatment of mice with dexamethasone promotes a dose-dependent increase in *Salmonella* Typhimurium growth within mouse livers and spleens. The increased numbers of bacteria described by Smyth et al. (2008) are probably the result of the immunosuppressive activity of glucocorticoids. Pigs are remarkably resistant to immunosuppression of dexamethasone, even at a high dose of 2 mg/kg body weight (Griffin, 1989; Roth and Flaming, 1990; Saulnier et al., 1991; Flaming et al., 1994). Therefore, the dexamethasone induced recrudescence of *Salmonella* Typhimurium in pigs is probably not the direct consequence of the immunosuppressive activity of dexamethasone (Figure 2).

We also demonstrated that this glucocorticoid mediated effect was not the result of a direct effect on the bacterium. Earlier research has shown that norepinephrine *in vitro* promotes the growth and the motility of *Salmonella enterica* (Bearson and Bearson, 2008; Methner et al., 2008). However, we provide evidence that cortisol does not cause an increase in growth in LB and DMEM medium, or any significant changes in the gene expression of *Salmonella* Typhimurium when grown in a complex medium, at a physiological stress concentration of 1  $\mu\text{M}$  (Wei et al., 2010; Figure 9; <http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30923>). In contrast to the absence of a direct effect on the bacterium, we showed that cortisol and dexamethasone promote intracellular proliferation of *Salmonella* Typhimurium in porcine macrophages, in a dose-dependent manner at concentrations (0.1 to 100  $\mu\text{M}$ ) that do not exert a notable effect on cell viability (Figure 3 and 8). Nevertheless, this increased survival was not observed 3D4/31 and IPEC-J2 cells (Figure 4). Although *Salmonella* is an extensively studied bacterium, still many questions remain about the intracellular environment of *Salmonella* within different host cells. After invasion, *Salmonella* resides within a *Salmonella* containing vacuole (SCV) which serves as a unique intracellular compartment where it resides and eventually replicates. Maturation of the SCV has been studied in different cell types and these studies indicate that the SCV biogenesis may not be generalized (Gorvel and Méresse, 2001). Possibly, cortisol affects the SCV biogenesis in primary macrophages and not in other cell types, which results in an increased survival of the bacterium in these primary macrophages.

Although we showed that catecholamines did neither affect the intracellular proliferation nor the invasion of *Salmonella* Typhimurium in primary macrophages and IPEC-J2 cells (Figure 6 and 7), catecholamines have been shown to promote the growth and motility of *Salmonella* (Toscano et al., 2007; Bearson and Bearson, 2008; Methner et al., 2008). Concentrations of the catecholamines were not determined in the *in vivo* trial since they have a half-life of approximately 3 min and because their serum levels change in matter of seconds (Whitby et al., 1961; Yamaguchi and Kopin, 1979). However, it is commonly known that a stress reaction also results in the release of catecholamines. Recently, Pullinger et al. (2010) demonstrated that the release of norepinephrine in pigs by administration of 6-hydroxydopamine, enhances the faecal excretion of *Salmonella* Typhimurium. Therefore, it is possible that catecholamines and glucocorticoids act in a synergistic way to cause a sudden increase of *Salmonella* Typhimurium shedding in stressed animals. Since stress is very common in food producing animals and since these stress hormones and derivatives are frequently used in human and animal medicine, their effects need further examination (Behrend and Kempainen, 1997; Lowe et al., 2008). The elucidation of the mechanisms through which stress and its hormones alter the susceptibility to an infection could help to improve the prevention and treatment of *Salmonella* Typhimurium infections in pigs, and as a consequence help to reduce the number of cases of human salmonellosis.

In conclusion, we showed that the glucocorticoid cortisol is involved in a stress induced recrudescence of *Salmonella* Typhimurium in carrier pigs. In addition to this, we pointed out that cortisol promotes the intracellular proliferation of *Salmonella* Typhimurium in porcine macrophages which is caused by an indirect effect through the cell.

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## CHAPTER 2:

### **Cortisol modifies protein expression of *Salmonella* Typhimurium infected porcine macrophages, associated with *scsA* driven intracellular proliferation**

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## Abstract

Persistently infected pigs with *Salmonella* Typhimurium re-excrete the bacterium during periods of stress leading to an increased cross-contamination, a higher degree of carcass contamination and consequently to higher numbers of foodborne salmonellosis in humans. The mechanism of this stress related recrudescence is poorly understood, but recently it has been associated with a cortisol induced increased intracellular survival of the bacterium in primary porcine macrophages. The aim of the present study was to unravel this cortisol induced mechanism. We showed that the cortisol induced increased survival of *Salmonella* Typhimurium in primary porcine macrophages was both actin and microtubule dependent. Proteomic analysis of *Salmonella* Typhimurium infected primary porcine macrophages revealed that cortisol caused an increased expression of cytoskeleton associated proteins, including a constituent of the SCV, a component of microtubules and proteins that regulate the polymerization of actin. Using *in vivo* expression technology, we established that the gene expression of intracellular *Salmonella* Typhimurium bacteria was altered during cortisol treatment of *Salmonella* Typhimurium infected primary porcine macrophages and we identified *scsA* as a major driver for the increased intracellular survival of *Salmonella* Typhimurium during cortisol exposure to these cells. We thus conclude that cortisol modifies host macrophage protein expression and affects intracellular *Salmonella* gene expression, resulting in *scsA* driven increased intracellular proliferation of the bacterium.

## Introduction

During the past decade, microbial endocrinology was introduced as a new research area where microbiology and neurophysiology intersect. Work from this field showed that bacteria, including *Salmonella* Typhimurium, can exploit the neuroendocrine alteration due to a stress reaction as a signal for growth and pathogenic processes (Verbrugghe et al., 2011a). Normally, pigs infected with *Salmonella* Typhimurium carry this bacterium asymptotically in their tonsils, gut and gut-associated lymphoid tissue for months resulting in so called *Salmonella* carriers (Wood et al., 1991). These persistently infected animals intermittently shed low numbers of *Salmonella* bacteria. Recently, we showed that a 24 hour feed withdrawal increased the intestinal *Salmonella* Typhimurium load in carrier pigs (Verbrugghe et al., 2011b). Since starvation stress results in the recrudescence of *Salmonella* bacteria, this could lead to an increased cross-contamination during transport and lairage and to a higher degree of carcass contamination. The consumption of contaminated pork meat is one of the major routes of human salmonellosis worldwide (Pires et al., 2011). Therefore, starvation stress induced recrudescence of *Salmonella* bacteria could lead to higher numbers of foodborne *Salmonella* infections in humans.

To date, most research in microbial endocrinology was limited to the influence of catecholamines on bacterial infections (Verbrugghe et al., 2011a). However, we recently highlighted the role of glucocorticoids in microbial endocrinology. We showed that the starvation stress induced recrudescence of *Salmonella* Typhimurium in pigs was correlated with increased serum cortisol levels and that recrudescence of *Salmonella* Typhimurium in pigs can be induced by a single intramuscular injection of the glucocorticoid dexamethasone (Verbrugghe et al., 2011b). Although cortisol did not cause an increased growth or significant changes in the gene expression of *Salmonella* Typhimurium when grown in a complex medium, the glucocorticoid promoted intracellular proliferation of *Salmonella* Typhimurium in primary porcine macrophages. Once *Salmonella* has crossed the intestinal epithelium, it encounters macrophages associated with Peyer's patches in the submucosal space (Ohl and Miller, 2001). By hiding, and even replicating in these cells, *Salmonella* bacteria can be spread throughout the body using the blood stream or the lymphatic fluids. In pigs, the colonization of *Salmonella* Typhimurium is mostly limited to the gastrointestinal tract, but these macrophages can be a reservoir of persistent infection.

Until now, the mechanism of cortisol induced increased intracellular survival of *Salmonella* Typhimurium in primary macrophages is unknown. Therefore, the aim of the present study was to unravel the mechanism of how the infected macrophages respond to cortisol exposure and to identify *Salmonella* Typhimurium genes responsible for the cortisol induced increased survival of the bacterium.

## Materials and Methods

### Bacterial strains and growth conditions

*Salmonella* Typhimurium strain 112910a, isolated from a pig stool sample and characterized previously by Boyen et al. (2008), was used as the wild type strain (WT). *Salmonella* Typhimurium deletion mutants  $\Delta scsA$  and  $\Delta cbpA$  were constructed according to the one-step inactivation method described by Datsenko and Wanner (2000) and slightly modified for use in *Salmonella* Typhimurium as described previously (Boyen et al., 2006). Primers used to create the gene-specific linear PCR fragments (*cbpA* and *scsA* forward and reverse) are given in Table 1. The targeted genes were completely deleted from the start codon through the stop codon, as confirmed by sequencing. Unless otherwise stated, the bacteria were generally grown overnight for 16 hours at 37 °C in 5 mL of LB broth with aeration to a stationary phase. To obtain highly invasive late logarithmic cultures, 2  $\mu$ L of a stationary phase culture were inoculated in 5 mL LB broth and grown for 5 hours at 37 °C without aeration (Lundberg et al., 1999).

Construction of the *Salmonella* Typhimurium *in vivo* expression technology (IVET) pool was previously described in Van Parys et al. (2011). The frozen aliquots were thawed and added to 5 mL LB broth supplemented with the additives, 50  $\mu$ g/mL ampicillin (Sigma-Aldrich), 20  $\mu$ g/mL nalidixic acid (Sigma-Aldrich), 1.35% (w/v) adenine (Sigma-Aldrich) and 0.337% (w/v) thiamine (Sigma-Aldrich) and the bacterial culture was grown for 3 hours with aeration at 37 °C.

**Table 1: Primers used in this study to create the deletion mutants  $\Delta scsA$  and  $\Delta cbpA$ .**

Primers	Sequences
<i>cbpA</i> forward	5'-GAAACCTTTTGGGGTCCCTTCTGTATGTATTGATTTAGCGAGATGATGCTTGTGTAGGCTGGAGCTGCTTC-3'
<i>cbpA</i> reverse	5'-GTGTGCAAACAAAATTCGGTGATGGTAAAGGTGACAGTGATGTTAGCCATCATATGAATATCCTCCTTAG-3'
<i>scsA</i> forward	5'-CAAACCGCGCCAGTGGCTAAGATAACTCGCGTTAAACAGTGAGGGCGCATGTGTAGGCTGGAGCTGCTTC-3'
<i>scsA</i> reverse	5'-ATTTTTTCTCCGTGAATGAGTAATTAACCGTTAGCAATAACCGGTCTGCATATGAATATCCTCCTTAG-3'



### **Effect of cortisol on the protein expression of *Salmonella* Typhimurium infected primary porcine macrophages**

A comparative proteome study was conducted to reveal the effects of cortisol on the protein expression of *Salmonella* Typhimurium infected primary porcine alveolar macrophages (PAM). We used a gel-free approach called isobaric tags for relative and absolute quantification (iTRAQ) in which four different isobaric labels are used to tag N-termini and lysine side chains of four different samples with four different isobaric reagents. Upon collision-induced dissociation during MS/MS, the isobaric tags are released, which results in four unique reporter ions that are used to quantify the proteins in the four different samples (Ross et al., 2004).

Sample preparation: PAM were isolated and cultured as described in Verbrugge et al. (2011b), they were seeded in 175 cm<sup>2</sup> cell culture flasks at a density of approximately 5 x 10<sup>7</sup> cells per flask and were allowed to attach for 2 hours. Subsequently, PAM were washed 3 times with Hank's buffered salt solution with Ca<sup>2+</sup> and Mg<sup>2+</sup> (HBSS+, Gibco) and a gentamicin protection invasion assay was performed as described by Boyen et al. (2009). Briefly, *Salmonella* was inoculated into the cell culture flasks at a multiplicity of infection (MOI) of 10:1. To synchronize the infection, the inoculated flasks were centrifuged at 365 x g for 10 min and incubated for 30 min at 37 °C under 5% CO<sub>2</sub>. Subsequently, the cells were washed 3 times with HBSS+ and fresh medium supplemented with 100 µg/mL gentamicin (Gibco) was added. After 1 hour, the medium was replaced by fresh medium containing 20 µg/mL gentamicin, with or without 1 µM cortisol (Sigma-Aldrich). Twenty-four hours after infection, the cells were washed 3 times with HBSS+ and treated with lysis buffer containing 1% (v/v) Triton X-100 (Sigma-Aldrich), 40 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris, Sigma-Aldrich), a cocktail of protease inhibitors (PIs; Sigma-Aldrich) and phosphatase inhibitors (PPI, Sigma-Aldrich), 172.6 U/mL deoxyribonuclease I (DNase I, Invitrogen, USA) and 100 mg/mL ribonuclease A (RNase A, Qiagen, Venlo, The Netherlands). Subsequently, cell debris and bacteria were removed by centrifugation at 2300 x g for 10 min at 4 °C. Two % (v/v) tributylphosphine (TBP, Sigma-Aldrich) was added to the supernatant followed by centrifugation at 17 968 x g for 10 min. The supernatant was held on ice until further use and the pellet was dissolved and sonicated (6 times 30 sec), using an ultrasonic processor XL 2015 (Misonix, Farmingdale, New York, USA), in reagent 3 of the Ready Prep Sequential extraction kit (Bio-Rad, Hercules, CA, USA). This was centrifuged at 17 968 x g for 10 min. Both supernatants were combined and a buffer switch to 0.01%

(w/v) SDS in H<sub>2</sub>O was performed using a Vivaspin column (5000 molecular weight cut off Hydrosarts, Sartorius, Germany). Protein concentration was determined using the Bradford Protein Assay (Thermo Fisher Scientific, Rockford, USA) according to the manufacturer's instructions.

*Trypsin digest and iTRAQ labeling:* Digest and labeling of the samples (100 µg proteins per sample) with iTRAQ reagents was performed according to the manufacturer's guidelines (AB Sciex, Foster City, CA, USA). Individual samples of cortisol treated or untreated PAM were analyzed in the same run, making paired comparisons possible and minimizing technical variation. Each condition was run in duplicate using different labels of the four-plex labeling kit. The experiment was conducted in twofold and the labeling of the samples was as follows: run 1 (untreated PAM sample 1: 114 – untreated PAM sample 2: 115 – treated PAM sample 1: 116 – treated PAM sample 2: 117) – run 2 (untreated PAM sample 3: 114 – untreated PAM sample 4: 115 – treated PAM sample 3: 116 – treated PAM sample 4: 117). After labeling, 6 µL of a 5% (v/v) hydroxylamine solution was added to hydrolyze unreacted label and after incubation at room temperature for 5 min, the samples were pooled, dried and resuspended in 5 mM KH<sub>2</sub>PO<sub>4</sub> (15% (v/v) acetonitrile) (pH 2.7). The combined set of samples was first purified on ICAT SCX cartridges, desalted on a C18 trap column and finally fractionated using SCX chromatography. Each fraction was analyzed by nano LC-MSMS as described by Bijttebier et al. (2009).

*Data analysis:* With no full pig protein database available, different search parameters and databases, both EST and protein, were validated to obtain maximum spectrum annotation. Best results (39% of spectra annotated above homology threshold with a 3.71% false discovery rate in the decoy database) were obtained when searching NCBI Mammalia. For quantification, data quality was validated using ROVER (Colaert et al., 2011). Based on this validation a combined approach was used to define recurrently different expression patterns. In a first approach, the four ratios that can be derived from each run (114/116, 115/117, 114/117 and 115/116) were log-transformed and a t-test was used to isolate protein ratios significantly different from 0 in each run. In a second approach, the two runs were merged into one file and the 114/116 and 115/117 ratios of each run were log-transformed and these ratios were multiplied (log\*log). Proteins with recurrent up- or downregulation result in positive log\*log protein ratios and those > 0.01 were retained and listed. Proteins that were

present in both lists were considered unequivocally differentially expressed. This combined approach allows defining proteins with relatively low, but recurrent expressional differences.

### **The contribution of the cytoskeleton to cortisol induced intracellular proliferation of *Salmonella* Typhimurium in primary porcine macrophages**

The contribution of the cytoskeleton during the cortisol induced increased proliferation of *Salmonella* Typhimurium in PAM was investigated using cytochalasin D (Sigma) for the inhibition of F-actin polymerization, and nocodazole (Sigma) as an inhibitor for microtubule formation. Therefore, PAM were seeded in 24-well plates at a density of approximately  $5 \times 10^5$  cells per well, allowed to attach for 2 hours and infected with *Salmonella*, as described in the iTRAQ analysis. To assess the intracellular proliferation, the medium containing 100 µg/mL gentamicin was replaced after 1 hour incubation with fresh medium containing 20 µg/mL gentamicin, with or without 1 µM cortisol, 2 µM cytochalasin D and/or 20 µM nocodazole. Twenty-four hours after infection, the number of viable bacteria was determined by plating 10-fold dilutions on Brilliant Green Agar (BGA, international medical products, Brussels, Belgium).

### **Screening for cortisol induced *Salmonella* Typhimurium genes in primary porcine macrophages**

IVET is a promoter-trapping method that can be used to identify bacterial promoters (genes) that are upregulated during interactions of the bacterium with its environment. An IVET transformants pool was used covering the major part of the *Salmonella* Typhimurium genome (Van Parys et al., 2011). Therefore, total genomic DNA of the WT *Salmonella* Typhimurium was isolated and digested. These DNA fragments were inserted in purified pIVET1 plasmids (one fragment per plasmid), in front of a promoterless *purA* gene, followed by a promoterless *lacZY* operon. After replication in *Escherichia coli*, these plasmids were inserted in *Salmonella* Typhimurium lacking the *purA* gene ( $\Delta purA$  *Salmonella* Typhimurium). This pIVET1 plasmid integrates in the *Salmonella* chromosome. *Salmonella* bacteria lacking the *purA* gene do not longer survive in macrophages. If the cloned DNA contains a promoter which is activated within the macrophage, the *purA* gene and the *lacZY* operon will be expressed and the bacterium will survive intracellularly.

We used this IVET transformants pool, to identify *Salmonella* Typhimurium genes that are intracellularly expressed in PAM after exposure to cortisol (Van Parys et al., 2011). For this purpose, PAM were seeded in 6-well plates at a density of approximately  $3 \times 10^6$

cells per well, a *Salmonella* Typhimurim IVET pool was inoculated into the 6-well plates at a multiplicity of infection of 50:1 and the infected cells were treated with medium with or without 1  $\mu$ M cortisol, as described in the iTRAQ analysis. Sixteen hours after infection, PAM were washed 3 times and lysed for 10 min with 500  $\mu$ L 1% (v/v) Triton X-100. This was added to 9.5 mL of LB broth enriched with the additives and grown with aeration at 37  $^{\circ}$ C. After 3 hours, the bacterial culture was centrifuged at 2300 x g for 10 min at 37  $^{\circ}$ C and the pellet was resuspended in 3 mL PAM medium without antibiotics. This was considered as one passage and in total three passages were performed. Finally, after the third passage of the *Salmonella* Typhimurium IVET pool in PAM whether or not treated with 1  $\mu$ M cortisol, the cells were lysed and plated on MacConkey agar (Oxoid) supplemented with the additives and 1% (w/v) filter-sterilized lactose (Merck KGaA) to assess the *lacZY* expression level of the IVET transformants. This allowed detection of IVET transformants containing promoters expressed intracellularly in PAM but not on MacConkey agar. These fusion strains formed white to pink colonies on MacConkey lactose agar (low-level *lacZY* expression), whereas fusion strains containing promoters that are constitutively expressed showed red colonies (high-level *lacZY* expression). As we were interested in genes that are intracellularly induced, but not extracellularly, all the colonies showing low-level *lacZY* expression were picked up, purified and sequenced as previously described (Van Parys et al., 2011).

### **Invasion and proliferation assays of *Salmonella* Typhimurium and its isogenic mutants $\Delta$ *scsA* and $\Delta$ *cbpA* in primary porcine macrophages**

Based on the IVET screen, the effect of cortisol on the intracellular proliferation of  $\Delta$ *scsA* and  $\Delta$ *cbpA* was determined in comparison to the WT strain. Therefore, PAM were seeded in 24-well plates at a density of approximately  $5 \times 10^5$  and inoculated with *Salmonella* Typhimurium WT,  $\Delta$ *scsA* or  $\Delta$ *cbpA*, as described in the iTRAQ analysis. The medium containing 100  $\mu$ g/mL gentamicin was replaced after 1 hour incubation with fresh medium containing 20  $\mu$ g/mL gentamicin with or without cortisol ranging from 0.001 to 100  $\mu$ M. After 24 hours, the cells were washed 3 times and lysed for 10 min with 1% (v/v) Triton X-100 and 10-fold dilutions were plated on BGA plates.

### **Comparison of the gene expression of *Salmonella* Typhimurium and its isogenic mutant $\Delta$ *scsA***

In order to uncover differences in gene expression between the *Salmonella* Typhimurium and its isogenic mutant  $\Delta$ *scsA*, RNA was isolated from *Salmonella*

Typhimurium WT and *Salmonella* Typhimurium  $\Delta scsA$  grown in LB medium at logarithmic and stationary growth phase (Lundberg et al., 1999). Two OD600 units were harvested and RNA was extracted and purified using SV Total RNA Isolation Kit (Promega Benelux bv, Leiden, The Netherlands) according to manufacturer's instructions. The quality and purity of the isolated RNA was determined using a Nanodrop spectrophotometer and Experion RNA StdSens Analysis kit (Bio-rad). The SALSA microarrays and protocols for RNA labeling, microarray hybridization and subsequent data acquisition have been described previously (Nagy et al., 2006). RNA (10  $\mu$ g) from 3 independent biological replicates of *Salmonella* Typhimurium WT (control) and *Salmonella* Typhimurium  $\Delta scsA$  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 of the  $\Delta scsA$  deletion mutant were assessed to be significantly differently expressed in comparison to the genes of *Salmonella* Typhimurium WT (control) by an analysis of variance test with a Benjamini and Hochberg false discovery rate of 0.05 and with a 1.5-fold change in the expression level. 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 GSE30924 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30924>).

### Statistical Analysis

All *in vitro* experiments were conducted in triplicate with 3 repeats per experiment, unless otherwise stated. All statistical analyses were performed using SPSS version 19 (SPSS Inc., Chicago, IL, USA). Normally distributed data were analyzed using unpaired Student's t-test or one-way ANOVA to address the significance of difference between mean values with significance set at  $p \leq 0.05$ . Bonferroni as post hoc test was used when equal variances were assessed. If equal variances were not assessed, the data were analyzed using Dunnett's T3 test. Not normally distributed data were analyzed using the non parametric Kruskal-Wallis analysis, followed by Mann-Whitney U test.

## Results

### **Differential protein expression of *Salmonella* Typhimurium infected primary porcine macrophages after exposure to cortisol**

Peptides from trypsin digested proteins were labeled with isobaric mass tag labels and analyzed by 2-D LC-MS/MS. Collision-induced dissociation results in the release of these isobaric tags, which allows relative quantification of the peptides. A broad comparison between cortisol treated and untreated *Salmonella* Typhimurium infected PAM, resulted in the identification of 23 proteins with relatively low, but recurrent expressional differences, as shown in Table 2. Two of these proteins showed higher levels in untreated PAM, whereas 21 of them were more abundant in cortisol treated PAM. Proteomic analysis revealed a cortisol increased expression of beta tubulin, capping protein beta 3 subunit, thymosin beta-4, actin-related protein 3B, tropomyosin 5, and elongation factor 1-alpha 1 isoform 4, which are 6 proteins that are involved in reorganizations of the cytoskeleton. Furthermore, cortisol caused an increased expression of transketolase, Cu-Zn superoxide dismutase, glutaredoxin and prostaglandin reductase 1 (15-oxoprostaglandin 13-reductase) which play a role in the macrophage defense mechanisms.

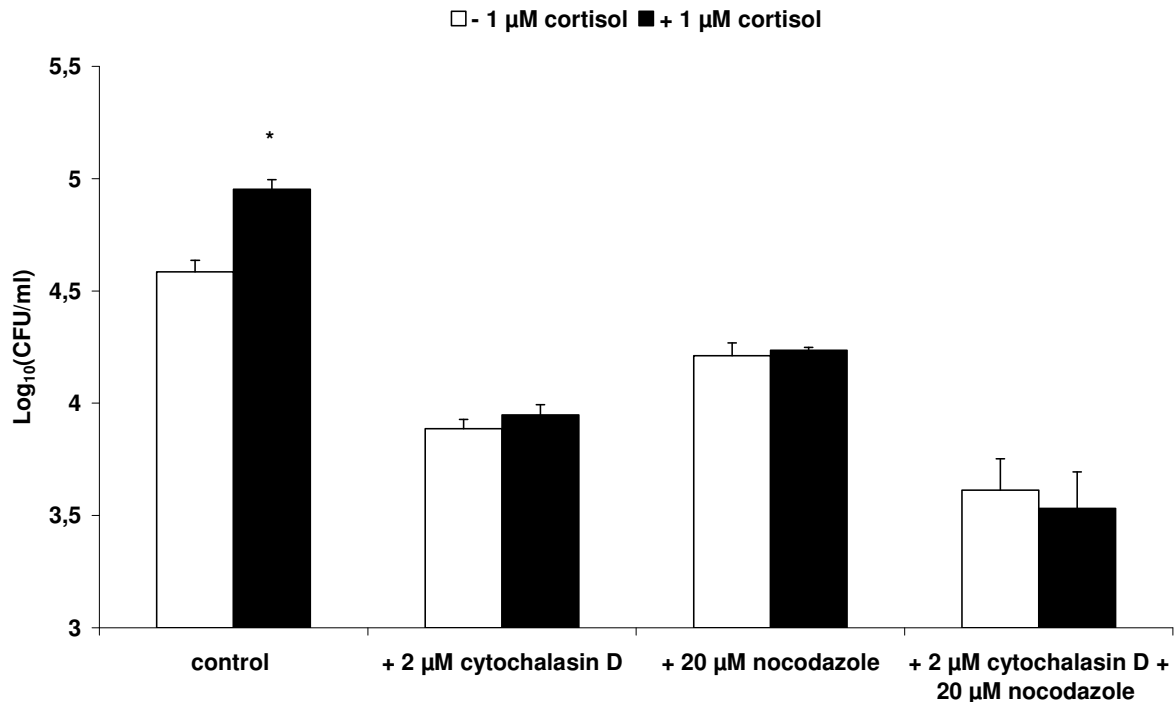
### **Cortisol induced increased survival of *Salmonella* Typhimurium is both microfilament and microtubule dependent**

As earlier described, exposure to 1  $\mu$ M cortisol for 24 hours led to a significant increase of the number of intracellular *Salmonella* Typhimurium bacteria compared to untreated PAM (Verbrugghe et al., 2011b). In the present study, we showed that this cortisol induced increased intracellular proliferation of *Salmonella* Typhimurium is microfilament and microtubule dependent. The treatment of *Salmonella* Typhimurium infected PAM with cytochalasin D and/or nocodazole resulted in the inhibition of the cortisol induced increased survival of the bacterium. Results are summarized in Figure 1.

**Table 2: Differential protein expression of *Salmonella* infected macrophages after exposure to cortisol.**

Protein name	Function*	Protein ratio treated/untreated PAM of the t-test approach	Protein ratio treated/untreated PAM of the log*log approach
Cytochrome c oxidase subunit 5B, mitochondrial	This protein is one of the nuclear-coded polypeptide chains of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport.	0.7	0.8
Pulmonary surfactant-associated protein B	Pulmonary surfactant-associated proteins promote alveolar stability by lowering the surface tension at the air-liquid interface in the peripheral air spaces.	0.7	0.8
Tropomyosin 5	Is an actin-binding protein that regulates actin mechanics.	1.2	1.2
Cathepsin B precursor	Thiol protease which is believed to participate in intracellular degradation and turnover of proteins.	1.2	1.2
Peptidyl-prolyl cis-trans isomerase B	Peptidyl-prolyl cis-trans isomerase B accelerates the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides.	1.2	1.2
Transketolase	Is an enzyme of the pentose phosphate pathway and the Calvin cycle that catalysis the conversion of Sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate to D-ribose 5-phosphate + D-xylulose 5-phosphate in both directions.	1.2	1.3
Translation elongation factor 1 alpha 2 isoform 1	This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.	1.3	1.3
L-lactate dehydrogenase A chain	Is an enzyme that catalyses the conversion from (S)-lactate + NAD <sup>+</sup> to pyruvate + NADH in the final step of anaerobic glycolysis.	1.2	1.2
Cu-Zn-superoxide dismutase	Is an enzyme that catalysis the dismutation of superoxide into oxygen and hydrogen peroxide.	1.2	1.2
Cytochrome c oxidase subunit IV	This protein is one of the nuclear-coded polypeptide chains of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport.	1.2	1.3
Malate dehydrogenase, mitochondrial	Is an enzyme in the citric acid cycle that catalyzes the conversion of (S)-malate + NAD <sup>+</sup> into oxaloacetate + NADH and vice versa	1.2	1.3
Elongation factor 1-alpha 1 isoform 4	This protein promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis.	1.2	1.3
Thymosin beta-4	Plays an important role in the organization of the cytoskeleton. Binds to and sequesters actin monomers (G actin) and therefore inhibits actin polymerization.	1.2	1.3
Capping protein beta 3 subunit	Cellular component of the F-actin capping protein complex that binds to and caps the barbed ends of actin filaments, thereby regulating the polymerization of actin monomers but not severing actin filaments.	1.2	1.3
Annexin A1	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis. This protein regulates phospholipase A2 activity.	1.3	1.3
Neutral alpha-glucosidase AB	Cleaves sequentially the 2 innermost alpha-1,3-linked glucose residues from the Glc2Man9GlcNAc2 oligosaccharide precursor of immature glycoproteins.	1.3	1.3
CD14 antigen	The protein is a surface antigen that is preferentially expressed on monocytes/macrophages. It cooperates with other proteins to mediate the innate immune response to bacterial lipopolysaccharide.	1.3	1.3
Beta tubulin	Tubulin is the major constituent of microtubules. It binds two moles of GTP, one at an exchangeable site on the beta chain and one at a non-exchangeable site on the alpha-chain.	1.3	1.4
Actin-related protein 3B	May function as ATP-binding component of the Arp2/3 complex which is involved in regulation of actin polymerization and together with an activating nucleation-promoting factor (NPF) mediates the formation of branched actin networks.	1.4	1.4
Granulins	Granulins have possible cytokine-like activity. They may play a role in inflammation, wound repair, and tissue remodeling.	1.4	1.5
Glutaredoxin	Is a redox enzyme that uses glutathione as a cofactor and which plays a role in cell redox homeostasis.	1.4	1.4
Vat1 protein	This protein belongs to the oxidoreductases that play a role in oxidation-reduction processes.	1.7	1.7
Prostaglandin reductase 1	Catalyzes the conversion of leukotriene B4 into 12-oxo-leukotriene B4. This is an initial and key step of metabolic inactivation of leukotriene B4.	1.8	1.8

Differentially expressed proteins identified in cortisol treated PAM in comparison to untreated PAM, by use of iTRAQ analysis coupled to 2-D LC-MS/MS. Superscript (\*) refers to protein description according to the UniProtKB/Swiss-Prot protein sequence database (<http://expasy.org/sprot/>).



**Figure 1: Effect of cortisol, cytochalasin and/or nocodazole on the intracellular proliferation of *Salmonella Typhimurium* in macrophages.** Number of intracellular *Salmonella Typhimurium* bacteria in PAM that were treated with control medium, 2  $\mu$ M cytochalasin D, 20  $\mu$ M nocodazole or the combination of both, for 24 hours after invasion. The white bars represent medium without cortisol and the black bars represent medium with 1  $\mu$ M cortisol. The log<sub>10</sub> values of the number of gentamicin protected bacteria + standard deviation are shown. Results are presented as a representative experiment conducted in triplicate. Superscript (\*) refers to a significant difference compared to the condition without cortisol ( $p \leq 0.05$ ).

### Differential gene expression of intracellular *Salmonella Typhimurium* WT bacteria after exposure to cortisol

All the colonies showing low-level *lacZY* expression were analysed to identify genes that were intracellularly expressed in PAM that might be essential for *Salmonella* survival in PAM. In total, we purified and sequenced 287 and 69 colonies from PAM whether or not treated with 1  $\mu$ M cortisol, respectively. An overview of the identified genes from 3 independent experiments is given in Table 3. Of all genes, only *STM4067* was found in all 3 independent experiments and in both conditions. *CbpA*, *pflC*, *pflD* and *scsA* were identified in all 3 independent experiments, however only in PAM that were treated with 1  $\mu$ M cortisol. These genes might thus be intracellularly cortisol induced genes of the bacterium.

### Deletion of *scsA* abolishes the cortisol induced increased intracellular proliferation of *Salmonella Typhimurium* in PAM

Following IVET screening and based on the literature, the effect of cortisol on the intracellular survival of *Salmonella Typhimurium*  $\Delta$ *scsA* and  $\Delta$ *cbpA* in comparison to the WT



strain was investigated. The results represented in Figure 2 show that the intracellular proliferation of *Salmonella* Typhimurium WT and  $\Delta cbpA$  was higher in cortisol treated PAM, for 24 hours, in comparison to untreated cells. Exposure to cortisol concentrations of respectively  $\geq 10$  nM and 500 nM led to a significant dose-dependent increase of the number of intracellular *Salmonella* Typhimurium WT or  $\Delta cbpA$  bacteria. In contrast, cortisol did not affect the intracellular proliferation of *Salmonella* Typhimurium  $\Delta scsA$  in PAM (Figure 2). This implies that the *scsA* gene is at least partly responsible for the increased intracellular survival of *Salmonella* WT in cortisol exposed PAM.

### **Deletion of *scsA* gene causes an upregulation of the *scsBCD* operon**

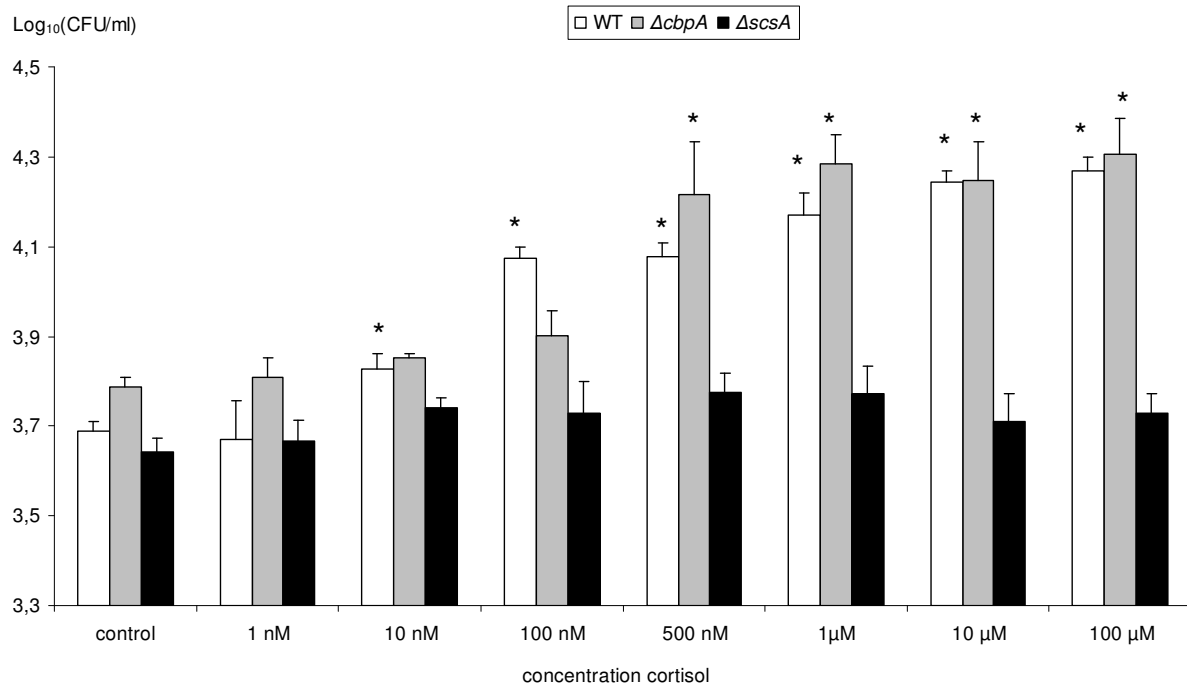
A transcriptomic comparison of the *Salmonella* Typhimurium  $\Delta scsA$  and WT strains grown to logarithmic and stationary phase revealed the differential expression of 57 and 19 genes respectively. The transcriptomic data showed that deletion of *scsA* resulted in the up-regulation of the entire *scsBCD* operon at both logarithmic and stationary phase. The *scsB*, *scsC* and *scsD* genes were up-regulated by 34.16, 19.63 and 6.50-fold respectively during stationary phase and 32.09, 19.90 and 6.33-fold respectively during logarithmic phase growth (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30924>).

In the stationary phase culture, an increased expression of *Salmonella* pathogenicity island (SPI-1) Type III Secretion system (T3SS) Needle Complex Protein PrgI (1.81) and the SPI-1 T3SS effector protein SipA (1.71) was observed. Furthermore, *Salmonella* Typhimurium  $\Delta scsA$  grown to a logarithmic phase culture showed an increased expression of the T3SS effector protein SipC (2.19). However, the invasion capacity of *Salmonella* Typhimurium  $\Delta scsA$  was not altered in comparison to the WT strain (unpublished data).

**Table 3: IVET screening for cortisol induced genes.**

Gene	Gene product description*	Frequency (+ 1 $\mu$ M cortisol)	Percentage (+ 1 $\mu$ M cortisol)	Frequency (- 1 $\mu$ M cortisol)	Percentage (- 1 $\mu$ M cortisol)
<i>cbpA</i>	curved DNA-binding protein CbpA	3/3	9.5		
<i>cmk</i>	cytidylate kinase	2/3	1.4		
<i>dnaC</i>	DNA replication protein DnaC	1/3	0.7		
<i>dnaK</i>	molecular chaperone DnaK			1/3	4.3
<i>dnaT</i>	primosomal protein DnaI	1/3	0.7		
<i>efp</i>	elongation factor P			1/3	2.9
<i>entF</i>	enterobactin synthase subunit F	1/3	0.7		
<i>eutA</i>	reactivating factor for ethanolamine ammonia lyase			1/3	1.4
<i>folA</i>	dihydrofolate reductase	1/3	0.7		
<i>gppA</i>	guanosine pentaphosphate phosphohydrolase	1/3	1.4		
<i>gyrB</i>	DNA gyrase, subunit B	1/3	0.3		
<i>lysS</i>	lysyl-tRNA synthetase	2/3	1.0		
<i>marC</i>	multiple drug resistance protein MarC	1/3	0.3		
<i>menA</i>	1,4-dihydroxy-2-naphthoate octaprenyltransferase	2/3	6.0		
<i>menG</i>	ribonuclease activity regulator protein RraA	2/3	4.6		
<i>nlpB</i>	lipoprotein			2/3	16.0
<i>parE</i>	DNA topoisomerase IV subunit B			1/3	17.5
<i>pfIC</i>	pyruvate formate lyase II activase	3/3	3.6		
<i>pfID</i>	formate acetyltransferase 2	3/3	3.6		
<i>prfC</i>	peptide chain release factor 3	1/3	0.3		
<i>proP</i>	proline/glycine betaine transporter	1/3	0.7		
<i>prpD</i>	2-methylcitrate dehydratase	2/3	0.7		
<i>prpE</i>	propionyl-CoA synthetase	2/3	0.7		
<i>ratB</i>	outer membrane protein			1/3	1.4
<i>rfaD</i>	ADP-L-glycero-D-mannoheptose-6-epimerase			1/3	2.9
<i>rnt</i>	ribonuclease T	1/3	2.9		
<i>rpoE</i>	RNA polymerase sigma factor RpoE	1/3	0.3		
<i>rpoN</i>	RNA polymerase factor sigma-54			1/3	1.4
<i>rpoZ</i>	DNA-directed RNA polymerase subunit omega	1/3	0.3		
<i>scsA</i>	suppression of copper sensitivity protein A	3/3	8.1		
<i>STM0014</i>	putative transcriptional regulator	1/3	0.3		
<i>STM0266</i>	putative cytoplasmic protein	1/3	0.3		
<i>STM0272</i>	putative chaperone ATPase	1/3	0.3		
<i>STM0409</i>	putative hypothetical protein	1/3	0.7		
<i>STM2314</i>	putative chemotaxis signal transduction protein	1/3	0.7		
<i>STM2840</i>	putative anaerobic nitric oxide reductase flavorubredoxin	1/3	0.3		
<i>STM4067</i>	putative ADP-ribosylglycohydrolase	3/3	36.3	3/3	21.7
<i>tolC</i>	outer membrane channel protein	1/3	0.7		
<i>torA</i>	trimethylamine N-oxide reductase subunit	1/3	1.0		
<i>trpS</i>	tryptophanyl-tRNA synthetase	1/3	0.7		
<i>yabN</i>	transcriptional regulator SgrR	1/3	0.7		
<i>ybdZ</i>	cytoplasmic protein	1/3	0.7		
<i>ycgB</i>	SpoVR family protein	1/3	0.3		
<i>yfeA</i>	hypothetical protein	1/3	0.3		
<i>yfeC</i>	negative regulator	1/3	0.3		
<i>ygdH</i>	nucleotide binding	1/3	0.7		
<i>ygfA</i>	ligase	1/3	0.3		
<i>yggE</i>	periplasmic immunogenic protein	2/3	3.9		
<i>yhbG</i>	ABC transporter ATP-binding protein YhbG			1/3	1.4
<i>yjbB</i>	transport protein	1/3	0.3	1/3	2.9
<i>yjjK</i>	RNA polymerase factor sigma-54			1/3	26.2
<i>yqjE</i>	inner membrane protein	1/3	1.0		
<i>yqjG</i>	glutathione S-transferase	1/3	1.7		

List of genes of *Salmonella* Typhimurium induced intracellularly in primary alveolar macrophages. The represented data are the result of 3 independent experiments for PAM whether (+ 1  $\mu$ M cortisol) or not (- 1  $\mu$ M cortisol) treated with cortisol. In total, we purified and sequenced 287 and 69 colonies from PAM whether or not treated with 1  $\mu$ M cortisol, respectively. The frequency shows the fraction of positive samples in relation to the total number of independent experiments. If an expressed gene was found more than once in cortisol treated PAM, then the contribution of the gene in relation to the total number of 287 tested colonies, is expressed as percentage. If an expressed gene was found more than once in untreated PAM, then the contribution of the gene in relation to the total number of 69 tested colonies, is expressed as percentage. Superscript (\*) refers to gene product description according to the National Center for Biotechnology Information ([http://www.ncbi.nlm.nih.gov/nucore/NC\\_003197](http://www.ncbi.nlm.nih.gov/nucore/NC_003197)).



**Figure 2: Effect of cortisol on the survival of *Salmonella* Typhimurium and its isogenic mutants  $\Delta cbpA$  and  $\Delta scsA$  in macrophages.** Number of intracellular *Salmonella* Typhimurium WT,  $\Delta cbpA$  or  $\Delta scsA$  bacteria in PAM that were treated with control medium or different concentrations of cortisol, for 24 hours after invasion. The log<sub>10</sub> values of the number of gentamicin protected bacteria + standard deviation are shown. Results are presented as a representative experiment conducted in triplicate. Superscript (\*) refers to a significant difference compared to the control ( $p \leq 0.05$ ) group.

## Discussion

As shown with iTRAQ analysis (Table 2), the increased proliferation of *Salmonella* Typhimurium in cortisol treated primary macrophages is associated with an increased expression of cytoskeleton-associated proteins. Intracellular replication of *Salmonella* Typhimurium is accompanied by a complex series of cytoskeletal changes, such as F-actin rearrangements and the formation of continuous tubular aggregates (Guignot et al., 2004; Guiney and Lesnick, 2005; Henry et al., 2006). These cytoskeletal events are important for the cytoplasmic transport and the establishment and the stability of the bacterial replicative niche, also called *Salmonella* containing vacuole (SCV) (Drecktrah et al., 2008; Henry et al., 2006; Kuhle et al., 2004). The capacity of *Salmonella* to replicate inside macrophages is based upon this establishment of a SCV (Schroeder et al., 2011). iTRAQ analysis revealed a cortisol increased expression of beta tubulin, a constituent of microtubules, capping protein beta 3 subunit, thymosin beta-4 and actin-related protein 3B, three proteins that regulate the polymerization of actin. In addition to this, cortisol induces an increased expression of

tropomyosin 5, an actin-binding protein that regulates actin mechanics, and which has been shown to be one of the constituents of the SCV in eukaryotic cells (Finlay et al., 1991). Furthermore, an increased expression of elongation factor 1-alpha 1 isoform 4 and translation elongation factor 1-alpha 2 isoform 1 was detected. Although these proteins play an important role during protein biosynthesis, in line with augmented expression of these cytoskeletal proteins, it has also been demonstrated that elongation factor 1-alpha appears to have a second role as a regulator of cytoskeletal rearrangements (Shiina et al., 1994; Yang et al., 1990).

Using cytochalasin D for the inhibition of F-actin polymerization, and nocodazole as an inhibitor for microtubule formation, we showed that this cortisol induced increased intracellular proliferation of *Salmonella* Typhimurium is microfilament and microtubule dependent (Figure 1). Together these results suggest that the induced cytoskeletal protein expression might facilitate SCV formation and stabilization.

In addition to this, iTRAQ analysis revealed that the increased proliferation of *Salmonella* Typhimurium in cortisol treated primary macrophages is also associated with an increased expression of proteins that are involved in macrophage defense mechanisms. Primary macrophages produce and release reactive oxygen species (ROS) in response to phagocytosis of *Salmonella*. Although the exact mechanisms by which ROS damage bacteria in the phagosome are unclear, it is known that ROS are essential components of the antimicrobial repertoire of macrophages (Slauch, 2011). Transketolase is an enzyme of the pentose phosphate pathway which is the major source of nicotinamide adenine dinucleotide phosphate (NADPH), a substrate for superoxide production (Pick et al., 1989). However, in order to protect themselves from the constant oxidative challenge, primary macrophages produce Cu-Zn superoxide dismutase as a key enzyme in the dismutation of superoxide into oxygen and hydrogen peroxide (Marikovsky et al., 2003). According to Gadgil et al. (2003), monocytes that were primed by lipopolysaccharide (LPS) showed an increased expression of transketolase and superoxide dismutase (Gadgil et al., 2003). In addition to this, glutaredoxin also plays a protective role in the response to oxidative stress (Bandyopadhyay et al., 1998; Davis et al., 1997; Luikenhuis et al., 1998; Rodríguez-Manzaneque et al., 1999). Therefore, the observed increased expression of transketolase, Cu-Zn superoxide dismutase and glutaredoxin in cortisol treated primary macrophages, might be the result of the increased intracellular proliferation of *Salmonella* Typhimurium in these cells.

Prostaglandin reductase 1 or 15-oxoprostaglandin 13-reductase is a protein which is involved in the metabolic inactivation of leukotriene B<sub>4</sub> (LTB<sub>4</sub>), a neutrophil chemoattractant. Since prostaglandin reductase 1 was more abundant in the cortisol treated primary

macrophages, cortisol treatment of infected macrophages could result in an increased inactivation of LTB<sub>4</sub>. It has been shown that LTB<sub>4</sub> enhances the phagocytosis and killing of *Salmonella* Typhimurium (Demitsu et al., 1989), offering a mechanistic explanation for the increased survival of *Salmonella* Typhimurium in cortisol treated primary macrophages.

Besides the altered protein expression of cortisol infected primary macrophages, IVET screening showed that the gene expression of intracellular *Salmonella* Typhimurium differs markedly in cortisol treated primary macrophages in comparison to untreated primary macrophages (Table 3). Recently we showed that cortisol did not cause any significant changes in the gene expression of *Salmonella* Typhimurium WT when grown in a complex medium (Verbrugghe et al., 2011b). This implies that the cortisol mediated increased intracellular proliferation of *Salmonella* Typhimurium is probably caused by an indirect effect through the cell. Of all identified genes, only *STM4067* was found in all 3 independent experiments and in both conditions. *STM4067* encodes the putative ADP-ribosylglycohydrolase, which was identified by Van Parys et al. (2011) as a factor for intestinal *Salmonella* Typhimurium persistence in pigs. *PflC* and *pflD* encode the pyruvate formate lyase activase II and the formate acetyltransferase 2, respectively. Both genes play a role in the anaerobic glucose metabolism (Sawers et al., 1998). *CbpA* encodes the curved DNA binding protein which is a molecular hsp40 chaperone that is involved in bacterial responses to environmental stress and which is homologous to *dnaJ* (Van Parys et al., 2011). *ScsA* encodes the suppressor of copper sensitivity protein and according to Gupta et al. (1997), it might function as a peroxidase by preventing the formation of free hydroxyl radicals resulting from the reaction of copper with hydrogen peroxide (Gupta et al., 1997). Deletion of *scsA* resulted in the inhibition of cortisol induced increased intracellular proliferation of *Salmonella* Typhimurium in primary macrophages (Figure 2). This implies that the *scsA* gene is at least partly responsible for the increased intracellular survival of *Salmonella* WT in cortisol exposed primary macrophages.

Deletion of the *scsA* gene results in the upregulation of the *scsBCD* operon, as assessed by microarray analysis (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30924>). According to Gupta et al. (1997), the *scs* locus consists of two operons, one operon consisting of the single *scsA* gene and another operon containing the *scsB*, *scsC* and *scsD* genes encoding proteins that may mediate copper tolerance in *Escherichia coli*, by catalyzing the correct folding of periplasmic copper-binding target proteins via a disulfide isomerise-like activity (Gupta et al., 1997). It is possible that *scsA* acts as a negative regulator

for the *scsBCD* operon or the upregulation of these genes could be a result of gene redundancy.

In conclusion, we showed the cortisol induced increased survival of *Salmonella* Typhimurium in primary macrophages is microfilament and microtubule dependent and that it coincides with an increased expression of cytoskeleton-associated proteins and proteins of the macrophage defense mechanism. These cortisol induced host-cell alterations are associated with modified intracellular gene expression of *Salmonella* Typhimurium resulting in a *scsA* dependent intracellular proliferation of *Salmonella* Typhimurium in pig macrophages.

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## CHAPTER 3:

### **T-2 toxin induced *Salmonella* Typhimurium intoxication results in decreased *Salmonella* numbers in the cecal contents of pigs, despite marked effects on *Salmonella*-host cell interactions**

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## Abstract

The mycotoxin T-2 toxin and *Salmonella* Typhimurium infections pose a significant threat to human and animal health. Interactions between both agents may result in a different outcome of the infection. Therefore, the aim of the presented study was to investigate the effects of low and relevant concentrations of T-2 toxin on the course of a *Salmonella* Typhimurium infection in pigs. We showed that the presence of 15 and 83 µg T-2 toxin per kg feed significantly decreased the amount of *Salmonella* Typhimurium bacteria present in the cecal contents, and a tendency to a reduced colonization of the jejunum, ileum, cecum, colon and colon contents was noticed. *In vitro*, proteomic analysis of porcine enterocytes revealed that a very low concentration of T-2 toxin (5 ng/mL) affects the protein expression of mitochondrial, endoplasmatic reticulum and cytoskeleton associated proteins, proteins involved in protein synthesis and folding, RNA synthesis, mitogen-activated protein kinase signaling and regulatory processes. Similarly low concentrations (1-100 ng/mL) promoted the susceptibility of porcine macrophages and intestinal epithelial cells to *Salmonella* Typhimurium invasion, in a SPI-1 independent manner. Furthermore, T-2 toxin (1-5 ng/mL) promoted the translocation of *Salmonella* Typhimurium over an intestinal porcine epithelial cell monolayer. Although these findings may seem in favour of *Salmonella* Typhimurium, microarray analysis showed that T-2 toxin (5 ng/mL) causes an intoxication of *Salmonella* Typhimurium, represented by a reduced motility, a downregulation of metabolic and *Salmonella* Pathogenicity Island 1 genes. This study demonstrates marked interactions of T-2 toxin with *Salmonella* Typhimurium pathogenesis, resulting in bacterial intoxication.

## Introduction

T-2 toxin is a type A trichothecene, produced by various *Fusarium* spp. such as *Fusarium acuminatum*, *F. equiseti*, *F. poae* and *F. sporotrichioides* (Moss, 2002). In moderate climate regions of North America, Asia and Europe, these moulds are common contaminants of cereals such as wheat, barley, oats, maize and other crops for human and animal consumption (Placinta et al, 1999). Since mycotoxins are very stable under normal food processing conditions, T-2 toxin can end up in the food and feed. With T-2 toxin being the most acute toxic trichothecene (Gutleb et al, 2002), this mycotoxin may pose a threat to human and animal health around the world. Pigs appear to be one of the most sensitive species to *Fusarium* mycotoxins (Hussein and Brasel, 2001). Moderate to high levels of T-2 toxin cause a variety of toxic effects including immunosuppression, feed refusal, vomiting, weight loss, reduced growth and skin lesions (Wu et al., 2010). Only little information is available on *in vivo* effects from humans with known exposure to T-2 toxin. Wang et al. (1993) reported an outbreak of human toxicosis in China caused by moldy rice contaminated with T-2 toxin at concentrations ranging from 180 to 420 µg T-2 toxin per kg, and the main symptoms were nausea, vomiting, abdominal pain, thoracic stuffiness and diarrhoea. Furthermore, it is suggested that alimentary toxic aleukia (ATA), which occurred in the USSR in the period 1941-1947, is related to the presence of T-2 toxin producing *Fusarium* spp. in over-wintered grain. Clinical symptoms include inflammation of gastric and intestinal mucosa, leukopenia, hemorrhagic diathesis, granulopenia, bone marrow aplasia and sepsis (Scientific Committee on Food, 2001). Although a tolerable daily intake (TDI) value for the sum of T-2 toxin and HT-2 toxin of 100 ng/kg has been set by the European Union (European Food Safety Authority, 2011), control of exposure is limited since no maximum guidance limits for T-2 toxin in food and feedstuff are yet established by the European Union. However, contamination of cereals with T-2 toxin is an emerging issue and concentrations up to 1810 µg T-2 toxin per kg wheat have been reported in Germany (Schollenberger et al., 2006).

Besides mycotoxins, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) infections are a major issue in swine production and one of the major causes of foodborne salmonellosis in humans (European Food Safety Agency, 2009). Pigs infected with *Salmonella* Typhimurium mostly carry this bacterium asymptotically in their tonsils, gut and gut-associated lymphoid tissue for weeks or even months (Wood et al.,

1989). These carrier pigs excrete very low numbers of *Salmonella* and are difficult to distinguish from uninfected pigs. However, at slaughter they can be a source of environmental and carcass contamination, leading to higher numbers of foodborne *Salmonella* infections in humans. Although nontyphoidal *Salmonella* infections in humans mostly result in gastroenteritis, it is still a major cause of morbidity and mortality worldwide. It is estimated that nontyphoidal *Salmonella* infections result in 93.8 million illnesses globally each year, of which 80.3 million are foodborne, and 155,000 result in death (Majowicz et al., 2010).

T-2 toxin is rapidly absorbed in the small intestine (Cavret and Lecoecur, 2006) and affects the porcine and human innate immune system at various levels (Rafai et al., 1995b; Kankkunen et al., 2009). Since the pathogenesis of a *Salmonella* infection is characterized by a systemic and an enteric phase of infection, T-2 toxin might interfere with the pathogenesis of *Salmonella* Typhimurium. However, until now, there are no data available describing an interaction between low concentrations of T-2 toxin and the pathogenesis of a *Salmonella* Typhimurium infection in pigs. Only some scarce results have been reported of an altered susceptibility to intestinal infections after ingestion of sometimes high and even irrelevant concentrations of certain mycotoxins. Feeding pigs with 5 mg T-2 toxin per kg feed, resulted in a substantial increase in aerobic bacterial counts in the intestine (Tenk et al., 1982). Tai and Pestka (1988) showed that the oral exposure of mice to T-2 toxin could result in an impaired murine resistance to *Salmonella* Typhimurium. Furthermore Oswald et al. (2003) showed that fumonisin B<sub>1</sub> (FB1) increases the intestinal colonization by pathogenic *Escherichia coli* in pigs. However, Tanguy et al. (2006) stated that feeding pigs with FB1 did not induce modifications in the number of *Salmonella* bacteria in the ileum, cecum and colon of pigs.

With T-2 toxin and *Salmonella* being two phenomenons to which pigs can be exposed during their lives, the aim of the presented study was to investigate the effects of low and in practice relevant concentrations of T-2 toxin on the course of a *Salmonella* Typhimurium infection in pigs and to elucidate if it alters bacterium-host cell interactions.

## Materials and methods

### Chemicals

T-2 toxin (Sigma-Aldrich, Steinheim, Germany) stock solution of 5 mg/mL was prepared in ethanol and stored at – 20 °C. Serial dilutions of T-2 toxin were prepared in Luria-Bertani broth (LB, Sigma-Aldrich) or in the corresponding cell culture medium, depending on the experiment.

### Bacterial strains and growth conditions

*Salmonella* Typhimurium strain 112910a, isolated from a pig stool sample and characterized previously by Boyen et al. (2008), was used as the wild type strain in which the spontaneous nalidixic acid resistant derivative strain (WT<sub>nal</sub>) was constructed. The construction and characterization of a deletion mutant in the gene encoding the SPI-1 regulator HilA has been described before (Boyen et al., 2006a). Unless otherwise stated, the bacteria were generally grown overnight (16 to 20 hours) as a stationary phase culture with aeration at 37 °C in 5 mL of LB broth. To obtain highly invasive late logarithmic cultures for invasion assays, 2 µL of a stationary phase culture were inoculated in 5 mL LB broth and grown for 5 hours at 37 °C without aeration (Lundberg et al., 1999).

For oral inoculation of pigs, the WT<sub>nal</sub> was used to provide a selectable marker for identification of experimentally introduced bacteria when plating tonsillar, lymphoid, intestinal and faecal samples. The bacteria were grown for 16 hours at 37 °C in 5 mL LB broth on a shaker, washed twice in Hank's buffered salt solution (HBSS, Gibco, Life Technologies, Paisley, Scotland) by centrifugation at 2300 x g for 10 min at 4 °C and finally diluted in HBSS to the appropriate concentration of 10<sup>7</sup> colony forming units (CFU) per mL. The number of viable *Salmonella* bacteria per mL inoculum was determined by plating 10-fold dilutions on Brilliant Green agar (BGA, international medical products, Brussels, Belgium) supplemented with 20 µg/mL nalidixic acid (BGA<sup>NAL</sup>) for selective growth of the mutant strains.

### Experimental infection with *Salmonella* Typhimurium of pigs fed T-2 toxin-supplemented diets

All animal experiments were carried out in strict accordance with the recommendations in the European Convention for the Protection of Vertebrate Animals used

for Experimental and other Scientific Purposes. The experimental protocols and care of the animals were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2010/049 + expansion 2010/101).

### Experimental design

Three-week-old piglets (commercial closed line based on Landrace) from a serologically *Salmonella* negative breeding herd (according to the Belgian *Salmonella* monitoring program) were used in this *in vivo* trial. The *Salmonella*-free status of the piglets was tested serologically using a commercially available *Salmonella* antibody test kit (IDEXX, Hoofddorp, The Netherlands), and bacteriologically via multiple faecal sampling. At arrival, the piglets were randomized into three groups of 5 piglets (Table 1) and each group was housed in separate isolation units at 26 °C under natural day-night rhythm with *ad libitum* access to feed and water. The first 6 days after arrival, all piglets received a commercial blank piglet feed (DANIS, Koolskamp, Belgium) that contains all the nutrients for proper growth, as an acclimatisation period. The feed was free from mycotoxin-contamination, as determined by multi-mycotoxin liquid chromatography tandem mass spectrometry (LC-MS/MS) (Monbaliu et al., 2010) and the composition of the feed is provided in the Table 1. The acclimatisation period was followed by a feeding period of 23 days with the experimental feed diets that were prepared by adding T-2 toxin to the blank feed. The first group received *ad libitum* blank feed (control group), the second group received feed contaminated with 15 µg/kg T-2 toxin (15 ppb group) and the third group received feed contaminated with 83 µg/kg T-2 toxin (83 ppb group). These concentrations were chosen based on previous measurements of T-2 toxin contamination of feed (Monbaliu et al., 2010). After a feeding period of 18 days, the pigs were orally inoculated with  $2 \times 10^7$  CFU of *Salmonella* Typhimurium WT<sub>nal</sub>. Five days after inoculation, the pigs were euthanized and samples of tonsils, ileocaecal lymph nodes, duodenum, jejunum, ileum, cecum, colon, contents of cecum and colon and rectal faeces were collected for bacteriological analysis to determine the number of *Salmonella* bacteria. To investigate the intestinal cytokine response, ileal fragments were immediately frozen in liquid nitrogen and stored at -70 °C until analysis. Furthermore, to determine the average weight gain (%) of the pigs, the animals were individually weighed after the acclimatization period and, in order to exclude a possible effect of the *Salmonella* infection on the weight gain, after a feeding period of 18 days.



### Bacteriological analysis

All tissues and samples were weighed and 10% (w/v) suspensions were prepared in buffered peptone water (BPW, Oxoid, Basingstoke, United Kingdom). The samples were homogenized with a Colworth stomacher 400 (Seward and House, London, United Kingdom) and the number of *Salmonella* bacteria was determined by plating 10-fold dilutions on BGA<sup>NAL</sup> plates. These were incubated for 16 hours at 37 °C. The samples were pre-enriched for 16 hours in BPW at 37 °C and, if negative at direct plating, enriched for 16 hours at 37 °C in tetrathionate broth (Merck KGaA, Darmstadt, Germany) and plated again on BGA<sup>NAL</sup>. Samples that were negative after direct plating but positive after enrichment were presumed to contain 83 CFU per gram tissue or contents (detection limit for direct plating). Samples that remained negative after enrichment were presumed to be free of *Salmonella* in 1 gram tissue or contents and were assigned value '1' prior to log transformation. Subsequently the number of CFU for all samples was converted logarithmically prior to calculation of the average differences between the log<sub>10</sub> values of the different groups and prior to statistical analysis.

**Table 1:** Composition of the blank piglet feed used in the *in vivo* assay.

<b>Composition piglet feed</b>	
Crude protein	17.70%
Crude fat	5.90%
Crude ash	5.30%
Crude fiber	
Phosphorus	0.56%
Lysine	1.30%
<b>Additives</b>	
Vitamin A	18500 IU/kg
Vitamin D3	2000 IU/kg
Vitamin E	100 mg/kg
Copper(II) sulfate pentahydrate	160 mg/kg
Ethoxyquin	
3-phytaseE.C.3,1,3,8(E1600)	500 FTU/kg
<b>Feedstuff</b>	
Barley	30%
Wheat	18%
Toasted soybeans	14%
Maize	13%
Soya meal	7%
Wheat gluten	4%
Monocalcium phosphate	0.50%
Natriumchloride	0.40%
Palm oil	0.30%

Intestinal cytokine response analysis

Total RNA from the intestinal samples was isolated using RNAzol<sup>®</sup>RT (MRC Inc., Cincinnati, USA) according to the manufacturer's instructions. Extracted RNA was resuspended in 20 µL ultra-pure water. The RNA concentration was measured by absorbance at 260 nm using a nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA) and the integrity of the RNA samples was checked using an Experion RNA StdSens Analysis kit (Biorad Laboratories, Hercules, CA, USA). The construction of cDNA and real-time quantitative PCR analysis to quantify interleukin (IL)-1 $\beta$ , IL-6, IL-8, IL-12/IL-23p40, IL-18, tumour necrosis factor alfa (TNF $\alpha$ ), interferon-gamma (IFN $\gamma$ ) and monocyte chemotactic protein-1 (MCP-1), were carried out as described by Vandenbroucke et al. (2011). IL23 is composed of a p19 subunit of IL-23 and the p40 subunit of interleukin 12. Therefore, changes in IL-12p40 expression reflect changes of both IL-23 and IL-12 expression. Primers used for the amplification are given in Table 2. Hypoxanthine phosphoribosyltransferase (HPRT) and histone H3.3 (HIS) were used as housekeeping genes.

**Table 2:** List of genes and sequences of the primers used for quantitative PCR analysis.

Gene name	Forward primer (5' → 3')	Reverse primer (5' → 3')
HPRT	GAGCTACTGTAATGACCAGTCAACG	CCAGTGTCAATTATATCTTCAACAATCAA
HIS	AAACAGATCTGCGCTTCC	GTCTTCAAAAAGGCCAAC
IL-1 $\beta$	GGGACTTGAAGAGAGAAGTGG	CTTCCCTTGATCCCTAAGGT
IL-6	CACCGGTCTTGTGGAGTTTC	GTGGTGGCTTTGTCTGGATT
IL-8	TTCTGCAGCTCTCTGTGAGGC	GGTGGAAAGGTGTGGAATGC
IL-12/IL-23p40	CACTCCTGCTGCTTCACAAA	CGTCCGGAGTAATTCTTTGC
IL-18	ATGCCTGATTCTGACTGTTC	CTGCACAGAGATGGTTACTGC
TNF $\alpha$	CCCCCAGAAGGAAGAGTTTC	CGGGCTTATCTGAGGTTTGA
IFN $\gamma$	CCATTCAAAGGAGCATGGAT	GAGTTCACTGATGGCTTTGC
MCP-1	CAGAAGAGTCACCAGCAGCA	TCCAGGTGGCTTATGGAGTC

### Effects of T-2 toxin on host-pathogen interactions between *Salmonella* Typhimurium and porcine host cells

#### Cytotoxicity of T-2 toxin towards *Salmonella* Typhimurium infected porcine macrophages and intestinal epithelial cells

It is possible that T-2 toxin increases the toxicity of *Salmonella* Typhimurium for host cells, resulting in an increased cell death. Therefore, the cytotoxic effect of T-2 toxin on *Salmonella* Typhimurium infected primary porcine alveolar macrophages (PAM) and

intestinal porcine epithelial (IPEC-J2) cells was determined using the neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) uptake assay (Bouaziz et al., 2006). Both cell cultures were isolated and cultured as previously described (Verbrugge et al., 2011). PAM were seeded in a 96-well microplate at a density of approximately  $2 \times 10^5$  cells per well and were allowed to attach for 2 hours. The IPEC-J2 cells were seeded in a 96-well microplate at a density of approximately  $2 \times 10^4$  cells per well and allowed to grow for either 24 hours or 21 days, representing actively dividing and differentiated cells respectively. Subsequently, a *Salmonella* gentamicin protection invasion assay was performed as follows. The host cells were inoculated with *Salmonella* at a multiplicity of infection (MOI) of 10:1. To synchronize the infection, the inoculated multiwell plates were centrifuged at  $365 \times g$  for 10 min and incubated for 30 min at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ . Subsequently, the cells were washed 3 times with Hank's buffered salt solution with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (HBSS+, Gibco) and fresh medium supplemented with  $100 \mu\text{g/mL}$  gentamicin (Gibco) was added. Following a 1 hour incubation, the medium was replaced by fresh medium containing  $20 \mu\text{g/mL}$  gentamicin whether or not supplemented with different concentrations of T-2 toxin, for 24 hours. PAM, actively dividing and differentiated IPEC-J2 cells were subjected to T-2 toxin concentrations ranging from 0.250 to  $10 \text{ ng/mL}$ , 0.500 to  $10 \text{ ng/mL}$  and 0.500 to  $100 \text{ ng/mL}$ , respectively. To assess cytotoxicity,  $150 \mu\text{L}$  of freshly prepared neutral red solution ( $33 \mu\text{g/mL}$  in DMEM without phenol red), preheated to  $37^\circ\text{C}$ , was added to each well and the plate was incubated at  $37^\circ\text{C}$  for an additional 2 hours. The cells were then washed twice with HBSS+ and the dye was released from viable cells by adding  $150 \mu\text{L}$  of extracting solution ethanol/Milli-Q water/acetic acid, 50/49/1 (v/v/v) to each well. The plate was shaken for 10 min and 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:

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

Where a =  $\text{OD}_{540}$  derived from the wells incubated with T-2 toxin, b =  $\text{OD}_{540}$  derived from blank wells, c =  $\text{OD}_{540}$  derived from untreated control wells.

#### Effect of T-2 toxin on the invasion and intracellular survival of *Salmonella* Typhimurium in porcine macrophages and intestinal epithelial cells

To examine whether the ability of *Salmonella* Typhimurium to invade and proliferate in PAM and IPEC-J2 cells was altered after exposure of these cells to T-2 toxin, invasion and intracellular survival assays were performed.

For the invasion assays, PAM and IPEC-J2 cells were seeded in 24-well plates at a density of approximately  $5 \times 10^5$  and  $10^5$  cells per well, respectively. PAM were allowed to attach for 2 hours and IPEC-J2 cells were allowed to grow for either 24 hours or 21 days. Subsequently, PAM and actively dividing and differentiated IPEC-J2 cells were exposed to different concentrations of T-2 toxin ranging from 0.250 to 7.5 ng/mL, 0.500 to 10 ng/mL and 0.500 to 100 ng/mL, respectively. After 24 hours, a gentamicin protection assay was performed as mentioned above. In short, the cells were inoculated with *Salmonella* bacteria (WT or  $\Delta hilA$ ), whether or not grown in LB medium with T-2 toxin at concentrations ranging from 0.5 to 100 ng/mL, at a MOI of 10:1. Subsequently, the cells were washed and fresh medium supplemented with 100  $\mu\text{g/mL}$  gentamicin was added. After one hour, PAM and IPEC-J2 cells were washed 3 times and lysed for 10 min with 1% (v/v) Triton X-100 (Sigma-Aldrich) or 0.2% (w/v) sodium deoxycholate (Sigma-Aldrich), respectively, and 10-fold dilutions were plated on BGA plates.

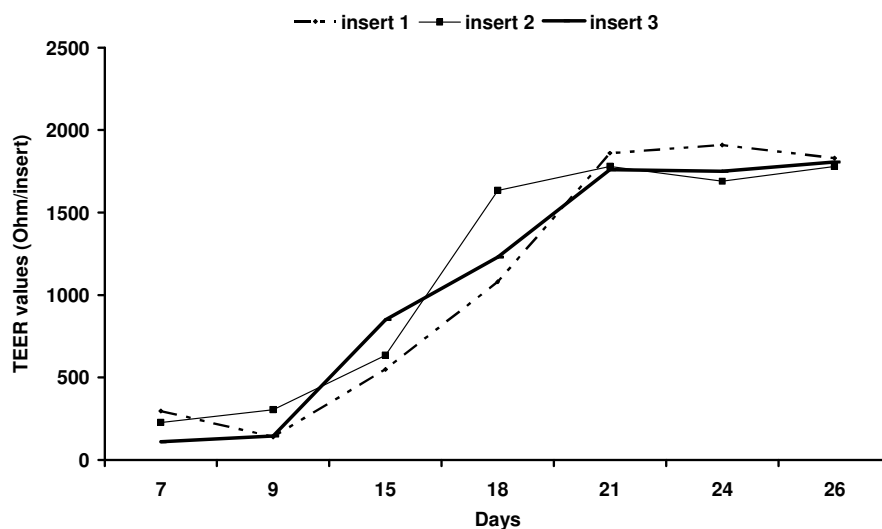
To assess intracellular growth, cells were seeded and inoculated with *Salmonella* Typhimurium as mentioned above, but the medium containing 100  $\mu\text{g/mL}$  gentamicin was replaced after 1 hour incubation with fresh medium containing 20  $\mu\text{g/mL}$  gentamicin, whether or not supplemented with different concentrations of T-2 toxin as described in the invasion assay. The number of viable bacteria was assessed 24 hours after infection.

#### Effect of T-2 toxin on the translocation of *Salmonella* Typhimurium through an intestinal epithelial cell layer

To examine whether T-2 toxin affects the transepithelial passage of *Salmonella* Typhimurium through IPEC-J2 cells, a translocation assay was performed. Prior to seeding IPEC-J2 cells, Transwell<sup>®</sup> polycarbonate membrane inserts with a pore size of 3.0  $\mu\text{m}$  and membrane diameter of 6.5 mm (Corning Costar Corp., Cambridge, MA) were coated using PureCol bovine purified collagen (Inamed Biomaterials, Fremont, California, USA). Collagen working solution was made using a 1:100 dilution of PureCol (2.9 mg/mL) in  $\text{H}_2\text{O}$ . Two hundred  $\mu\text{L}$  of the working collagen solution was added to each transwell and was allowed to air-dry in a laminar flow hood before being exposed to UV radiation for 20 min. After coating, IPEC-J2 cells were seeded on the apical side of these inserts at a density of  $2 \times 10^4$  cells/insert, cell medium was refreshed every 3 days and cells were cultured for 21 days in order to differentiate, which was determined by a preliminary experiment (Figure 1).

After 21 days, 200  $\mu\text{L}$  cell culture medium with T-2 toxin at concentrations of 0.750, 1, 2.5, 4 or 5 ng/mL was added to the apical side, while the basolateral side received 1 mL of

blank culture medium. After 24 hours of treatment with T-2 toxin, the Transwell® inserts were washed three times with HBSS+. Then,  $5 \times 10^6$  CFU of *Salmonella* Typhimurium were added to the apical compartment, suspended in IPEC-J2 medium without antibiotics, but supplemented with the respective concentrations of T-2 toxin. The basolateral compartment was filled with antibiotic-free IPEC-J2 medium. After 15, 30, 45 and 60 min at 37 °C and 5% CO<sub>2</sub>, the number of bacteria (CFU/mL) was determined in the basolateral compartment by plating 10-fold dilutions on BGA plates. In addition, transepithelial electrical resistance (TEER) measurements were performed before and after the incubation with T-2 toxin in order to evaluate the cell barrier integrity. This was done by transferring the inserts to an insert chamber (EndOhm-6, World Precision Instruments, Sarasota, Florida, USA) and measuring the TEER via an epithelial voltohmmeter (World Precision Instruments).



**Figure 1:** The progression of TEER values of IPEC-J2 cells, seeded at a density of  $2 \times 10^4$  cells, on collagen coated Transwell® polycarbonate membrane inserts (pore size =  $3.0 \mu\text{m}$  and membrane diameter =  $6.5 \text{ mm}$ ).

### Effects of T-2 toxin on porcine host cells

In order to elucidate the underlying mechanism of T-2 toxin induced increased invasion and translocation of *Salmonella* Typhimurium in and over porcine host cells, the effects of T-2 toxin on porcine host cells were assessed.

### Cytotoxicity of T-2 toxin towards porcine macrophages and intestinal epithelial cells

In order to determine the toxic character of T-2 toxin on porcine host cells and to determine whether it increases the toxicity of *Salmonella* Typhimurium for these porcine host

cells, the cytotoxicity of T-2 toxin on uninfected PAM and IPEC cells was determined as described in the neutral red assay.

#### Effect of T-2 toxin on porcine enterocyte ultrastructure

Since the invasion assay pointed out that the T-2 toxin induced increased invasion of *Salmonella* Typhimurium was the highest in differentiated IPEC-J2 cells, transmission electron microscopy (TEM) was performed to characterize the effects of T-2 toxin on the ultrastructure of differentiated IPEC-J2 cells. The effect of 5 ng/mL T-2 toxin was investigated because this concentration significantly increases the invasion of the bacterium, without affecting the cell viability.

IPEC-J2 cells were seeded in 24-well plates at a density of approximately  $10^5$  cells per well and were allowed to grow for 21 days. Samples for TEM were collected 24 hours after treatment with 5 ng/mL T-2 toxin or blank medium as a control. After treatment with T-2 toxin, the wells were washed three times with HBSS+, after which the cells were fixed in 4% formaldehyde in a 0.121 M Na-cacodylate buffer (pH 7.4) containing 1% (w/v)  $\text{CaCl}_2$  for 24 h. After fixation, the wells were rinsed and subsequently dehydrated by adding successively 50%, 70%, 90% and 100% ethanol to the wells. Next, the cells were embedded in LX-112 resin (Ladd Research Industries, Burlington, Vermont) and cut with an ultratome (Ultracut E, Reichert Jung, Nussloch, Germany). The sections were examined under a Jeol EX II transmission electron microscope (Jeol, Tokyo, Japan) at 80 kV.

#### Effect of T-2 toxin on the protein expression of porcine enterocytes

Based on the results of the invasion assay, a comparative proteome study was conducted to reveal the effects of 5 ng/mL T-2 toxin on the protein expression of differentiated IPEC-J2 cells. We used a gel-free approach called isobaric tags for relative and absolute quantification (iTRAQ) in which four different isobaric labels are used to tag N-termini and lysine side chains of four different samples with four different isobaric reagents. Upon collision-induced dissociation during MS/MS, the isobaric tags are released, which results in four unique reporter ions that are used to quantify the proteins in the four different samples (Ross et al., 2004).

Sample preparation: IPEC-J2 cells were seeded in  $175 \text{ cm}^2$  cell culture flasks at a density of approximately  $2 \times 10^6$  cells per flask and were allowed to grow for 21 days. Subsequently, IPEC-J2 cells were washed 3 times with HBSS+ and either incubated with 5 ng/mL T-2 toxin

or left untreated. After 24 hours, the cells were washed 3 times with HBSS+ and were scraped off the bottom of the flask using a cell scraper. After washing the cells by centrifugation at 2300 x g for 10 min at 4 °C, they were finally resuspended in 500 µL lysis buffer containing 40 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris, Sigma-Aldrich), a cocktail of protease inhibitors (PIs; Sigma-Aldrich) and phosphatase inhibitors (PPI, Sigma-Aldrich), 172.6 U/mL deoxyribonuclease I (DNase I, Invitrogen, USA), 100 mg/mL ribonuclease A (RNase A, Qiagen, Venlo, The Netherlands) and 2% (v/v) tributylphosphine (TBP, Sigma-Aldrich). The cells were sonicated (6 times 30 sec), using an ultrasonic processor XL 2015 (Misonix, Farmingdale, New York, USA), followed by centrifugation at 17,968 x g for 10 min. The supernatant was held on ice until further use and the pellet was dissolved and sonicated (6 times 30 sec), in reagent 3 of the Ready Prep Sequential extraction kit (Bio-Rad, Hercules, CA, USA). This was centrifuged at 17,968 x g for 10 min. Both supernatants were combined and a buffer switch to 0.01% (w/v) SDS in H<sub>2</sub>O was performed using a Vivaspinn column (5000 molecular weight cut off Hydrosarts, Sartorius, Germany). Protein concentration was determined using the Bradford Protein Assay (Thermo Fisher Scientific, Rockford, USA) according to the manufacturer's instructions.

*Trypsin digest and iTRAQ labeling:* Digest and labeling of the samples (100 µg proteins per sample) with iTRAQ reagents was performed according to the manufacturer's guidelines (AB Sciex, Foster City, CA, USA). Individual samples of T-2 toxin treated or untreated IPEC-J2 cells were analyzed in the same run, making pairwise comparisons possible and minimizing technical variation. Each condition was run in duplicate using different labels of the four-plex labeling kit. The experiment was conducted in twofold and the labeling of the samples was as follows: run 1 (untreated IPEC-J2 cells sample 1: 114 – untreated IPEC-J2 cells sample 2: 115 – treated IPEC-J2 cells sample 1: 116 – treated IPEC-J2 cells sample 2: 117) – run 2 (untreated IPEC-J2 cells sample 3: 114 – untreated IPEC-J2 cells sample 4: 115 – treated IPEC-J2 cells sample 3: 116 – treated IPEC-J2 cells sample 4: 117). After labeling, 6 µL of a 5% (v/v) hydroxylamine solution was added to hydrolyze unreacted label and after incubation at room temperature for 5 min, the samples were pooled, dried and resuspended in 5 mM KH<sub>2</sub>PO<sub>4</sub> (15% (v/v) acetonitrile) (pH 2.7). The combined set of samples was first purified on ICAT SCX cartridges, desalted on a C18 trap column and finally fractionated using SCX chromatography. Each fraction was analyzed by nano LC-MS/MS as described by Bijttebier et al. (2009).

Data analysis: With no full pig protein database available, different search parameters and databases, both EST and protein, were validated to obtain maximum spectrum annotation. Best results (39% of spectra annotated above homology threshold with a 3.71% false discovery rate in the decoy database) were obtained when searching NCBI Mammalia. For quantification, data quality was validated using ROVER (Colaert et al., 2011). Based on this validation a combined approach was used to define recurrently different expression patterns. In a first approach, the four ratios that can be derived from each run (114/116, 115/117, 114/117 and 115/116) were log-transformed and a t-test was used to isolate protein ratios significantly different from 0 in each run. In a second approach, the two runs were merged into one file and the 114/116 and 115/117 ratios of each run were log-transformed and these ratios were multiplied (log\*log). Proteins with recurrent up- or downregulation result in positive log\*log protein ratios and those > 0.01 were retained and listed. Proteins that were present in both lists were considered unequivocally differentially expressed. This combined approach allows defining proteins with relatively low, but recurrent expressional differences.

### **Effect of T-2 toxin on the growth, gene expression and motility of *Salmonella* Typhimurium**

Not only porcine host cells, but also *Salmonella* bacteria come in contact with T-2 toxin. Therefore, it is possible that T-2 toxin affects the bacterium and by doing so, alters the pathogenesis of a *Salmonella* Typhimurium infection in pigs.

#### Effect of T-2 toxin on the gene expression of *Salmonella* Typhimurium

To test whether T-2 toxin affected the gene expression of *Salmonella* Typhimurium, a microarray analysis was performed on RNA isolated from cultures of *Salmonella* Typhimurium grown for 5 hours to a logarithmic phase in the presence or absence of 5 ng/mL of T-2 toxin.

Two OD600 units were harvested and RNA was extracted and purified using the 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 T-2 toxin treated and untreated (control)



logarithmic 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 T-2 toxin treated and untreated controls by an analysis of variance test with a Benjamini and Hochberg false discovery rate of 0.05 and with a 1.5-fold change in the expression level. 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 GSE30925 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30925>).

#### Effect of T-2 toxin on the growth of *Salmonella* Typhimurium

The effect of T-2 toxin (0, 0.04, 0.31, 2.5 and 20 µg/mL) on the growth of *Salmonella* Typhimurium was examined during 24 hours. For this purpose, *Salmonella* Typhimurium was grown in LB broth whether or not supplemented with T-2 toxin. The number of CFU/mL was determined at different time points (t = 0, 3, 6, 8 and 24 hours) by titration of 10-fold dilutions of the bacterial suspensions on BGA.

#### Effect of T-2 toxin on the motility of *Salmonella* Typhimurium

One µL of an overnight culture of *Salmonella* Typhimurium was spotted in the middle of a swim plate (Difco Nutrient Broth (Becton, Dickinson and Company, Sparks, USA), 0.5% (w/v) glucose, 0.25% agar), whether or not supplemented with T-2 toxin (0, 100, 500, 1000 ng/mL). The plates were allowed to dry for 1 hour at room temperature, after which they were incubated at 37 °C for 16 hours.

### **Statistical Analysis**

All *in vitro* experiments were conducted in triplicate with 3 repeats per experiment, unless otherwise stated. All statistical analyses were performed using SPSS version 19 (SPSS Inc., Chicago, IL, USA). Normally distributed data were analyzed using unpaired Student's t-test or one-way ANOVA to address the significance of difference between mean values with significance set at  $p \leq 0.05$ . Bonferroni as post hoc test was used when equal variances were assessed. If equal variances were not assessed, the data were analyzed using Dunnett's T3 test. Not normally distributed data were analyzed using the non parametric Kruskal-Wallis analysis, followed by a Mann-Whitney U test.

## Results

### T-2 toxin decreases the amount of *Salmonella* Typhimurium bacteria present in the cecal contents of pigs and causes a reduction in weight gain

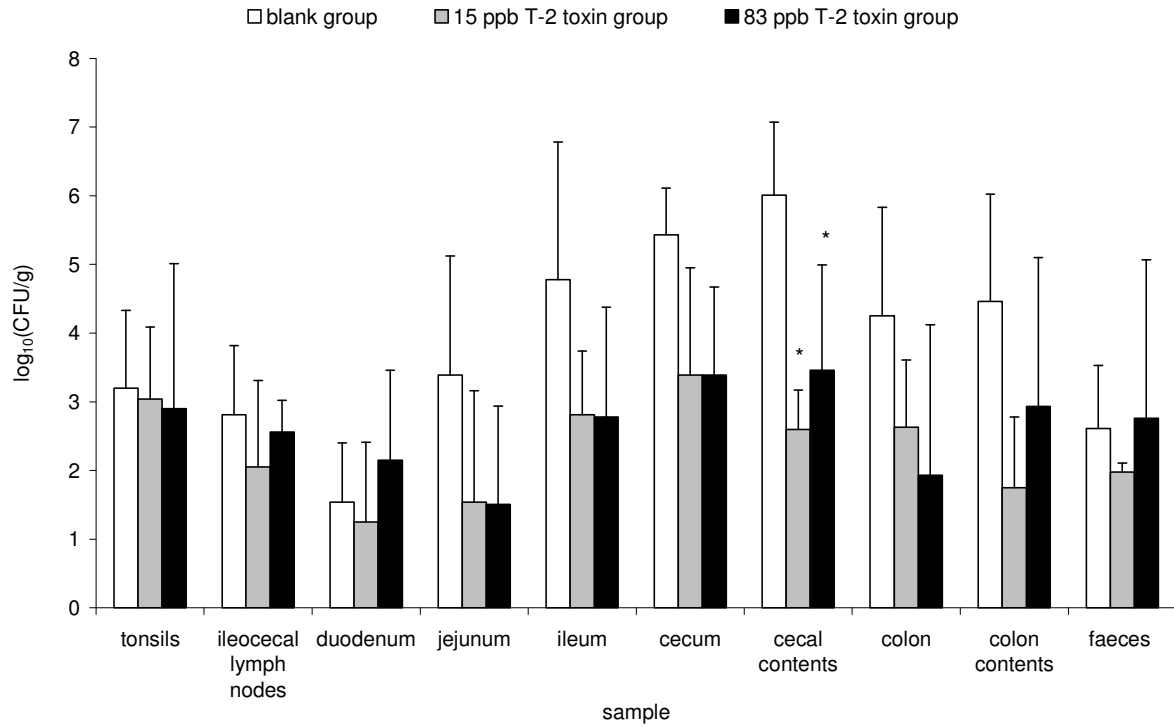
The presented study shows the effects of low and relevant concentrations of T-2 toxin on the course of a *Salmonella* Typhimurium infection in pigs. Animals that received feed contaminated with 15 µg or 83 µg T-2 toxin per kg feed for 23 days had lower numbers of *Salmonella* Typhimurium per gram in their bowel contents and organs in comparison to the control group that received non-contaminated feed for 23 days. As illustrated in Figure 2, this decrease was significant in the cecal contents for both the 15 ppb and 83 ppb group ( $p = 0.001$  and  $p = 0.011$ , respectively). A tendency to reduced colonization of the jejunum, ileum, cecum, colon and colon contents was noticed, although not significantly.

As shown in Table 3, the addition of 83 µg T-2 toxin per kg feed resulted in a significantly reduced weight gain in comparison to the control group that had a mean weight gain (%)  $\pm$  standard deviation, during 18 days, of  $102.1 \pm 17.3$  ( $p = 0.016$ ). Pigs that were fed 15 or 83 µg T-2 toxin per kg feed, for 18 days had a mean weight gain (%)  $\pm$  standard deviation of  $104.4 \pm 14.2$  and  $70.9 \pm 11.3$ , respectively. This corresponds to a mean weight gain  $\pm$  standard deviation of  $0,326 \pm 0,08$  kg per day for the control group,  $0,322 \pm 0,08$  kg per day for the 15 ppb group and  $0,239 \pm 0,04$  kg per day for the 83 ppb group.

**Table 3:** Distribution of the sexes of the pigs that received, during 18 days, blank feed (control group), feed contaminated with 15 µg T-2 toxin per kg feed (15 ppb group) or feed contaminated with 83 µg T-2 toxin per kg feed (83 ppb group), their respective weight at the beginning of the experiment and their average weight gain.

		distribution of sexes	weight at beginning of the experiment (kg)	Average weight gain per group (kg/day)	Average weight gain during 18 days per group (%)
control group	piglet 1	female	6,5	0.326 $\pm$ 0.08	102 $\pm$ 17.3
	piglet 2	female	5,5		
	piglet 3	female	5,5		
	piglet 4	male	5,0		
	piglet 5	male	6,0		
15 ppb group	piglet 6	female	4,5	0.322 $\pm$ 0.08	104 $\pm$ 14.2
	piglet 7	male	7,0		
	piglet 8	female	5,0		
	piglet 9	male	4,5		
	piglet 10	male	7,0		
83 ppb group	piglet 11	female	5,5	0.239 $\pm$ 0.04	70.9 $\pm$ 11.3*
	piglet 12	male	7,5		
	piglet 13	female	6,0		
	piglet 14	male	6,5		
	piglet 15	male	5,0		

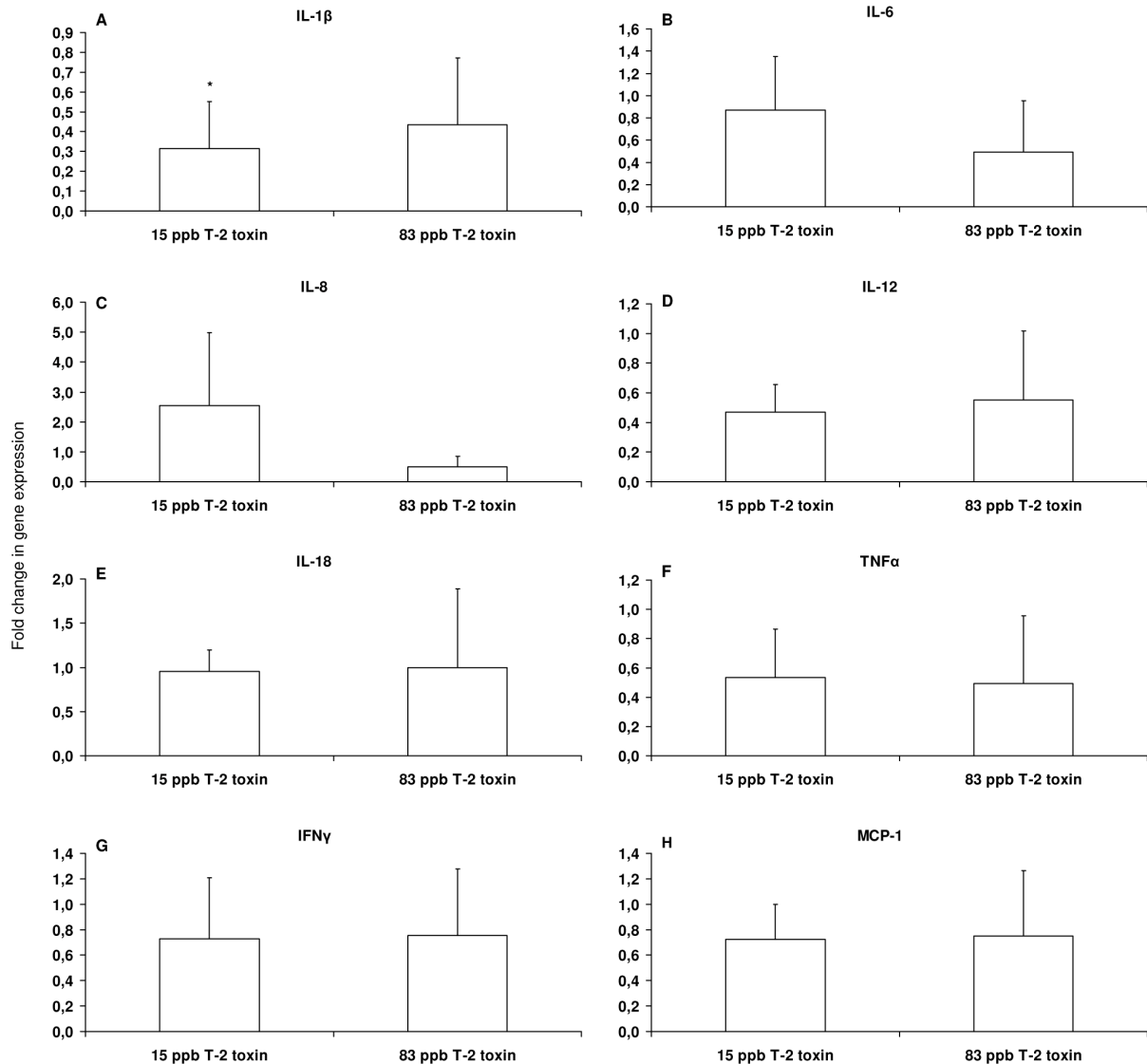
Superscript (\*) refers to a significant difference compared to the control group ( $p < 0.05$ ).



**Figure 2: Effect of T-2 toxin on the colonization by *Salmonella Typhimurium* in pigs.** Recovery of *Salmonella Typhimurium* bacteria from various organs and gut contents of pigs that received, during 23 days, blank feed (control group, white bars), feed contaminated with 15 µg T-2 toxin per kg feed (15 ppb group, grey bars) or feed contaminated with 83 µg T-2 toxin per kg feed (83 ppb group, black bars), respectively. Five days after inoculation with  $2 \times 10^7$  CFU of *Salmonella Typhimurium*, the pigs (n = 5) were euthanized and the log<sub>10</sub> value of the ratio of CFU per gram sample is given as the mean + standard deviation. Superscript (\*) refers to a significant difference compared to the control group (p < 0.05).

### The addition of T-2 toxin (15 µg/kg) to the feed caused a decreased expression of IL-1β

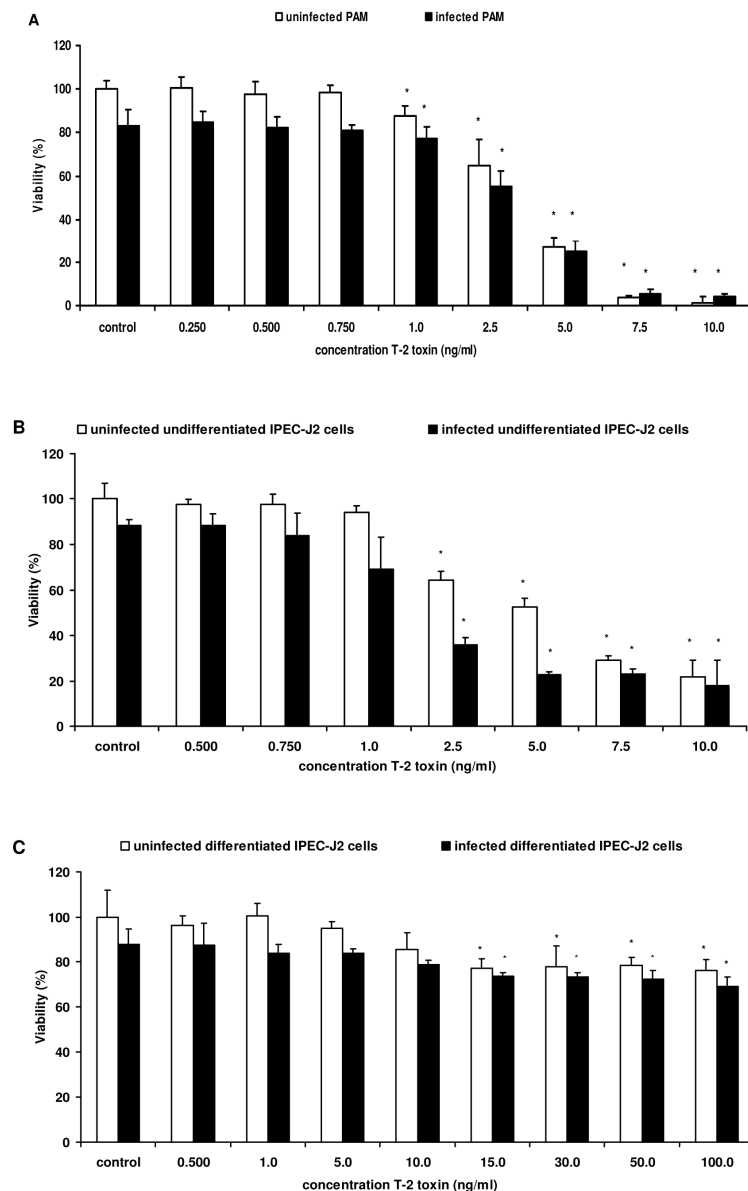
The effect of a 23 day feeding period with 15 µg or 83 µg of T-2 toxin per kg feed on the intestinal mRNA expression levels of the cytokines (IL-1β, IL-6, IL-12/IL-23p40, IL-18, IFNγ and TNFα) and chemokines (IL-8 and MCP-1) was examined 5 days post inoculation with *Salmonella* Typhimurium. The results are illustrated in Figure 3. For IL-1β, a significant decreased fold change was noticed in pigs exposed to 15 µg T-2 toxin per kg feed compared to the *Salmonella* Typhimurium positive control pigs ( $p = 0.027$ ).



**Figure 3: Effect of T-2 toxin on the intestinal inflammatory response.** Fold change in cytokine gene expression of the porcine ileum of *Salmonella* Typhimurium positive pigs that received feed contaminated with 15 µg T-2 toxin per kg feed (15 ppb T-2 toxin) or feed contaminated with 83 µg T-2 toxin per kg feed (83 ppb T-2 toxin), relative to *Salmonella* Typhimurium positive pigs that received blank feed (control group), during 23 days. Five days after inoculation with  $2 \times 10^7$  CFU of *Salmonella* Typhimurium, the pigs ( $n = 5$ ) were euthanized and the cytokine gene expression levels (A: IL-1β, B: IL-6, C: IL-8, D: IL-12/IL-23p40, E: IL-18, F: TNFα, G: IFNγ and H: MCP-1) were determined. The data represent the normalized target gene amount relative to the control group which is considered 1. The results are presented as means + standard deviation for a total of 5 pigs per test condition. Superscript (\*) refers to a significant difference compared to the control group ( $p < 0.05$ ).

### T-2 toxin is cytotoxic to porcine macrophages and intestinal epithelial cells

The cytotoxic effect of T-2 toxin on PAM, undifferentiated and differentiated IPEC-J2 cells as determined using the neutral red assay, is shown in Figure 4. The viability of both uninfected and infected PAM, undifferentiated and differentiated IPEC-J2 cells was significantly decreased by exposure to concentrations of T-2 toxin  $\geq 1$  ng/mL,  $\geq 2.5$  ng/mL and  $\geq 15$  ng/mL, respectively. IC50 values of T-2 toxin for the different cell types were determined by linear regression and are presented in Table 4.



**Figure 4: The effect of T-2 toxin on the cell viability.** Percentage viability (%) of *Salmonella* Typhimurium infected and uninfected (A) PAM exposed to different concentrations of T-2 toxin (0.250-10 ng/mL), (B) undifferentiated IPEC-J2 cells exposed to different concentrations of T-2 toxin (0.500-10 ng/mL), (C) differentiated IPEC-J2 cells exposed to different concentrations of T-2 toxin (0.500-100 ng/mL). Twenty-four hours after incubation with T-2 toxin, the cytotoxic effect was determined by neutral red assay. Results represent the means of 3 independent experiments conducted in triplicate and their standard deviation. Superscript (\*) refers to a significant difference compared to the control group ( $p < 0.05$ ).

**Table 4:** IC50 values of T-2 toxin for PAM, undifferentiated and differentiated IPEC-J2 cells, either or not infected with *Salmonella* Typhimurium.

Cell type	T-2 toxin concentration (ng/mL)
Uninfected PAM	4.3
Infected PAM	4.4
Uninfected undifferentiated IPEC-J2 cells	5.7
Infected undifferentiated IPEC-J2 cells	4.7
Uninfected differentiated IPEC-J2 cells	185
Infected differentiated IPEC-J2 cells	212.8

### Treatment of porcine macrophages and intestinal epithelial cells with T-2 toxin promotes the invasion of *Salmonella* Typhimurium

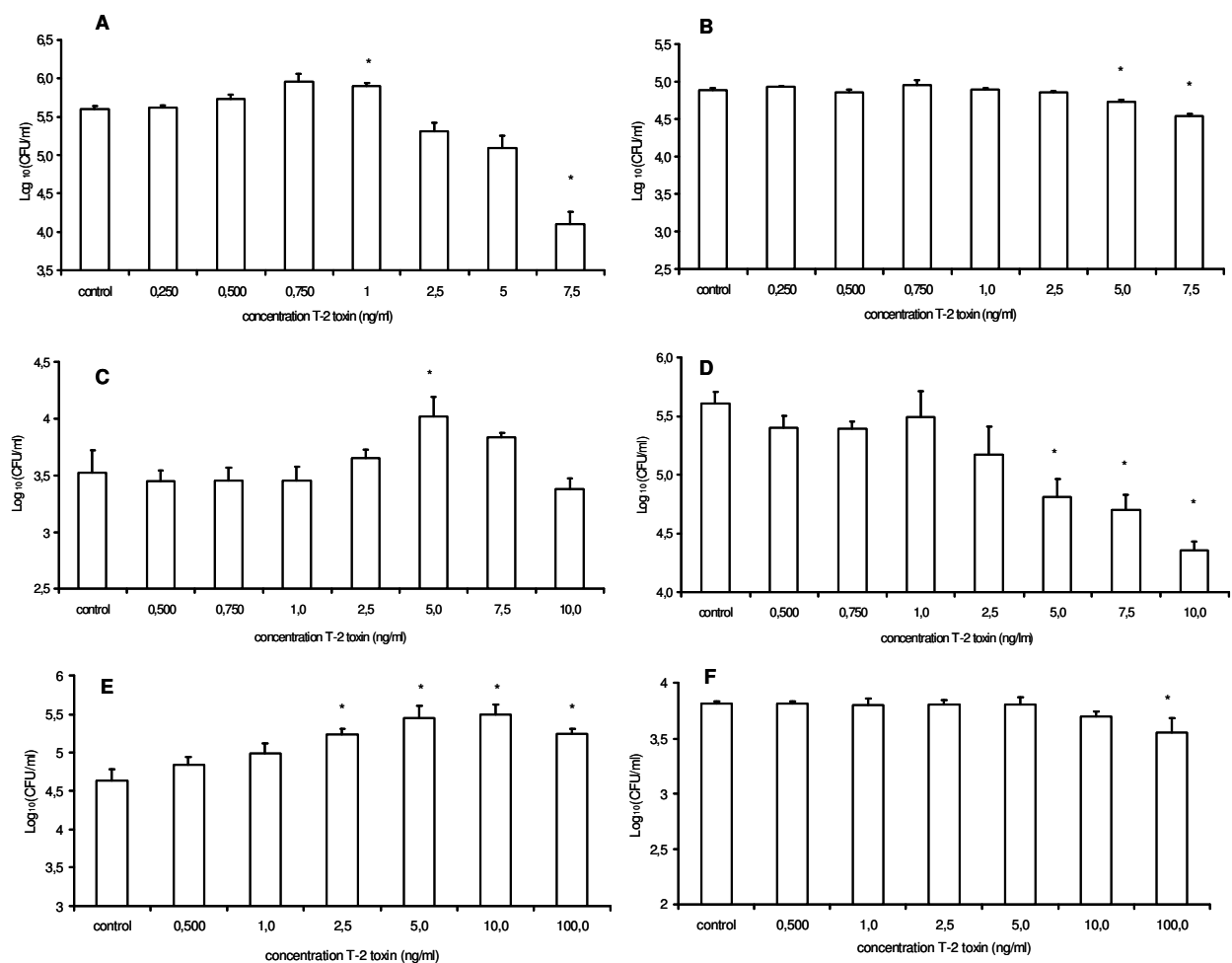
Altered host-pathogen interactions between *Salmonella* Typhimurium and porcine host cells could account for the reduced numbers of *Salmonella* Typhimurium present in the cecal contents. Therefore, the effect of T-2 toxin on host-pathogen interactions was investigated. The results of the invasion and intracellular survival assays of *Salmonella* Typhimurium in PAM, undifferentiated and differentiated IPEC-J2 cells with or without exposure to T-2 toxin are summarized in Figure 5.

The invasion of *Salmonella* Typhimurium was higher in PAM, undifferentiated and differentiated IPEC-J2 cells that were treated with T-2 toxin, for 24 hours, in comparison to non-treated cells. Exposure of PAM, undifferentiated and differentiated IPEC-J2 cells to T-2 toxin concentrations of 1, 5 and  $\geq 2.5$  ng/mL, respectively, led to a significant increase in the number of intracellular *Salmonella* Typhimurium bacteria. Due to the toxicity of T-2 toxin, exposure of PAM to T-2 toxin concentrations  $\geq 7.5$  ng/mL, resulted in a significant decrease in the number of intracellular bacteria. As shown in Figure 6, similar results were obtained using the deletion mutant  $\Delta hila$ , where a significant increased invasion was seen at T-2 toxin concentrations  $\geq 5$  ng/mL.

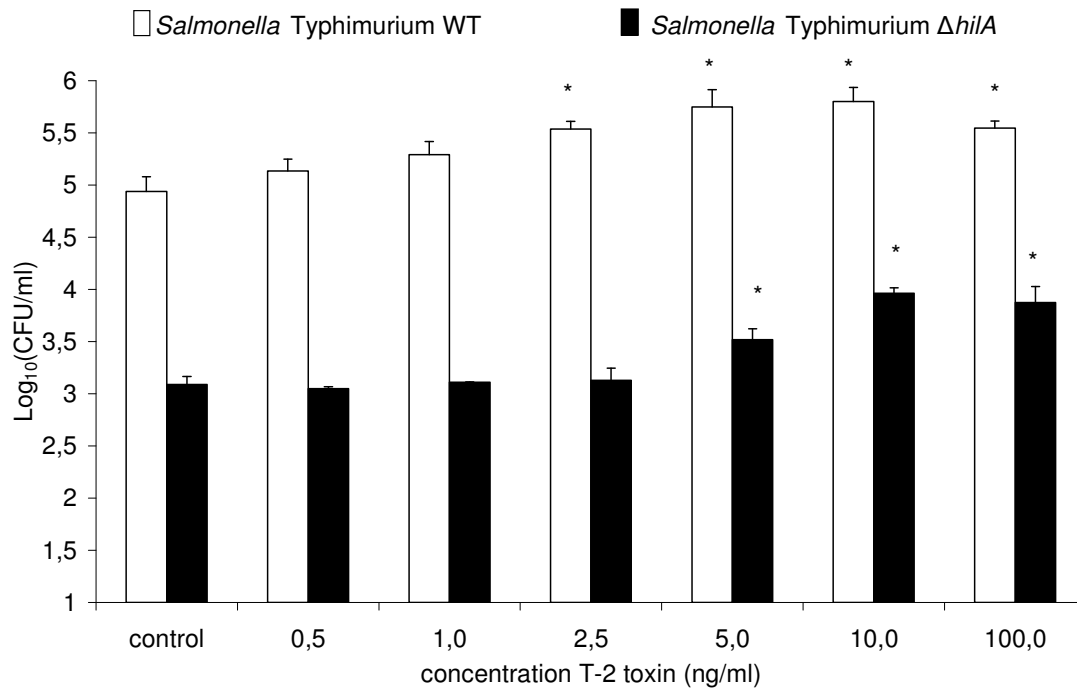
A 24 hour treatment of *Salmonella* infected PAM, undifferentiated and differentiated IPEC-J2 cells with non-cytotoxic concentrations of T-2 toxin, did not affect the intracellular proliferation of *Salmonella* Typhimurium in these cells. However, treatment with toxic concentrations of T-2 toxin resulted in a significantly decreased survival of *Salmonella* Typhimurium in PAM and undifferentiated IPEC-J2 cells at T-2 toxin concentrations  $\geq 5$  ng/mL and in differentiated IPEC-J2 cells at concentrations  $\geq 100$  ng/mL T-2 toxin.

### T-2 toxin promotes the transepithelial passage of *Salmonella* Typhimurium through the intestinal epithelium

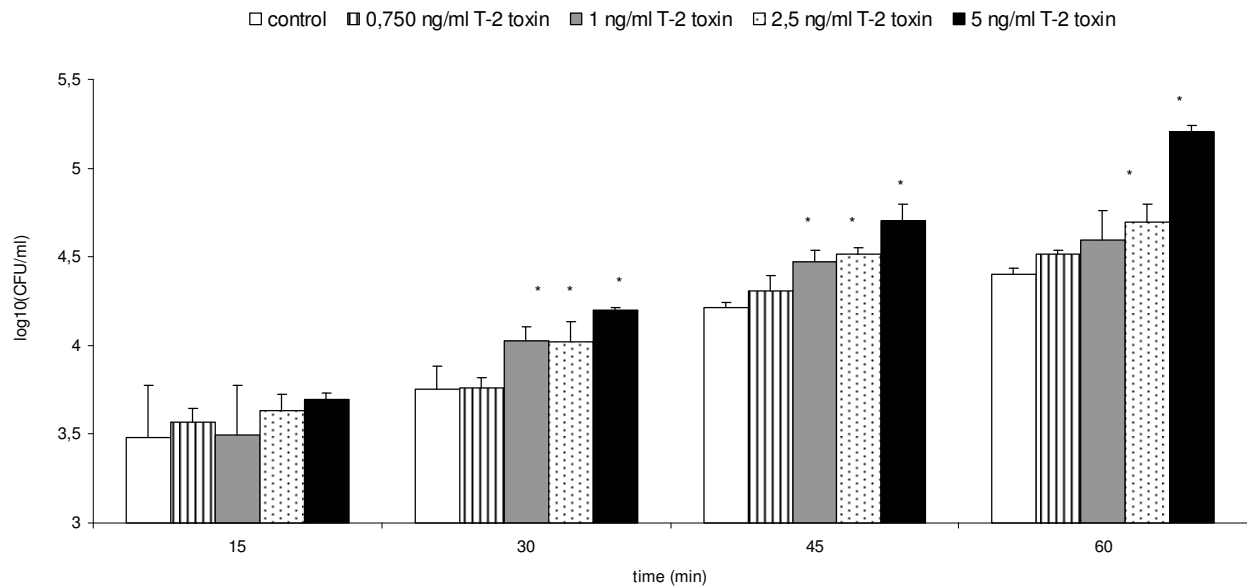
The passage of *Salmonella* Typhimurium through 21-days-old IPEC-J2 cells treated for 24 hours with non-cytotoxic concentrations of T-2 toxin varying from 0.750 to 5 ng/mL is shown in Figure 7. Already after 30 minutes, treatment of the IPEC-J2 cell monolayer with T-2 toxin concentrations  $\geq 1$  ng/mL resulted in a significant increase in the number of translocated bacteria in comparison to non-treated IPEC-J2 cells. Exposure to concentrations of T-2 toxin varying from 0.750 to 5 ng/mL, for 24 hours, did not lead to a decrease in TEER (Table 5) indicating no loss of integrity of the epithelial monolayer and suggesting an increased transcellular passage of the bacteria.



**Figure 5: Effect of T-2 toxin treatment of porcine cells on the invasion and intracellular proliferation of *Salmonella* Typhimurium.** The invasiveness is shown of *Salmonella* Typhimurium in (A) PAM, (C) undifferentiated and (E) differentiated IPEC-J2 cells whether or not exposed to different concentrations of T-2 toxin (0.250-7.5, 0.500-10 or 0.500-100 ng/mL respectively). The survival of *Salmonella* Typhimurium, 24 hours after invasion in (B) PAM, (D) undifferentiated and (F) differentiated IPEC-J2 cells whether or not exposed to different concentrations of T-2 toxin (0.250-7.5, 0.500-10 or 0.500-100 ng/mL respectively) is given. The log<sub>10</sub> values of the number of gentamicin protected bacteria + standard deviation are given. Results are presented as a representative experiment conducted in triplicate. Superscript (\*) refers to a significant difference compared to the control group (p < 0.05).



**Figure 6: Effect of T-2 toxin treatment of differentiated IPEC-J2 cells, on the invasion of *Salmonella* Typhimurium WT and  $\Delta$ hilA.** The invasiveness is shown of *Salmonella* Typhimurium WT (white bars) and *Salmonella* Typhimurium  $\Delta$ hilA (black bars) in differentiated IPEC-J2 cells whether or not exposed to different concentrations of T-2 toxin (0.500-100 ng/mL). The log<sub>10</sub> values of the number of gentamicin protected bacteria + standard deviation are given. Results are presented as a representative experiment conducted in triplicate. Superscript (\*) refers to a significant difference compared to the control group (p < 0.05).



**Figure 7: The influence of T-2 toxin treatment of an on IPEC-J2 monolayer on the transepithelial passage of *Salmonella* Typhimurium.** IPEC-J2 cells seeded onto inserts for 21 days until differentiation were either exposed to blank medium or treated with different concentrations of T-2 toxin (0.750, 1, 2.5 or 5 ng/mL) for 24 h, prior to measuring the transepithelial passage of *Salmonella* Typhimurium. The translocation of the bacteria was measured 15, 30, 45 and 60 minutes after inoculation. Results are presented as a representative experiment conducted in triplicate. Superscript (\*) refers to a significantly higher translocation of the bacteria compared to the unexposed control wells (p < 0.05).



**Table 5:** TEER values of IPEC-J2 cells, 21 days after seeding them at a density of  $2 \times 10^4$  cells, on collagen coated Transwell® polycarbonate membrane inserts. After 21 days, the cells were exposed to different concentrations of T-2 toxin ranging from 0 to 5 ng/mL, during 24 hours.

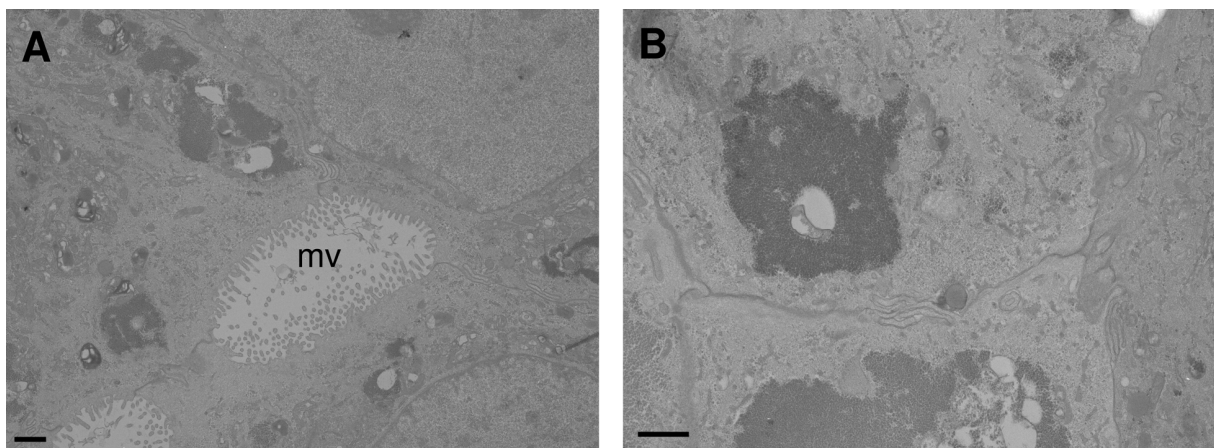
TEER values	At day 21 (Ohm/insert)	At day 22 (Ohm/insert)
control 1	1650	1666
control 2	1430	1532
control 3	1510	1536
0.750 ng/mL T-2 toxin 1	1320	1465
0.750 ng/mL T-2 toxin 2	1489	1502
0.750 ng/mL T-2 toxin 3	1399	1466
1.0 ng/mL T-2 toxin 1	1678	1674
1.0 ng/mL T-2 toxin 2	1723	1836
1.0 ng/mL T-2 toxin 3	1985	2010
2.5 ng/mL T-2 toxin 1	1328	1401
2.5 ng/mL T-2 toxin 2	1504	1506
2.5 ng/mL T-2 toxin 3	1863	1935
5 ng/mL T-2 toxin 1	1652	1702
5 ng/mL T-2 toxin 2	1423	1536
5 ng/mL T-2 toxin 3	1489	1497

### **T-2 toxin affects the protein expression of differentiated IPEC-J2 cells at a concentration that does not cause morphological changes**

In order to elucidate the possible mechanism of the T-2 toxin increased invasion in differentiated IPEC-J2 cells, iTRAQ analysis was performed on T-2 toxin treated porcine cells. Based on the invasion assay results, we opted to investigate the effect of T-2 toxin at a concentration of 5 ng/mL on differentiated IPEC-J2 cells. Peptides from trypsin digested proteins were labeled with isobaric mass tag labels and analyzed by 2-D LC-MS/MS. Collision-induced dissociation results in the release of these isobaric tags, which allows relative quantification of the peptides. A broad comparison between 5 ng/mL T-2 toxin treated and untreated differentiated IPEC-J2 cells, resulted in the identification of 21 proteins with relatively low, but recurrent expressional differences, as shown in Table 6. Eight of these proteins showed higher levels in untreated IPEC-J2 cells, whereas 13 of them were more abundant in T-2 toxin treated IPEC-J2 cells.

Proteomic analysis established a T-2 toxin induced upregulation of predicted nucleolin-related protein isoform 3 which is involved in ribosome biogenesis (Ginisty et al., 1999), elongation factor 1-beta which is involved in protein synthesis, peptidyl-prolyl cis-trans isomerise which is essential for protein folding (Hayano et al., 1991; Carr-Schmid et al., 1999) and glutathione S-transferase P which is an inhibitor for the c-Jun N-terminal kinase signalling (Wang et al., 2001). Furthermore, T-2 toxin increased the expression of pre-mRNA splicing factor heterogeneous nuclear ribonucleoprotein F, 14-3-3 sigma, branched-chain-

amino-acid aminotransferase, heat shock protein 60, heat shock protein 10 and thioredoxin-related transmembrane protein 1, highlighting the toxic character of 5 ng/mL T-2 toxin. In contrast, T-2 toxin caused a decreased expression of proteins involved in membrane functions, mitochondrial proteins and endoplasmic reticulum (ER) related proteins, namely annexin A4, cytochrome c oxidase subunit VIIc, chain A mitochondrial F1-ATPase complexed with aurovertin B, S-transferase 3 and S100-A16. These are involved in membrane bilayer function (Li et al., 2003), mitochondrial electron transport (Iwaashi et al., 2008), adenosine triphosphate (ATP) production (Van Raaij, et al., 1996), the cellular defense against oxygen-free radicals (Thameem et al., 2003) or  $\text{Ca}^{2+}$  homeostasis, cell proliferation, migration, differentiation, apoptosis and transcription (Heizmann et al., 2002; Sturchler et al., 2006), respectively. Moreover, T-2 toxin affects the expression of cytoskeleton associated proteins. It causes a decreased expression of cytokeratin 18, myristoylated alanine-rich C-kinase substrate and putative beta-actin and an increased expression of thymosin beta-10, cysteine and glycine-rich protein 1 isoform 1 and profilin. Generally, these data showed that even a low concentration of 5 ng/mL T-2 toxin damages the porcine enterocyte and affects cytoskeletal proteins. TEM pointed out that these changes in protein expression are not correlated with morphological changes (Figure 8).



**Figure 8: The effect of T-2 toxin on the morphology of differentiated IPEC-J2 cells.** Transmission electron micrographs of differentiated IPEC-J2 cells fixed 24 hours after exposure to (A) control medium or (B) 5 ng/mL T-2 toxin. These pictures serve as a representative for a confluent monolayer of IPEC-J2 cells and no differences were seen on the ultrastructure of T-2 toxin (5 ng/mL) treated IPEC-J2 cells in comparison to untreated cells. Scale bar = 1  $\mu\text{M}$ ; mv = microvilli.

**Table 6: Differential protein expression of differentiated IPEC-J2 cells after exposure to T-2 toxin.**

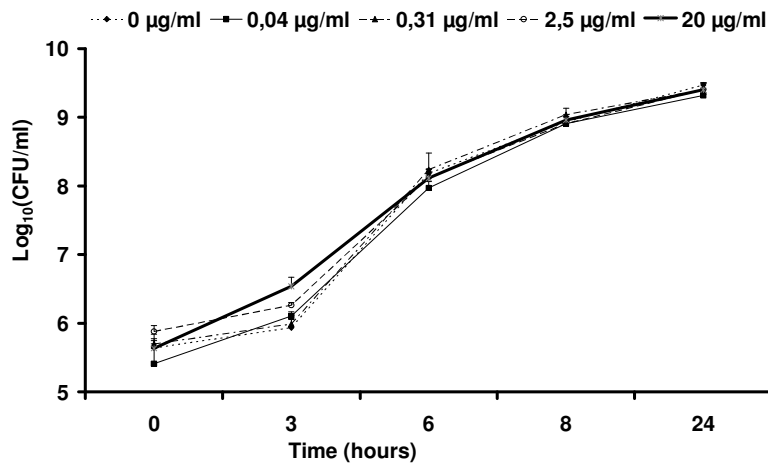
Protein name	Function*	Protein ratio treated/untreated IPEC-J2 cells on the t-test approach	Protein ratio treated/untreated IPEC-J2 cells on the log*log approach
Cytochrome c oxidase subunit VIIc {N-terminal}	This protein is one of the nuclear-coded polypeptide chains of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport.	0.6	0.6
Microsomal glutathione S-transferase 3	Functions as a glutathione peroxidase.	0.6	0.6
PREDICTED: similar to Keratin, type I cytoskeletal 18 (Cytokeratin 18)	When phosphorylated, plays a role in filament reorganization.	0.7	0.7
Myristoylated alanine-rich C-kinase substrate	Myristoylated alanine-rich C-kinase substrate is a filamentous (F) actin cross-linking protein.	0.7	0.7
Annexin A4	Calcium/phospholipid-binding protein which promotes membrane fusion and is involved in exocytosis.	0.7	0.7
Chain A, Bovine Mitochondrial F1-ATPase Complexed With Aurovertin B	Mitochondrial membrane ATP synthase (F <sub>1</sub> F <sub>0</sub> ATP synthase or Complex V) produces ATP from ADP in the presence of a proton gradient across the membrane.	0.7	0.8
Protein S100-A16	Calcium-binding protein. Binds one calcium ion per monomer.	0.8	0.8
Putative beta-actin	Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously expressed in all eukaryotic cells.	0.8	0.7
Cysteine and glycine-rich protein 1 isoform 1	Encodes a member of the cysteine-rich protein (CSRP) family that includes a group of LIM domain proteins, which may be involved in regulatory processes important for development and cellular differentiation.	1.2	1.2
Heat shock protein 60	Implicated in mitochondrial protein import and macromolecular assembly.	1.2	1.2
PREDICTED: similar to nucleolin-related protein isoform 3	Plays a role in different steps in ribosome biogenesis.	1.2	1.2
Heterogeneous nuclear ribonucleoprotein F	Component of the heterogeneous nuclear ribonucleoprotein (hnRNP) complexes which provide the substrate for the processing events that pre-mRNAs undergo before becoming functional, translatable mRNAs in the cytoplasm.	1.2	1.2
Heat shock protein 10	Essential for mitochondrial protein biogenesis, together with chaperonin 60.	1.2	1.2
Thymosin beta-10	Binds to and sequesters actin monomers (G actin) and therefore inhibits actin polymerization.	1.2	1.2
Thioredoxin-related transmembrane protein 1	May participate in various redox reactions.	1.3	1.3
Glutathione S-transferase P	Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles.	1.3	1.3
14-3-3 protein sigma	Adapter protein implicated in the regulation of a large spectrum of both general and specialized signalling pathway. of G2/M progression.	1.3	1.3
Elongation factor 1-beta	Elongation factor 1-beta and Elongation factor 1-delta stimulate the exchange of GDP bound to Elongation factor 1-alpha to GTP.	1.3	1.3
Profilin	Binds to actin and affects the structure of the cytoskeleton.	1.5	1.5
Cyclophilin A or Peptidyl-prolyl cis-trans isomerase A	Peptidyl-prolyl isomerase accelerates the folding of proteins.	1.6	1.6
Branched-chain-amino-acid aminotransferase, cytosolic	Catalyzes the first reaction in the catabolism of the essential branched chain amino acids leucine, isoleucine, and valine.	1.6	1.6

Differentially expressed proteins identified in T-2 toxin (5 ng/mL) treated IPEC-J2 cells in comparison to untreated IPEC-J2 cells by use of iTRAQ analysis coupled to 2-D LC-MS/MS. Superscript (\*) refers to the protein description according to the UniProtKB/Swiss-Prot protein sequence database (<http://expasy.org/sprot/>).

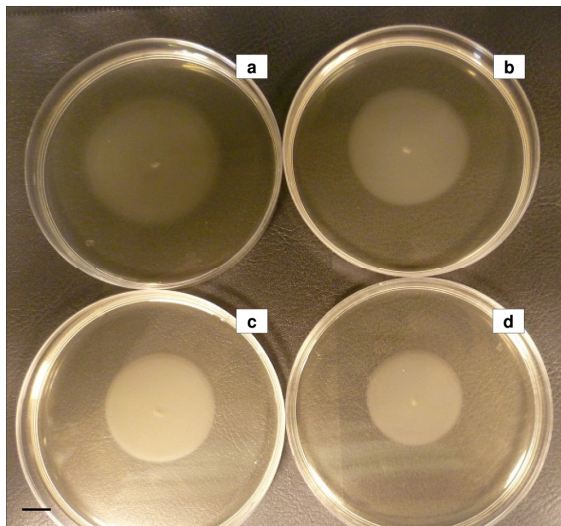
**T-2 toxin does not affect the growth, but causes a downregulation of metabolic genes, decreases the motility and invasiveness of *Salmonella* Typhimurium, resulting in a stressed bacterium**

Preliminary experiments showed that T-2 toxin up to 20 µg/mL had no observable effect on the growth of *Salmonella* Typhimurium (Figure 9). In order to look further for any possible effects of T-2 toxin on *Salmonella* Typhimurium, a microarray study was carried out with RNA isolated from logarithmic phase cultures grown for 5 hours in the presence or absence of 5 ng/mL T-2 toxin. It was found that expression of 262 genes was repressed and 352 genes induced following exposure to T-2 toxin ([http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE30925](http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE30925)). In general, exposure of *Salmonella* Typhimurium to T-2 toxin resulted in a small but significant reduction in the expression of key metabolic genes including 8 glycolytic genes, and genes encoding cytochrome o and d terminal oxidases, succinate dehydrogenase, NADH dehydrogenase and ATP synthase. Similarly, T-2 toxin exposure resulted in reduced expression of genes encoding both 30S and 50S ribosomal proteins. In addition, it was noted that expression of 5 flagella biosynthesis genes was reduced as was expression of 16 of the *Salmonella* Pathogenicity Island 1 (SPI-1) genes. Consistent with the observed reduction in flagella gene expression, motility of *Salmonella* Typhimurium on swarm plates was found to be reduced by T-2 toxin in a dose dependent manner, however at concentrations of T-2 toxin  $\geq 100$  ng/mL (Figure 10). Furthermore, in line with the reduced expression of the *Salmonella* SPI-1 genes, concentrations of T-2 toxin  $\geq 10$  ng/mL significantly decreased the invasion capacity of *Salmonella* Typhimurium in differentiated IPEC-J2 cells (Figure 11A). However, if both *Salmonella* bacteria and differentiated IPEC-J2 cells are exposed to T-2 toxin ( $\geq 2.5$  ng/mL), increased invasion in differentiated IPEC-J2 cells was observed (Figure 11B).

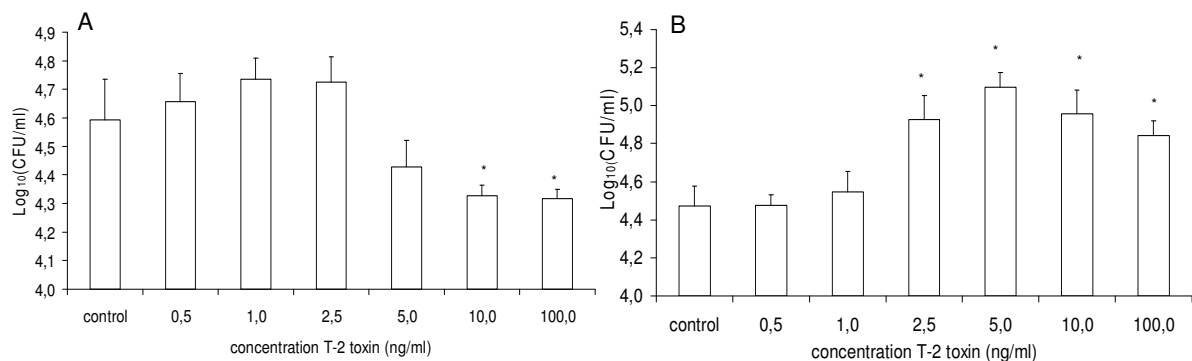
Of the genes found to be upregulated following T-2 toxin exposure many (36) were related to cell-envelope and outer membrane biogenesis suggesting that the toxin may cause membrane or cell wall damage. Expression of 61 genes involved in signal transduction and transcription was increased, suggesting the bacteria were undergoing a global stress response to deal with the toxic insult. Consistent with this, both the *emrAB* and *bcr* multidrug efflux systems and the *marAB* multi-antibiotic efflux system were upregulated as were several other detoxification systems and the *yehYXW* proline/glycine betaine transport systems involved in osmoprotection. Overall the transcriptomic data reveals a bacterium under stress, up-regulating stress response systems and downregulating its metabolic functions.



**Figure 9:** Effect of T-2 toxin on the growth of *Salmonella* Typhimurium in LB broth. The log<sub>10</sub> values of the CFU/mL + standard deviation are given at different time points (t = 0, 2.5, 5, 7.5, 24 hours). *Salmonella* Typhimurium growth was examined in LB medium, whether or not supplemented with T-2 toxin (0.04-20 µg/mL). Results are presented as a representative experiment conducted in triplicate.



**Figure 10:** Effect of T-2 toxin on the swarming capacity of *Salmonella* Typhimurium. Swarming capacity of *Salmonella* Typhimurium after overnight incubation at 37 °C on semi-solid agar plates supplemented with (a) 0 ng/mL T-2 toxin, (b) 100 ng/mL T-2 toxin, (c) 500 ng/mL T-2 toxin, or (d) 1000 ng/mL T-2 toxin. The diameter of the circle is a measure for the motility of the bacteria. Scale bar = 1 cm.



**Figure 11:** The influence of T-2 toxin treatment of differentiated IPEC-J2 cells and/or *Salmonella* Typhimurium bacteria, on the invasion in these cells. The invasiveness is shown of *Salmonella* Typhimurium bacteria grown for 5 hours in LB medium with T-2 toxin (0.500-100 ng/mL), in (A) untreated differentiated IPEC-J2 cells and (B) T-2 toxin (0.500-100 ng/mL) treated differentiated IPEC-J2 cells, for 24 hours. The log<sub>10</sub> values of the number of gentamicin protected bacteria + standard deviation are given. Results are presented as a representative experiment conducted in triplicate. Superscript (\*) refers to a significant difference compared to the control group (p < 0.05).

## Discussion

The ingestion of 83  $\mu\text{g}$  T-2 toxin per kg feed by pigs resulted in a significant reduction of weight gain, compared to control pigs that received blank feed (Table 3). To our knowledge, this is the first time such an effect has been reported due to a low concentration of T-2 toxin. Since contamination of human foodstuff with T-2 toxin is an emerging issue and concentrations up to 1810  $\mu\text{g}$  T-2 toxin per kg wheat have been reported in Germany (Schollenberger et al., 2006), it is feasible that T-2 toxin may also affect human metabolism. Different studies describe that, at high doses, T-2 toxin affects the intestinal absorption of nutrients and reduces the daily feed intake, resulting in a reduced body weight gain (Harvey et al., 1990, 1994; Rafai et al., 1995a). However, due to the housing conditions of the animals, we were not able to record the daily feed intake of the animals. Therefore, we cannot conclude whether the reduced weight gain of the pigs was the result of a decreased daily feed intake.

iTRAQ analysis showed that even an extreme low concentration of 5 ng/mL T-2 toxin affects protein expression in differentiated IPEC-J2 cells compared to untreated cells (Table 6). The main mechanism by which T-2 toxin causes its toxic effects is through inhibition of protein synthesis, leading to a ribotoxic stress response. This activates c-Jun N-terminal kinase (JNK)/p38 MAPKs and as a consequence modulates numerous physiological processes including cellular homeostasis, cell growth, differentiation and apoptosis (Shifrin et al., 1999). Proteomic analysis showed an upregulation of proteins involved in ribosome biogenesis, protein synthesis, protein folding and c-Jun N-Terminal kinase signalling. The increased expression of these proteins could be a rescue mechanism, highlighting that even a low concentration of 5 ng/mL T-2 toxin leads to a ribotoxic stress response in differentiated IPEC-J2 cells. The toxic character of T-2 toxin was also shown by the upregulation of heat shock proteins, pre-mRNA splicing factor heterogeneous nuclear ribonucleoprotein F, which could be a mechanism to increase mRNA stability (Yang et al., 2008), and 14-3-3 sigma. The protein 14-3-3 sigma is a p53-regulated inhibitor of G<sub>2</sub>/M progression (Hermeking et al., 1997) and its upregulation might emphasize the DNA damage caused by T-2 toxin (DiPaola, 2002). Overall, these iTRAQ data might indicate that T-2 toxin damages the porcine enterocyte, and by doing so, harms the absorption of nutrients with a reduced weight gain as result.

In contrast to other *Fusarium* mycotoxins, there is no guidance value set by the European Commission for the amount of T-2 toxin in complementary and complete feed for

pigs. As shown by a neutral red assay, T-2 toxin affects cell viability at very low concentrations (Figure 4). The *in vitro* viability of porcine macrophages, undifferentiated and differentiated porcine intestinal epithelial cells was significantly decreased at concentrations  $\geq 1$  ng/mL,  $\geq 2.5$  ng/mL and  $\geq 15$  ng/mL, respectively. Taking into account that such low concentrations negatively affect cell viability *in vitro*, and that these concentrations are relevant in practice (Schollenberger et al., 2006; Monbaliu et al., 2010), it is of utmost importance that maximum levels are set for this mycotoxin as well.

Ingestion of low and relevant concentrations of T-2 toxin (15 and 83  $\mu$ g/kg) results in reduced numbers of *Salmonella* Typhimurium bacteria in the cecal contents of pigs, and a tendency to a reduced colonization of the jejunum, ileum, cecum, colon and colon contents (Figure 2). With T-2 toxin and *Salmonella* Typhimurium being major problems in swine industry and with salmonellosis being one of the most important zoonotic bacterial diseases in both developed and developing countries, we aimed at evaluating the effect of T-2 toxin on the pathogenesis of a *Salmonella* Typhimurium infection in pigs. Until now, conflicting results have been published concerning the effect of mycotoxins on the susceptibility to intestinal infections and still little is known about the effects of low concentrations of these mycotoxins (Tenk et al., 1982; Tai et al., 1988; Ziprin and McMurray, 1988; Kubena et al., 2001; Oswald et al., 2003; Waché et al., 2009). According to Ziprin and McMurray (1988), T-2 toxin did not affect the course of salmonellosis in mice. In the present study, we provide evidence that these data cannot be extrapolated to a pig host. Since the porcine intestine shows physiological, anatomical and pathological similarities to the human gut (Almond, 1996), it is not unlikely that T-2 toxin similarly affects the pathogenesis of a *Salmonella* infection in the human host as in the pig host.

The ingestion of 15  $\mu$ g T-2 toxin per kg feed resulted in a significant decreased expression of IL-1 $\beta$  (Figure 3). Once *Salmonella* has invaded the intestinal epithelium, the innate immune system is triggered and the porcine gut will react with the production of several cytokines (Skjolaas et al., 2006). Both *Salmonella* and mycotoxins affect the innate immune system. Zhou et al. (1998) found that deoxynivalenol (DON) increases the expression of TGF- $\beta$  and IFN- $\gamma$  in the small intestine of mice. Recently, Kruber et al. (2011) established that T-2 toxin strongly induces IL-8 production in a Caco-2 intestinal epithelial cell line. According to Vandenbroucke et al. (2011), DON and *Salmonella* Typhimurium synergistically potentiate intestinal inflammation in an ileal loop model of pigs. As our control group is *Salmonella* positive, we cannot conclude whether the decreased expression of IL-1 $\beta$  in the T-2 toxin treated pigs is caused by the effects of T-2 toxin on the innate immune

system, the reduced numbers of *Salmonella* Typhimurium in the gut, or a combination of both. Furthermore, by the use of ELISA analysis, Maresca et al. (2008) showed that DON caused a biphasic effect on IL-8 secretion by Caco-2 cells. They also pointed out that this biphasic effect was not observed at mRNA level, where a dose-dependent increase in IL-8 mRNA was noticed (Maresca et al., 2008). These data implicate that in order to obtain results about the secretion of IL-1 $\beta$ , ELISA analysis on the ileum should be performed.

In order to elucidate how T-2 toxin causes reduced numbers of *Salmonella* Typhimurium bacteria in the cecal contents of pigs, and a tendency to a reduced colonization of the jejunum, ileum, cecum, colon and colon contents, we investigated the effects of T-2 toxin on the interactions of *Salmonella* Typhimurium with porcine macrophages and intestinal epithelial cells, two cell types that play an important role in the pathogenesis of a *Salmonella* infection. *In vitro* treatment of the host cells with T-2 toxin rendered them more susceptible to invasion, in a SPI-1 independent manner, and increased the transepithelial passage of the bacterium (Figure 5, 6 and 7). This is in accordance with Vandebroucke et al. (2009, 2011) who showed that DON promotes the invasion and translocation of *Salmonella* Typhimurium over porcine host cells, by a mechanism that is not SPI-1 dependent. The results obtained by Maresca et al. (2008) also confirm our results since they pointed out that DON concentrations that do not compromise the barrier function, significantly increase the passage of non-invasive *Escherichia coli* bacteria through Caco-2 inserts. As reviewed by Maresca and Fantini (2010) such increase in bacterial passage through intestinal epithelial cells could be involved in inducing inflammatory bowel diseases. Extrapolating these results to the *in vivo* situation, one would expect an increased colonization by *Salmonella* in pigs. However, we showed that ingestion of low and relevant concentrations of T-2 toxin resulted in a significantly decreased amount of *Salmonella* Typhimurium bacteria in the cecal contents and in a tendency to reduced colonization of the jejunum, ileum, cecum, colon and colon contents. *In vitro*, T-2 toxin decreased the intracellular survival of *Salmonella* Typhimurium in PAM, undifferentiated IPEC-J2 cells and differentiated IPEC-J2 cells (Figure 5) at concentrations which significantly reduced the cell viability (Figure 4). Possibly this reduced survival plays an important role in the *in vivo* outcome. However, whether this reduced survival is due to a decrease in viable cells, a diminished replication capacity of the bacterium or a combination of both, is unknown.

Invasion of *Salmonella* in nonphagocytic cells involves a series of cytoskeletal changes, characterized by actin polymerization and the formation of membrane ruffles. These cytoskeletal changes are important for the uptake and the cytoplasmic transport of the



bacterium, as well as for the establishment and the stability of the bacterial replicative niche, also called *Salmonella* containing vacuole (SCV) (Vandenbroucke et al., 2009). By the use of iTRAQ analysis, we demonstrated that 5 ng/mL T-2 toxin induces alterations in the expression of proteins that are involved in the cytoskeleton formation of differentiated IPEC-J2 cells. T-2 toxin causes a decreased expression of cytokeratin 18, a member of the intermediate filament network that provides support and integrity to the cytoskeleton (Singh and Gupta, 1994), of myristoylated alanine-rich C-kinase substrate, a filamentous actin crosslinking protein (Hartwig et al., 1992) and of putative beta-actin, which is a major component of the cytoskeleton. Furthermore, T-2 toxin causes an increased expression of thymosin beta-10, an actin-sequestering protein involved in cytoskeleton organization and biogenesis (Sribenja et al., 2009), of cysteine and glycine-rich protein 1 isoform 1, a regulator for actin filament bundling (Tran et al., 2005) and of profilin, an actin-binding protein that can sequester G-actin or actively participate in filament growth (Gutsche-Perelroizen et al., 1999). According to Vandenbroucke et al. (2009), low concentrations of DON can modulate the cytoskeleton of macrophages resulting in an enhanced uptake of *Salmonella* Typhimurium in porcine macrophages. The observed changes in protein expression are not sufficient to induce morphological changes, as assessed with TEM (Figure 8). However, the T-2 toxin induced altered expression of cytoskeleton associated proteins could influence the interactions between IPEC-J2 cells and *Salmonella*. Thus T-2 toxin and *Salmonella* Typhimurium appear to act synergistically, inducing cytoskeleton reorganizations which increase the invasion of the bacterium.

We also examined the effects of T-2 toxin on *Salmonella* Typhimurium gene expression. Microarray analysis revealed that T-2 toxin caused a general downregulation of *Salmonella* Typhimurium metabolism and notably of ribosome synthesis. To our knowledge, this is the first time it has been shown that T-2 toxin affects ribosomal gene expression in both eukaryotic (Van Kol et al., 2011) and prokaryotic cells. Microarray analysis also showed that T-2 toxin causes a downregulation of flagella gene expression and consequently resulted in decreased motility of *Salmonella* Typhimurium (Figure 10). Motility of *Salmonella* increases the probability that the bacterium will reach suitable sites for invasion and successful infections (Shah et al., 2011). Transcriptomic analysis furthermore demonstrated that exposure to T-2 toxin results in reduced expression of many SPI-1 genes. According to Boyen et al. (2006b), SPI-1 plays a crucial role in the invasion and colonization of the porcine gut and in the induction of influx of neutrophils. Shah et al. (2011) indicated that the pathogenicity of *Salmonella* Enteritidis isolates is associated with both motility and secretion

of the type III secretion system (TTSS) effector proteins. Isolates with low invasiveness had impaired motility and impaired secretion of FlgK, FljB and FlfL or TTSS secreted SipA and SipD. Therefore, a T-2 toxin induced downregulation of SPI-1 and motility genes and a reduced motility may lead to a reduced colonization by the bacterium in pigs.

In conclusion, we showed that the presence of low and in practice relevant concentrations of T-2 toxin in the feed causes a decrease in the amount of *Salmonella* Typhimurium bacteria present in the cecal contents of pigs, and a tendency to a reduced colonization of the jejunum, ileum, cecum, colon and colon contents. *In vitro*, T-2 toxin causes an increased invasion and transepithelial passage of the bacterium in and through T-2 toxin treated porcine cells, in a SPI-1 independent manner. However, T-2 toxin significantly reduces the SPI-1 gene expression, invasiveness and motility of the bacterium. Therefore, *in vivo*, the effect of T-2 toxin on the bacterium is probably more pronounced than the host cell-mediated effect.

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## CHAPTER 4:

### **A modified glucomannan feed additive counteracts the reduced weight gain and diminishes cecal colonization of *Salmonella* Typhimurium in T-2 toxin exposed pigs**

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## Abstract

Salmonellosis is one of the most important zoonotic bacterial diseases and pigs are considered one of the main sources of human salmonellosis. Besides *Salmonella* infections, T-2 toxin contamination of various food and feed commodities, poses a serious threat to human and animal health, especially to pigs. A strategy for reducing the exposure to mycotoxins is the use of mycotoxin-adsorbing agents in the feed. Some of these mycotoxin-adsorbing agents might affect the pathogenesis of a *Salmonella* Typhimurium infection. The objective of this study was, therefore, to investigate the effect of a modified glucomannan feed additive, which is claimed to be a mycotoxin detoxifying agent, on the course of a *Salmonella* Typhimurium infection in T-2 toxin exposed and unexposed pigs. An *in vivo* trial was performed in which four different pig diets were provided during 23 days: a diet which was free of mycotoxins, a diet containing 1 g modified glucomannan feed additive per kg feed, a diet containing 83 µg T-2 toxin per kg feed and a diet containing 83 µg T-2 toxin per kg feed supplemented with 1 g modified glucomannan feed additive per kg feed. At day 18 of the feeding period, all pigs were inoculated with *Salmonella* Typhimurium and five days later the pigs were euthanized. The addition of the feed additive to T-2 toxin contaminated feed counteracted the reduced weight gain of pigs caused by T-2 toxin and there are indications that the modified glucomannan reduces the intestinal colonization of *Salmonella* Typhimurium, however not significantly. Furthermore, *in vitro* findings suggest that the modified glucomannan feed additive binds *Salmonella* bacteria ( $p < 0.001$ ). We thus conclude that the feed additive tested here, not only counteracts T-2 toxin induced weight loss, but possibly also captures *Salmonella* bacteria, resulting in a reduced intestinal colonization.

## Introduction

Worldwide, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the predominant serovar isolated from slaughter pigs (Fisher and participants, 2004). The bacterium is generally asymptotically present in carrier animals, that can be a source of environmental and carcass contamination, leading to higher numbers of foodborne *Salmonella* infections in humans (Boyen et al., 2008a). Since *Salmonella* Typhimurium is one of the major causes of foodborne salmonellosis in humans, there is an increasing need to control *Salmonella* infections in pigs.

Besides *Salmonella* infections, T-2 toxin contamination of cereals such as wheat, barley, oats and maize is an emerging issue (van der Fels-Klerx, 2010). With T-2 toxin being the most acute toxic among the trichothecenes, this mycotoxin may pose a threat to human and animal health and especially to pigs which seem to be one of the most sensitive species to *Fusarium* mycotoxins (Hussein and Brasel, 2001). Moderate to high levels of T-2 toxin cause immunosuppression, feed refusal, vomiting, weight loss, reduced growth and skin lesions (Wu et al., 2010). Recently, we showed that a low T-2 toxin concentration (83 µg/kg feed) causes a reduced weight gain and that the presence of 15 and 83 µg T-2 toxin per kg feed significantly decreased the amount of *Salmonella* Typhimurium bacteria present in the cecal contents (Verbrugghe et al., 2012). Until now, the no-observed-adverse-effect-level (NOAEL) of T-2 toxin in pigs is unknown and no maximum guidance limits for T-2 toxin in food and feedstuff are yet established by the European Union.

A strategy for reducing the exposure to mycotoxins is the use of mycotoxin-adsorbing agents that theoretically reduce the absorption and distribution of the mycotoxin. Some mycotoxin detoxifying agents, like glucomannans, are derived from yeast cell walls that contain  $\alpha$ -D-mannans and  $\beta$ -D-glucans. It has been described that  $\alpha$ -D-mannan binds with mannose-specific lectin-type receptors, such as type 1 fimbriae of *Salmonella* (Firon et al., 1983). According to Lowry et al. (2005), purified  $\beta$ -glucan is able to decrease the incidence of *Salmonella enterica* serovar Enteritidis organ invasion in immature chickens.

Our hypothesis was that the addition of a modified glucomannan feed additive to pig feed could not only counteract the negative effects of T-2 toxin, but could also reduce the colonization of *Salmonella* Typhimurium in pigs. Therefore, the aim of the present study was to investigate the effect of a modified glucomannan feed additive on the course of a *Salmonella* Typhimurium infection in T-2 toxin exposed and unexposed pigs.

## Materials and methods

### Chemicals, bacterial strains and growth conditions

A T-2 toxin (Sigma-Aldrich, Steinheim, Germany) stock solution of 250 µg/mL was prepared in ethanol and stored at – 20 °C. A modified glucomannan mycotoxin feed additive (Mycosorb<sup>®</sup>, trademark of Alltech, Inc., Nicholasville, Kentucky, that has been registered as a mycotoxin-binding agent with the U.S. Trademark), derived from the cell wall of *Saccharomyces cerevisiae*, was used at a concentration of 1 g per kg feed. *Salmonella* Typhimurium strain 112910a, isolated from a pig stool sample and characterized previously by Boyen et al. (2008b), was used as the wild type strain in which the spontaneous nalidixic acid resistant derivative strain (WT<sub>nal</sub>) was constructed. For oral inoculation of pigs, the WT<sub>nal</sub> was grown as described in Verbrugghe et al. 2012.

### Experimental *Salmonella* Typhimurium infection of pigs, fed a modified glucomannan and/or T-2 toxin supplemented diet

All animal experiments were carried out in strict accordance with the recommendations in the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes. The experimental protocols and care of the animals were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2010/049 + expansion 2010/101).

### Experimental design

Three-week-old piglets were randomized into four groups of 5 piglets and each group was housed in separate isolation units. The *Salmonella*-free status of the piglets was tested serologically using a commercially available *Salmonella* antibody test kit (IDEXX, Hoofddorp, The Netherlands), and bacteriologically via multiple faecal sampling, as described in Verbrugghe et al., 2012. The first 6 days after arrival, all piglets received a commercial control piglet feed (DANIS, Koolskamp, Belgium) of which the composition is provided in Table 1 and 2. The control feed was free from mycotoxin contamination, as determined by multi-mycotoxin liquid chromatography tandem mass spectrometry (LC-MS/MS) (Monbaliu et al., 2010). The acclimatization period was followed by an *ad libitum* feeding period of 23 days with the experimental feed diets: control group: control feed; feed additive group: control feed supplemented with 1 g modified glucomannan feed additive per

kg feed; T-2 toxin group: control feed contaminated with 83 µg T-2 toxin per kg feed; T-2 toxin + feed additive group: control feed contaminated with 83 µg T-2 toxin per kg feed and supplemented with 1 g modified glucomannan feed additive per kg feed. At day 18, by the use of 5 ml syringe, the pigs were orally inoculated with  $2 \times 10^7$  CFU of *Salmonella* Typhimurium WT<sub>nal</sub>. Five days after inoculation, the pigs were euthanized and gut contents and tissue samples were collected for bacteriological analysis, as described in Verbrugge et al., 2012. Furthermore, the animals were individually weighed after the acclimatization period, after the feeding period of 18 days and at euthanasia. Due to the housing conditions of the animals, we were not able to record the daily feed intake of the animals.

**Table 1:** Composition of the commercial control piglet feed (DANIS, Koolskamp, Belgium) (g/kg).

Components	Control pig feed
Maize	130
Wheat	180
Barley	300
Palm oil	3
Soybean meal	70
Toasted soybeans	140
Wheat gluten	40
Monocalcium phosphate	5
Natriumchloride	4
Premix*	128

\* One kg of premix contains Vitamin A - 18500 IU, Vitamin D3 - 2000 IU, Vitamin E - 100 mg, Copper(II)sulfate pentahydrate - 160 mg

**Table 2:** Chemical composition of commercial control piglet feed (DANIS, Koolskamp, Belgium) (g/kg).

Item	Control pig feed
Crude protein	177
Crude fat	59
Crude ash	53
Crude fibre	36.5
Phosphorus	5.6
Lysine	13

### Preparation of experimental diets

The concentration of T-2 toxin in the feed was chosen based on previous measurements of T-2 toxin contamination of feed (Monbaliu et al., 2010). To produce feed contaminated with 100 µg T-2 toxin per kg feed and/or 1 g modified glucomannan feed additive per kg feed, 40 mL of a stock solution of 250 µg T-2 toxin per mL ethanol, and/or 100 g of the feed additive was added to 500 g of control feed. This premix was then mixed by

hand with 5 kg of control feed to assure a homogeneous distribution of the toxin. The final premix was mixed by the use of a vertical feed mixer with archimedes screw for 20 min in 100 kg feed. To test T-2 toxin homogeneity in the feed, samples were taken at four different locations in the batch and analysed with LC-MS/MS which revealed the final presence of 83 µg T-2 toxin per kg feed.

### **Effect of a modified glucomannan feed additive on the amount of *Salmonella* bacteria in a minimal medium**

*Salmonella* Typhimurium bacteria ( $10^4$  CFU) were added to 1 mL HPLC H<sub>2</sub>O, as a minimal medium, with or without 20 mg of the modified glucomannan feed additive and incubated at 37 °C on a shaker. Immediately and, in order to avoid growth of *Salmonella* Typhimurium, 4 hours after incubation, the samples were submitted to static incubation for 1 min in order to allow precipitation of feed additive-*Salmonella* complexes and the number of *Salmonella* bacteria in the supernatant was assessed by plating 10-fold dilutions on BGA plates.

### **Statistical Analysis**

Weight gain and colonization data were evaluated statistically by two-factorial ANOVA using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). The differences for all parameters were tested according to the following statistical model:

$$y_{jik} = \mu + a_i + b_j + (ab)_{ij} + e_{ijk}$$

where  $\mu$  is the overall mean,  $a_i$  is the effect for the presence of T-2 toxin,  $b_j$  is the effect for the presence of the feed additive,  $(ab)_{ij}$  is the interaction effect, and  $e_{ijk}$  is the error term. Differences between treatment means were analysed for significance ( $p < 0.01$  or  $p < 0.05$ ) using Scheffé test. The binding capacity of the mycotoxin feed additive for *Salmonella* bacteria was analysed by a Paired Sample t-test.

## Results

The addition of the feed additive to feed contaminated with 83 µg T-2 toxin per kg feed neutralizes the reduced weight gain caused by T-2 toxin. After a feeding period of 18 days, two-factorial ANOVA revealed an interaction ( $p = 0.017$ ) between the presence of T-2 toxin and the feed additive for the daily weight gain of the pigs. After a feeding period of 18 days, the effect of the mycotoxin-adsorbing agent tested here, was however not statistically significant. Five days after infection with *Salmonella* Typhimurium and after a feeding period of 23 days, two-factorial ANOVA also revealed an interaction ( $p = 0.003$ ) between the presence of T-2 toxin and the feed additive for the weight gain. There was a difference in the average weight gain ( $p = 0.009$ ) per day between the T-2 toxin group and T-2 toxin + feed additive group.

As demonstrated earlier, supplementation of pig feed with 83 µg T-2 toxin per kg feed reduced the intestinal *Salmonella* load in pigs (Verbrugghe et al., 2012), Table 3. We now showed that the addition of the feed additive to T-2 toxin contaminated feed, also significantly reduced the amount of *Salmonella* Typhimurium bacteria present in the cecum ( $p = 0.003$ ) and cecal contents ( $p = 0.04$ ), in comparison to control pigs (Table 1). Moreover, the addition of the modified glucomannan to T-2 toxin contaminated feed seems to enhance the reduction in *Salmonella* bacteria. An overall reduced intestinal colonization was observed for the feed additive group, however not statistically significant. Two-factorial ANOVA showed that there was no interaction between the presence of T-2 toxin and the feed additive for the colonization of *Salmonella* Typhimurium. This indicates that both T-2 toxin and the modified glucomannan are able to reduce the colonization of *Salmonella*.

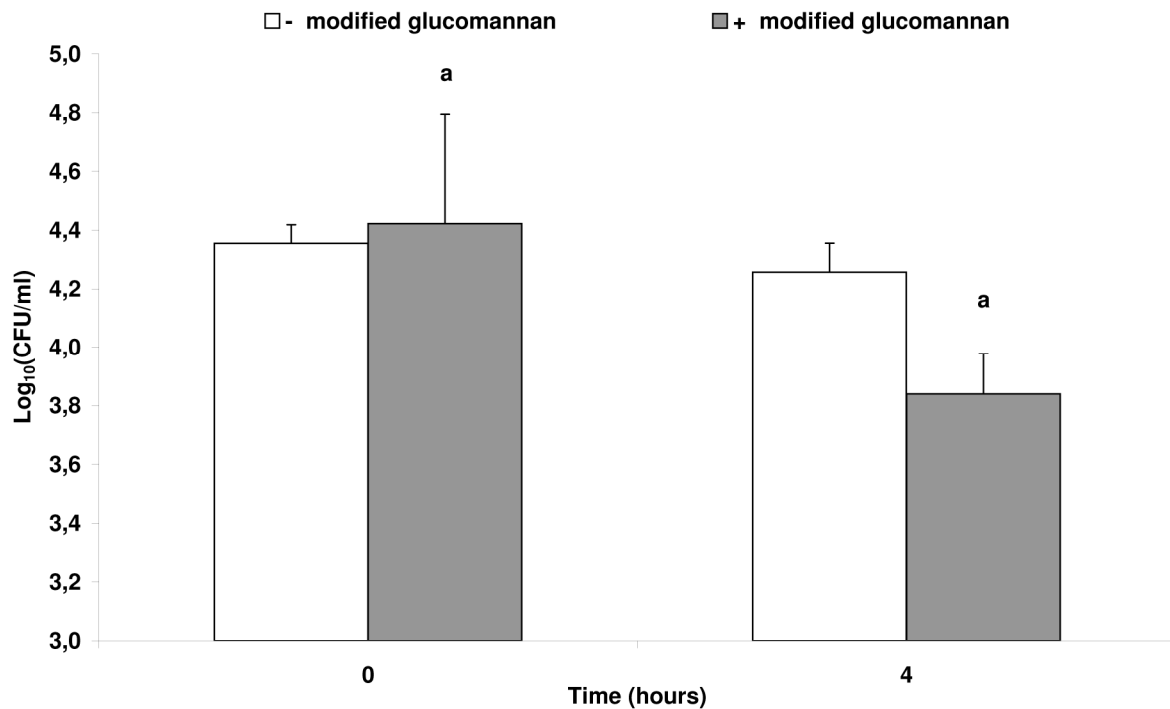
As shown in Figure 1, after 4 hours, the modified glucomannan feed additive reduced ( $p < 0.001$ ) the number of *Salmonella* bacteria in the supernatant in comparison to the start of the incubation period. It has been described that  $\alpha$ -D-mannan binds with mannose-specific lectin-type receptors, such as type 1 fimbriae of *Salmonella* (Firon et al., 1983). Prior to colonization, *Salmonella* uses its fimbriae to bind to the mannose-rich epithelial surface of the gut. Therefore, it is not unlikely that the modified glucomannan feed additive used here captures *Salmonella* bacteria, possibly through the interaction between  $\alpha$ -D-mannan with the fimbriae of *Salmonella* Typhimurium, resulting in a reduced colonization of the pathogen in pigs.

**Table 3:** The average weight gain and recovery of *Salmonella* Typhimurium bacteria from various organs and gut contents of pigs that received different pig diets.

Feeding group treatments	Start weight (kg)	Average weight gain (kg/day)		Number of <i>Salmonella</i> Typhimurium bacteria (Log <sub>10</sub> (CFU/g))									
		After 18 days	After 23 days	Tonsils	Ileocecal lymph nodes	Duodenum	Jejunum	Ileum	Cecum	Cecal contents	Colon	Colon contents	Faeces
Control group	5.7	0.326	0.330 <sup>a</sup>	3.2	2.8	1.5	3.4	4.8	5.4 <sup>a,B</sup>	6.0 <sup>a,b</sup>	4.3	4.5	2.6
Feed additive group	5.3	0.258	0.302	3.0	3.2	2.3	2.8	3.1	3.7	3.8	2.8	3.3	2.8
T-2 toxin group	6.1	0.239	0.222 <sup>a,B</sup>	2.9	2.6	2.2	1.5	2.8	3.4 <sup>a</sup>	3.5 <sup>a</sup>	1.9	2.9	2.8
T-2 toxin + feed additive group	5.6	0.322	0.352 <sup>B</sup>	1.7	3.0	2.0	2.2	2.8	2.6 <sup>B</sup>	3.1 <sup>b</sup>	2.4	1.9	1.9
Standard error of the mean (SEM)		0.016	0.015	0.362	0.155	0.197	0.308	0.349	0.340	0.425	0.368	0.398	0.296
p-value for the feed additive		0.785	0.042	0.320	0.243	0.424	0.943	0.198	0.036	0.096	0.447	0.160	0.615
p-value for T-2 toxin		0.696	0.221	0.290	0.505	0.674	0.047	0.087	0.010	0.036	0.062	0.067	0.559
p-value for the interaction		0.017	0.003	0.501	0.926	0.293	0.281	0.206	0.399	0.239	0.173	0.914	0.414

Differences in column signed with a,b: significant by  $p < 0.05$ ; signed with A, B: significant by  $p < 0.01$ .





**Figure 1:** The effect of a modified glucomannan feed additive on the amount of *Salmonella* bacteria in a minimal medium is presented before incubation ( $t = 0$ ) and after 4 hours ( $t = 4$ ). Results are presented as the mean  $\log_{10}$  values of the CFU/mL + standard deviation (SD) of three independent experiments conducted in triplicate. Superscript (a) refers to a significant difference with  $p < 0.001$ .

## Discussion

In literature, contradictory results have been published concerning the protective role of glucomannans. Aravind et al., (2003) showed that the addition of esterified glucomannan (0.05%) to a naturally contaminated diet (including T-2 toxin), was effective in counteracting the toxic effects, such as growth depression of broilers. Our data (Table 3) also indicate a neutralizing effect of the modified glucomannan feed additive (0.1%) tested here, towards the reduced weight gain in pigs caused by T-2 toxin. By contrast, Swamy et al. (2003) stated that the supplementation of a polymeric glucomannan mycotoxin adsorbent (0.2%) to mycotoxin contaminated feed did not prevent the mycotoxin-induced growth depression in pigs. These dissimilarities can be the result of the differences in glucomannan detoxifying agents used in these experiments. Probably, the degree of mycotoxin contamination and the daily feed intake also play a part.

$\beta$ -D-glucan, the second major polysaccharide of the yeast cell wall, may also play a role in the reduced colonization of *Salmonella* Typhimurium. It has been shown that some  $\beta$ -D-glucans can enhance the functional status of cells of the pig immune system. Sonck et al.

(2010) showed that  $\beta$ -glucans from algae (*Euglena gracilis*) and glucan preparations from baker's yeast (Macrogard, *Saccharomyces cerevisiae* and Zymosan) activated ROS-production of porcine monocytes and neutrophils. Particulate  $\beta$ -glucans stimulated the proliferation of pig lymphocytes and, except for Laminarin, most  $\beta$ -glucans gave rise to TNF- $\alpha$  and IL-1 $\beta$  secretion. These authors also showed that Macrogard induces a significant phenotypic maturation of porcine monocyte derived dendritic cells. Some  $\beta$ -glucans can improve the T-cell-stimulatory capacity of porcine monocyte derived dendritic cells. However, only curdlan induced a significant higher expression level of IL-1 (Sonck et al., 2011). Lowry et al. (2005) showed that addition of a purified  $\beta$ -glucan to the feed significantly decreased *Salmonella* Enteritidis organ invasion in experimentally infected immature chickens. The decreased colonization of *Salmonella* Typhimurium in pigs may thus be the result of an interplay between the immunomodulating and binding activity of the yeast cell wall polysaccharides.

Alternatively, it is possible that these glucomannans improve the intestinal microflora of piglets. Oligosaccharides are considered to improve host health by being a substrate for potentially beneficial bacteria such as lactobacilli and bifidobacteria (Gibson and Roberfroid, 1995). Therefore, it is not unlikely that the addition of substances rich in mannose, such as a modified glucomannan mycotoxin-adsorbing agent could have a bioprotective effect against intestinal infections caused by *Salmonella* Typhimurium. However, since we did not investigate the effects of the mycotoxin-adsorbing agent tested here, on the intestinal microflora of pigs, this assumption is only speculative.

In conclusion, we highlighted the protective role of the feed additive tested here, against the weight loss in pigs caused by T-2 toxin and we provided evidence that it may reduce the intestinal colonization of *Salmonella* Typhimurium in pigs.

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# **General discussion**



**... Stress induced recrudescence of a *Salmonella* Typhimurium infection in pigs ...**

For a long time, it has been known that stress can influence the outcome of a bacterial infection, including a *Salmonella* Typhimurium infection in pigs. Stress situations caused by inadequate housing conditions, overcrowding, heat, cold, feed deprivation before slaughter and transportation have been linked to increased disease susceptibility, pathogen carriage and pathogen shedding (Burkholder et al., 2008; Rostagno, 2009). This could lead to increased carcass contamination during slaughtering. *Salmonella* bacteria are present in latent carriers and stress induced pathogen shedding could result in an increased transmission of the bacterium and as a consequence lead to higher numbers of foodborne *Salmonella* infections in humans (Wood et al., 1991). Therefore, the elucidation of the mechanisms by which stress and its associated hormones induce recrudescence of the infection, would help us in the mitigation of a *Salmonella* Typhimurium infection in pigs and consequently reduce the cases of human salmonellosis.

**Cortisol induced increased replication of *Salmonella* in macrophages: modification of the *Salmonella* containing vacuole?**

The eukaryotic cytoskeleton is composed of actin filaments, intermediate filaments and microtubules and it is crucial for the cell shape, division and function (Wickstead and Gull, 2011). Upon intracellular growth of *Salmonella* Typhimurium, this cytoskeleton undergoes a complex series of changes, such as the formation of an actin meshwork around the *Salmonella* containing vacuoles and the accumulation of microtubules around *Salmonella* Typhimurium microcolonies (Guignot et al., 2003; Méresse et al., 2001). We showed that the cortisol induced increased intracellular proliferation of *Salmonella* Typhimurium is actin and microtubule dependent and that it results in an increased expression of cytoskeleton associated proteins, including a constituent of the *Salmonella* containing vacuole, a component of microtubules and proteins that regulate the polymerization of actin. Possibly, cortisol exerts its effect on the intracellular *Salmonella* bacteria through the modulation of the host cell cytoskeleton and more specific, the *Salmonella* containing vacuole. By doing so, the *Salmonella* containing vacuole and *Salmonella* Typhimurium collaborate in order to enhance bacterial replication.

Besides the maturation of the *Salmonella* containing vacuole, also the microenvironment within this *Salmonella* containing vacuole during the course of the

infection influences the intracellular proliferation of *Salmonella* bacteria (García-del Portillo, 2001). Within the *Salmonella* containing vacuole of epithelial cells, the amount of  $Mg^{2+}$  and  $Fe^{2+}$  is limited (García-del Portillo et al., 1999).  $Mg^{2+}$  limitation leads to the activation of the PhoP-PhoQ regulatory system (Garcia-Vescovi et al., 1996). Normally, the growth of *phoP* and *phoQ* mutants is limited in minimal salt-medium containing low  $Mg^{2+}$ . However, within epithelial cells, these mutants grow efficiently, suggesting that other nutrients are present within the *Salmonella* containing vacuole of epithelial cells. It has been shown that the microenvironment within epithelial cells contains histidine but lacks purines, pyrimidines and aromatic amino acids (Finlay et al., 1991). Information on putative nutrients within the microenvironment of the *Salmonella* containing vacuole in macrophages is limited. Rathman et al. (1996) showed that after uptake of *Salmonella* Typhimurium in murine macrophages, the *Salmonella* containing vacuole acidifies to pH 4.0 - 5.0, indicating that low pH might be a potential signal to direct intracellular growth (Beuzon et al., 1999). Factors determining the extent of intracellular proliferation of *Salmonella* Typhimurium remain poorly known. In certain cases, *Salmonella* intracellular proliferation is restrained.  $IFN\gamma$  is a host factor that activates macrophages and which restricts the intracellular growth rate (García-del Portillo, 2001). However, it is possible that cortisol influences the composition of the microenvironment and by doing so stimulates the intracellular *Salmonella* bacteria to grow instead of long-term parasitism within this *Salmonella* containing vacuole. Possibly *Salmonella* bacteria respond with an increased replication and recrudescence. As a result, *Salmonella* can escape the stressed host in order to infect new and healthy hosts.

### **Cortisol induced increased replication of *Salmonella* in macrophages: cause of increased *Salmonella* shedding?**

Stress induced shedding of *Salmonella* Typhimurium is associated with increased serum cortisol levels in pigs and cortisol increases the intracellular proliferation of *Salmonella* Typhimurium in macrophages. However, how this increased replication in macrophages results in an increase in intestinal numbers of *Salmonella* and subsequent shedding of the bacterium by pigs, remains unknown. Possibly, these *Salmonella* infected macrophages migrate out of the lamina propria back to the gut lumen. The lamina propria contains extracellular matrix including the interstitial matrix and the basement membrane, and it is closely associated with several cell types such as macrophages and lymphocytes (Louvard et



al., 1992). According to Poussier and Julius (1994), T-lymphocytes may migrate from the lamina propria into the epithelium. Also macrophages have been shown to migrate from the lamina propria to the intestinal lumen. Heatley and Bienenstock (1982) indicated the migration of lymphocytes and macrophages from the gut-associated lymphoid tissue towards the intestinal lumen of rabbits. Using normal human colonic biopsies, Mahida et al. (1997) showed that large numbers of lymphocytes, macrophages and eosinophils migrate out of the intestinal lamina propria following removal of the surface epithelium of human mucosal strips. These authors revealed that the migration occurs via tunnels in the extracellular matrix that end as discrete pores in the basement membrane. Although this mechanism of macrophage migration has been linked to a host defense mechanism elicited following injury and loss of epithelial cells, it is possible that following a stress period, *Salmonella* Typhimurium infected macrophages move back to the intestinal lumen. Pyroptosis of these macrophages can result in an increased amount of *Salmonella* bacteria present in the intestinal lumen. Alternatively, a direct transmigration of *Salmonella* bacteria from the lamina propria over the intestinal epithelium towards the intestinal lumen may occur. However, until now such a migration mechanism for bacteria has not yet been described.

Another scenario is that *Salmonella* bacteria reach the gut through biliary excretion. Bile is produced in the liver and is composed of various bile salts. These bile salts induce DNA damage to *Salmonella* bacteria since they increase the frequency of nucleotide substitutions, frameshifts and chromosomal rearrangements (Prieto et al., 2004; Merritt and Donaldson, 2009). However, *Salmonella* Typhimurium can be highly resistant against these bile salts (van Velkinburgh and Gunn, 1999). Antunes et al. (2011) showed that *Salmonella* Typhimurium can grow in physiological murine bile and survive in the lumen of the gallbladder of mice. In humans, *Salmonella* Typhi can colonize the gallbladder and persist in an asymptomatic carrier state that is frequently associated with the presence of gallstones (Crawford et al., 2010). In pigs, however, it remains to be determined if *Salmonella* Typhimurium colonizes and persists within the gallbladder.

### **Stress induced shedding of *Salmonella* Typhimurium: one man show?**

Cortisol is probably not the sole key player in the stress induced shedding of *Salmonella* Typhimurium by pigs. It has been shown that pre-treatment of mice with norepinephrine results in an enhanced systemic spread of *Salmonella* Typhimurium (Williams et al., 2006). McCuddin et al. (2008) showed that norepinephrine is needed for *Salmonella*

Saintpaul, Montevideo and Enteritidis, to gain access to the systemic circulation and to induce encephalopathy in calves. These data indicate that stress induced secretion of norepinephrine can influence the systemic phase of a *Salmonella* infection. In pigs however, the colonization of *Salmonella* Typhimurium is mostly limited to the gastrointestinal tract and conflicting results have been published concerning the effect of norepinephrine on the outcome of a *Salmonella* Typhimurium infection in pigs (Toscano et al., 2007; Pullinger et al., 2010).

Although we showed that epinephrine, norepinephrine and dopamine do not influence the invasion and intracellular survival of *Salmonella* Typhimurium in porcine macrophages and epithelial cells, several *in vitro* studies have shown that catecholamines can alter the growth and/or virulence of *Salmonella*, and as a consequence may influence bacterium-host interactions (Bearson and Bearson, 2008; Bearson et al., 2008; Methner et al., 2008). Possibly, catecholamines and glucocorticoids collaborate in order to aggravate a *Salmonella* infection in pigs. It is feasible that a catecholamine induced increased growth of the bacterium and a glucocorticoid induced recrudescence of the infection act in concert and consequently result in an increased shedding of *Salmonella* Typhimurium by pigs.

### **Stress induced recrudescence of the infection: universal mechanism?**

There is increasing evidence that stress also promotes the colonization of farm animals by other enteric pathogens such as *Escherichia coli* (*E. coli*) and *Campylobacter* (Rostagno, 2009). *In vivo* research showed that exposure to various stressors, such as feed withdrawal and handling, increases fecal shedding of *E. coli* in beef cattle (Brownlie and Grau, 1967; Reid et al., 2002), sheep (Grau et al., 1969), and young piglets (Dowd et al., 2007), as well as shedding of enterohemorrhagic *E. coli* in calves (Brown, et al., 1997; Cray et al., 1998). Besides the effect on *E. coli*, *in vivo* trials showed that transportation stress causes an increased colonization and shedding of *Campylobacter* in broiler chickens (Stern et al., 1995; Line et al., 1997; Whyte et al., 2001; Wesley et al., 2005). Furthermore, Byrd et al. (1998) demonstrated that preharvest feed withdrawal increases the frequency of *Campylobacter* crop contamination in broiler chickens. Wesley et al. (2009) confirmed these results in turkeys by demonstrating that transportation stress increases the number of *Campylobacter* bacteria in crop contamination.

*In vitro*, mainly the role of catecholamines has been described. For both pathogenic and commensal *E. coli* (Lyte and Ernst, 1992, 1993; Lyte et al., 1996a,b 1997a,b; Freestone et al., 2002; Chen et al., 2003; Sandrini et al., 2010), as well as for *Campylobacter* (Zeng et al.,

2009), catecholamines have been shown to cause an increased growth of the bacterium through the supply of iron. The increased availability of iron, which is supplied through the intervention of stress hormones, probably plays a key role in the effects of stress on the outcome of an infectious disease, but provides only a partial explanation for the *in vivo* effects. The role of glucocorticoids or the interplay between different stress hormones still remains unknown.

We identified *scsA* as a major driver for the increased intracellular replication of *Salmonella* Typhimurium in cortisol exposed primary porcine alveolar macrophages. *Salmonella* is a common facultative intracellular pathogen of which the intracellular survival and replication in macrophages are important virulence determinants (Ibarra and Steele-Mortimer, 2009). Intracellular multiplication in host cells has also been described for some other enteric bacteria including *Campylobacter* spp. (Day et al., 2000) and enteroinvasive *E. coli* strains (Götz and Goebelt, 2010). However, no homology for *scsA* has been described in these bacteria and glucocorticoid induced recrudescence has not yet been reported.

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### ... The effect of T-2 toxin on the pathogenesis of a *Salmonella* Typhimurium infection in pigs ...

Not only stress, but also mycotoxins are omnipresent in the pig industry. It is thought that fungi produce mycotoxins in order to cope with reactive oxygen species as a result of environmental stress (Reverberi, et al., 2010). The trichothecenes are a large group of structurally related mycotoxins that comprise the largest group of *Fusarium* mycotoxins found in Europe (Binder et al., 2007). According to van der Fels-Klerx (2010), cereal contamination with T-2 toxin is an emerging issue. With T-2 toxin and *Salmonella* Typhimurium being two phenomena to which pigs can be exposed during their lives, a possible interaction between these two is not excluded.

#### **T-2 toxin: promising novel antimicrobial agent from a theoretical point of view?**

Since we showed that T-2 toxin disrupted *Salmonella* Typhimurium gene expression, significantly decreased the amount of *Salmonella* Typhimurium bacteria present in the cecal contents, one could theoretically hypothesize whether T-2 toxin could be used as an antimicrobial agent against bacterial infections.

As shown by microarray analysis, even a low concentration of T-2 toxin affects the protein synthesis in *Salmonella* Typhimurium, causing a general downregulation of its metabolism and notably of the ribosome synthesis. In comparison to eukaryotic ribosomes that consist of 60S and 40S subunits, the ribosome subunits in prokaryotes are of 50S and 30S, yielding intact 70S subunits. These subunits are ribonucleoprotein complexes made up of specific ribosomal RNAs and ribosomal proteins (Madigan et al., 2000). Due to these differences in structure, some antibiotics such as aminoglycosides, lincosamides, macrolides and tetracyclines target ribosomes of bacteria at distinct locations within functionally relevant sites, while leaving eukaryotic ribosomes unaffected (Yonath, 2005). Similarly, T-2 toxin exposure of *Salmonella* Typhimurium bacteria resulted in reduced expression of genes encoding both 30S and 50S ribosomal proteins, indicating that T-2 toxin affects ribosome biogenesis of the bacterium. However, the toxicity of T-2 toxin in eukaryotic cells is based on a non-competitive inhibition of the protein synthesis (Cole and Cox, 1981). T-2 toxin binds to the 60S subunit of the ribosomes, and thereby inhibits the peptidyl transferase activity at the transcription site (Cundliffe et al., 1974; Hobden and Cundliffe, 1980; Yagen and Bialer, 1993). Although there are structural differences in the ribosome subunits between eukaryotic

and prokaryotic cells, T-2 toxin is able to interfere with both. We indeed showed that the inhibition of the protein synthesis leads to a high toxicity and eventually death in eukaryotic cells, whereas for *Salmonella* Typhimurium, it leads to a general downregulation of its metabolism and motility, but without affecting its viability and growth.

Only if T-2 toxin could be modified to such an extent that its specificity for prokaryotic ribosomes increases, without targeting eukaryotic ribosomes, then it could be used as an antimicrobial agent. Since the 12,13-epoxide ring is responsible for the toxicity of trichothecenes for eukaryotic cells, de-epoxidation of T-2 toxin would lead to a significant loss of toxicity for eukaryotic cells (Yagen and Bialer, 1993). However, whether the deepoxidized T-2 toxin still targets *Salmonella* Typhimurium bacteria is unknown.

### **T-2 toxin contamination: do we overlook HT-2 toxin and co-occurrence with other mycotoxins?**

T-2 toxin is rapidly metabolized to HT-2 toxin. The acute toxicity of both toxins is within the same range. Therefore, the toxicity of T-2 toxin might be partly attributed to HT-2 toxin. In a recent study of the European Food Safety Authority, it was shown that occurrence of T-2 toxin and HT-2 toxin in food and feed is about the same and co-occurrence of both damaging agents often happens (European Food Safety Authority, 2011).

Besides co-occurrence of T-2 toxin and HT-2 toxin, most of the *Fusarium* species are capable of producing more than one mycotoxin (Eriksen and Alexander, 1998). In contrast to HT-2 toxin, the acute toxicity of T-2 toxin and other mycotoxins varies and possibly, they interfere with each other. Thomson and Wannemacher (1986) investigated the effects of co-occurrence of several trichothecenes on the protein synthesis in Vero cells. Remarkably, deoxynivalenol appeared to be slightly antagonistic in an equimolar combination with T-2 toxin. Thuvander et al. (1999) examined the inhibitory effect of combined mycotoxins on the proliferation of human peripheral lymphocytes. Combinations of deoxynivalenol with T-2 toxin and/or diacetoxyscirpenol resulted in an antagonistic effect to the toxicity produced when exposed to T-2 toxin or diacetoxyscirpenol alone. In both cases, no synergistic action was noticed. The effects of co-exposure of several mycotoxins in pigs, was investigated by Friend et al. (1992). Twelve week old pigs were fed a diet containing 2.5 mg DON/kg feed and/or T-2 toxin at concentrations ranging from 0.1 to 3.2 mg T-2 toxin/kg feed. A reduced feed consumption was observed in the group fed deoxynivalenol alone and the highest level of T-2 toxin. Upon combined exposure, a reduced feed intake and body weight were observed

at the lowest and the highest dose of T-2 toxin, but not at the intermediate T-2 toxin dose. Co-exposure of broiler chickens to deoxynivalenol (16 mg/kg feed) and T-2 toxin (4 mg/kg) reduced the total body weight gains, whereas given alone, this was not observed (Kubena et al., 1989). Additive effects of T-2 toxin and diacetoxyscirpenol have been observed on lethality of broiler chickens (Hoerr et al., 1980) and for reduced feed consumption and incidence of oral lesions in laying hens (Diaz et al., 1994).

In conclusion, both additive and antagonistic effects have been noticed after combined exposure of animals to T-2 toxin with other mycotoxins. Therefore, the exact outcome cannot be predicted, but it is clear that co-occurrence of several mycotoxins can influence their action.

### **T-2 toxin: tolerable daily feed intake?**

In 2001, the Joint FAO/WHO Expert Committee on Food additives established a provisional maximum tolerable daily intake value of 60 ng/kg body weight per day for humans, for the sum of T-2 and HT-2 toxin (Anonymous, 2001). Provisional maximum tolerable daily intake values are based on animal studies and are calculated by dividing the NOAEL (no-observed-adverse-effect-level) or the LOAEL (lowest-observed-adverse-effect-level) by a safety factor. The provisional maximum tolerable daily intake of 60 ng/kg body weight per day was based on the haematotoxicity and immunotoxicity of T-2 toxin in pigs, since these are one of the most sensitive species to T-2 toxin. In a short term study (3 weeks) of Rafai et al. (1995), reduced number of leukocytes and lymphocytes, reduced antibody production against horse globulin, and a decrease in the lobule size of thymus and spleen was noticed in pigs at a LOAEL of 30 µg T-2 toxin per kg body weight per day. Since similar effects were not observed in other studies in pigs neither at this nor even higher doses, the Joint FAO/WHO Expert Committee on Food additives established that it would be very likely that the LOAEL in the study of Rafai et al. (1995) is close to the NOAEL. However, in order to account for the use of the LOAEL and study deficiencies, an uncertainty factor of 500 was included. This leads to a provisional maximum tolerable daily intake of 60 ng/kg body weight per day. In line with these results, the Scientific Committee on Food concluded in 2001 that the general toxicity, haematotoxicity and immunotoxicity of T-2 toxin are the critical effects and established a combined temporary tolerable daily intake for the sum of T-2 toxin and HT-2 toxin of 60 ng T-2 toxin/kg body weight (Anonymous, 2001).

Recently the Panel on Contaminants in the Food Chain established a new group tolerable daily intake of 100 ng/kg body weight for the sum of T-2 and HT-2 toxin (European Food Safety Authority, 2011). A feeding trial during 28 days conducted by Meissonnier et al. (2009) investigated the effects of 0, 0.54, 1.32 and 2.10 mg T-2 toxin per kg feed on pigs with a starting body weight of 11.4 kg. Diets containing 2.10 mg T-2 toxin/kg significantly decreased the live weight gain. Immunoglobulin concentrations in plasma of the pigs were not altered, except a significantly increased level of IgA on day 7 in the group fed the highest contaminated diet. In conclusion, the most sensitive endpoints that have been reported are still the immunological or haematological effects that occur from doses of 30 µg T-2 toxin/kg body weight per day (equivalent to 500 µg T-2 toxin/kg feed) (Rafai et al., 1995). However, since still no NOEL is available, alternative to the NOEL-LOEL, the Panel on Contaminants in the Food Chain conducted a benchmark dose analysis. Based on the results of Rafai et al. (1995) and Meissonnier et al. (2009), a 95% lower confidence limit for the benchmark dose response of 5% (BMDL05) was calculated for anti-horse globulin titre. An uncertainty factor of 100 was applied to the BMDL05 of 10 µg T-2 toxin/kg body weight per day. Therefore, the Panel on Contaminants in the Food Chain concluded that a full tolerable daily intake of 100 ng/kg body weight can now be established.

This tolerable daily intake of 100 ng/kg body weight is however a guidance value for human exposure. Due to the limited knowledge on the effects of T-2 and HT-2 toxins on farm and companion animals, and the absence of a comprehensive database on feed consumption by livestock in the European Union, the risks of these toxins on animal health have not been properly assessed. Moreover, there is still a need for more adequate information on the exposure of T-2 toxin and HT-2 toxin, i.e. occurrence in food and feed commodities and intakes. According to the Panel on Contaminants in the Food Chain, comparison of the estimates of exposure based on the reported levels of the sum of T-2 and HT-2 toxin in feeds to the BMDL05 for pigs of 10 µg T-2 toxin/kg body weight per day, the risk of adverse health effects as a result of consuming feed containing T-2 and HT-2 toxins is low (European Food Safety Authority, 2011).

Nevertheless, we showed that the addition of 83 µg T-2 toxin per kg feed results in a significant reduced average weight gain (%) during a feeding period of 18 days. Due to the housing conditions, we were unable to record the daily feed intake accurately. However, Carr (1998) estimates that a pig eats 4% of its body weight per day. The pigs that received 83 µg T-2 toxin per kg feed had an average initial weight of 6.1 kg, resulting in an average 0.244 kg feed consumption per day. This corresponds to an intake of 20.3 µg T-2 toxin per day for a

piglet of 6.1 kg or a daily intake of 3.3 µg T-2 toxin per kg body weight. According to these data, even a concentration below the BMDL05 for pigs results in a reduced average weight gain (%). We should not neglect the fact that this is only an estimation because the actual daily feed intake was not recorded. However, we indicate that consuming feed containing T-2 at concentrations below the BMDL05 for pigs is detrimental for the animals. Therefore, in our opinion there is still a need to establish maximum guidance limits in the feed for this mycotoxin, as is already the case for other mycotoxins.

### **Mycotoxin binders: the solution for mycotoxin contamination?**

In experimental study 4, we indicated that the addition of the mycotoxin detoxifying agent to T-2 toxin contaminated feed counteracted the reduced weight gain of pigs caused by T-2 toxin. However, whether this is the result of the actual binding of T-2 toxin by the modified glucomannan feed additive is unknown. Only recently the European Food Safety Authority implemented that *in vivo* trials to test the efficacy and safety of these mycotoxin detoxifying agents should be performed (European Food Safety Authority, 2010). This is why the *in vivo* efficacy of a lot of mycotoxin-adsorbing agents is yet unknown.

According to the European Food Safety Authority, the efficacy of the additive should be evaluated at the existing maximum or guidance levels of the mycotoxin (European Food Safety Authority, 2010). However, no maximum levels are yet established for T-2 toxin in pig feed. Furthermore, recently it was shown that when pigs received an oral bolus of 100 µg/kg T-2 toxin, even after 10 minutes, no T-2 toxin could be detected in the blood plasma of these pigs. Possibly T-2 toxin is very rapidly metabolised and as a consequence, it can not be detected in the blood plasma (De Baere et al., 2011). Consequently, it is difficult to determine the efficacy of mycotoxin detoxifying agents in the presence of T-2 toxin.

Since the *in vivo* efficacy of the modified glucomannan feed additive was not tested, it is not excluded that the mycotoxin detoxifying agent tested here, does not really bind T-2 toxin. Due to policy reasons of the company, the exact composition of the modified glucomannan feed additive is undisclosed. However, we know that it is derived from the cell wall of *Saccharomyces cerevisiae*, containing α-D-mannans and β-D-glucans. It has been described that α-D-mannan binds with type 1 fimbriae of *Salmonella* (Firon et al., 1983), which can lead to a decreased attachment and colonization by pathogens. For some β-glucans, it was shown that they can enhance the functional status of cells of the porcine immune system (Sonck et al., 2009; 2010). Furthermore, feed supplementation with Alphamune®



accelerated the gastrointestinal maturation in turkey poult (Solis de los Santos, et al., 2007). Alphamune<sup>®</sup> is a yeast-extract feed additive derived from *Saccharomyces cerevisiae* that also contains  $\alpha$ -mannans and  $\beta$ -glucans. Therefore, it is not unlikely that the modified glucomannan mycotoxin feed additive exerts its positive effects by improving the gut health of pigs instead of really binding the mycotoxin.

### **T-2 toxin: does it interfere with the serological response of pigs against *Salmonella* Typhimurium?**

We showed that T-2 toxin affects the colonization of *Salmonella* Typhimurium in pigs, but we did not investigate the effects of T-2 toxin on the serological response of pigs against *Salmonella* Typhimurium. However, several studies describe an altered vaccinal serological response of pigs after ingestion of certain mycotoxins. Pinton et al. (2008) showed that deoxynivalenol (2.2-2.5 mg/kg feed) increases the concentration of ovalbumin-specific IgA and IgG in ovalbumin immunized pigs. In contrast, dose-dependent decreases in antibody formation were seen in immunised pigs (horse globulin) that received T-2 toxin contaminated feed at several concentrations ranging from 0.5-3.0 mg/kg feed (Rafai et al., 1995). This was confirmed by Meissonier et al. (2009) who showed that pigs fed T-2 toxin contaminated feed exhibited reduced anti-ovalbumin antibody production.

An altered serological response against *Salmonella* Typhimurium could, however, interfere with the European *Salmonella* serosurveillance programmes that are mostly based on the detection of antibodies against the lipopolysaccharides of *Salmonella* (Abrahantes et al., 2009). To control *Salmonella* at the pre-harvest stage, surveillance and control programmes have been established in the different EU Member States. Since 2005, the Belgian Federal Agency for the Safety of the Food Chain implemented the *Salmonella* Action Plan (SAP) to control *Salmonella* in pig production, which became compulsory by means of a Royal decree in July 2007 (Dierengezondheidszorg Vlaanderen, accessed on 27/01/2012). This implies that based on serological analysis of blood samples collected from the fattening pigs, Belgian pig farms can be assigned as *Salmonella*-risk farms. If T-2 toxin would interfere with the antibody formation in pigs against *Salmonella* Typhimurium, than this could lead to false negative serum samples and as a consequence, an underestimation of the number of *Salmonella* positive pig farms.

Furthermore, we showed that the addition of 15 and 83  $\mu$ g T-2 toxin per kg feed significantly decreased the amount of *Salmonella* Typhimurium bacteria present in the cecal

contents. A reduced colonization of the jejunum, ileum, cecum, colon and colon contents was also noticed, however not significantly. This could lead to the development of carrier pigs instead of pigs that constantly shed *Salmonella* Typhimurium in their faeces. As shown by Österberg and Wallgren (2008), seroconversion of pigs that intermittently shed *Salmonella* Typhimurium, is delayed in comparison of constant shedders. Possibly, the presence of T-2 toxin in the feed does not only interfere with the antibody formation against *Salmonella* Typhimurium in pigs, but also may stimulate the formation of carrier pigs. Consequently, these carrier pigs can be susceptible to stress induced recrudescence of the infection.

### **The effects of T-2 toxin on the immune system: a model for other mycotoxins?**

The immune system is one of the main targets of mycotoxins and it can affect both the humoral and cellular immune response. In literature, the effects of mycotoxins on the immune system of pigs are mostly described for aflatoxins and deoxynivalenol. Aflatoxins have little or no effects on swine humoral immune responses (Miller et al., 1981; Panangala et al., 1986), but they affect the cellular immune system (Miller et al., 1987; van Heugten et al., 1994). Aflatoxins decrease the lymphocyte blastogenic response to mitogens and reduce macrophage migration (Miller et al., 1987; van Heugten et al., 1994). In contrast to these aflatoxins, T-2 toxin affects the humoral immune response by decreasing antibody formation in immunised pigs (Rafai et al., 1995; Meissonier et al., 2009). Depletion of lymphoid elements in the thymus and spleen was noticed and the leukocyte counts and portion of T lymphocytes were decreased in all exposure groups (Rafai et al., 1995, Schuhmacher-Wolz et al., 2010).

Ingestion of a low dose of fumonisin B1 (0.5 mg/kg body weight during 7 days) decreases the expression of IL-8 mRNA in the ileum of piglets (Bouhet et al., 2006). This decrease in IL-8 may lead to a reduced recruitment of inflammatory cells in the intestine and may participate in the increased susceptibility to intestinal infections (Oswald et al., 2003). Recently Devriendt et al. (2009) provided evidence that fumonisin B1 (1 mg/kg body weight during 10 days) reduces the intestinal expression of IL-12/IL-23p40, resulting in a reduced antigen presenting cell maturation and prolonged F4<sup>+</sup> enterotoxigenic *Escherichia coli* infection. In experimental study 3, we showed that IL-12/IL-23p40 expression is reduced after T-2 toxin treatment, however not significantly. If we extrapolate the results of Devriendt et al. (2009) to our results, a reduced maturation of *in vivo* antigen presenting cells could also be expected. However, in contrast to the prolonged F4<sup>+</sup> enterotoxigenic *Escherichia coli* infection, we noticed a reduced colonization of *Salmonella* Typhimurium.

For deoxynivalenol, it is known that it can either be immunostimulatory or immunosuppressive. Low doses of deoxynivalenol act immunostimulating by increasing production and secretion of pro-inflammatory cytokines. High doses of deoxynivalenol act on the other hand immunosuppressive by causing apoptosis of leucocytes (Yang et al., 2000). Deoxynivalenol affects the humoral immune response by increasing IgA in the serum of pigs, as well as the levels of expression of several cytokines such as IL-6, IL-10, IFN $\gamma$  and TNF- $\alpha$ . (Bergsjö et al., 1993; Grosjean et al., 2002; Swamy et al., 2002; Pinton et al., 2004). Using a porcine ligated intestinal ileal loop model, Vandebroucke et al. (2011) demonstrated that low doses of deoxynivalenol enhanced the intestinal inflammatory response to *Salmonella* Typhimurium. Co-exposure of pig loops to 1 mg/mL of deoxynivalenol and *Salmonella* Typhimurium compared to loops exposed to *Salmonella* Typhimurium alone, caused an increased expression of IL-12/IL-23p40 and TNF- $\alpha$ . Exposure of the ileal loops to deoxynivalenol alone did not alter the mRNA expression level of IL-1 $\beta$ , IL-6, IL-8, IL-12/IL-23p40, IL-18, TNF $\alpha$ , IFN $\gamma$  and monocyte chemoattractant protein-1 (MCP-1). By the use of *in vitro* tests, Vandebroucke et al. (2011) showed that deoxynivalenol renders the intestinal epithelium more susceptible to invasion and translocation of *Salmonella* Typhimurium. Possibly, deoxynivalenol stimulates the intestinal colonization of *Salmonella* Typhimurium in pigs, leading to an increased inflammatory response.

In contrast to the results obtained by Vandebroucke et al. (2011), we showed that the ingestion of low T-2 toxin concentrations (15 or 83  $\mu\text{g}/\text{kg}$ ) results in reduced amounts of *Salmonella* Typhimurium in cecal contents of pigs. A reduced colonization of the ileum, cecum and colon was also observed, however not significantly. Furthermore, we pointed out that the addition of T-2 toxin (15  $\mu\text{g}/\text{kg}$ ) resulted in a significant reduced expression of IL-1 $\beta$ . The effect of T-2 toxin on uninfected animals was not investigated. Thus, we do not know whether T-2 toxin, at a low dose (15 or 83  $\mu\text{g}/\text{kg}$ ), acts as an immunostimulator or an immunosuppressor in *Salmonella* negative pigs. Possibly, the decreased expression is a direct result of the reduced colonization of the gut by *Salmonella* Typhimurium. In general, the results in pigs obtained with T-2 toxin cannot be extrapolated to other mycotoxins.

**... Stress and T-2 toxin as two factors influencing a *Salmonella* Typhimurium infection in pigs ...**

In this thesis we showed that both stress and T-2 toxin can alter the pathogenesis of a *Salmonella* Typhimurium infection in pigs. Stress can cause recrudescence of a chronic infection and low doses of T-2 toxin can decrease the amount of *Salmonella* bacteria in the cecal contents of pigs. However, these two factors can be simultaneously present in pig farms. Until now, the combined effect of both factors has not been studied. Thus, one could wonder what the outcome would be on a *Salmonella* Typhimurium infection if both stress and T-2 toxin are present.

**Stress and T-2 toxin: what would be the outcome on the colonization of *Salmonella* Typhimurium in pigs?**

We showed that although T-2 toxin increases bacterial invasion and translocation *in vitro*, it also affects the gene expression and motility of *Salmonella* Typhimurium resulting in a reduced colonization of the bacteria in pigs. The effect of stress and its stress hormone cortisol on the colonization of *Salmonella* Typhimurium in pigs was not investigated in this thesis. However, we showed that cortisol does not affect the gene expression of extracellular *Salmonella* bacteria. Furthermore, cortisol, epinephrine, norepinephrine and dopamine do not affect the invasion of *Salmonella* Typhimurium in porcine macrophages and enterocytes. Taken these results into account, one could hypothesize that stress does not influence the colonization of *Salmonella* Typhimurium in pigs. Consequently the effect of T-2 toxin would probably dominate, with a reduced colonization of *Salmonella* Typhimurium in pigs as a result. However, other factors such as the timing of T-2 toxin exposure and other stress hormones may also play a role.

Studies in mice showed that depending on the timing of T-2 toxin exposure, a different outcome on bacterial infections could be achieved. Corrier and Ziprin demonstrated that short-term preinoculation with T-2 toxin enhanced the resistance to *Listeria monocytogenes*. On the other hand, when mice were first inoculated with this bacterium and thereafter with T-2 toxin, this resulted in immunosuppression (Corrier and Ziprin, 1986a, 1986b; Corrier et al., 1987a, 1987b). A significant increase in phagocytic activity occurred in macrophages from mice treated with T-2 toxin and subsequently sensitized with sheep red blood cells. In contrast, phagocytosis of sheep red blood cells was suppressed in cells from mice treated with

T-2 toxin after sensitization. Therefore, it has been suggested that the enhanced resistance to *Listeria monocytogenes* is associated with an increased migration of macrophages and an elevated phagocytic activity, which may have been mediated by altered T-regulatory cell activity (Corrier et al., 1987a). Possibly, in our experiment pre-exposure of T-2 toxin also caused an activation of the immune system, making the colonization of the already weakened bacterium more difficult. It can not be excluded that an increased colonization would be observed if we started the T-2 toxin treatment after infection with *Salmonella* Typhimurium.

Stress results in the release of a variety of neurotransmitters, peptides, cytokines, hormones and other factors into the circulation or tissues. Although we showed that cortisol does not affect extracellular *Salmonella* Typhimurium bacteria, it has been described that norepinephrine promotes growth, and motility of *Salmonella* (Bearson and Bearson, 2008; Bearson et al., 2008; Methner et al., 2008). An increased motility and growth could result in an increased invasion of *Salmonella* Typhimurium in pigs. It can thus not be excluded that although cortisol does not affect the extracellular bacterium nor the invasion in host cells, stress in general affects colonization of *Salmonella* Typhimurium in pigs.

Taking all these assumptions into account, drawing a general conclusion on the colonization of *Salmonella* Typhimurium is very hard without investigating the simultaneous effects of both stress and T-2 toxin *in vitro* and *in vivo*.

### **Stress and T-2 toxin: what would be the outcome on a chronic *Salmonella* Typhimurium infection in pigs?**

We showed that starvation stress and glucocorticoids (dexamethasone) are able to cause recrudescence of a latent *Salmonella* Typhimurium infection. The effect of T-2 toxin on the course of a chronic *Salmonella* Typhimurium infection was not investigated. However, *in vitro*, we showed that non-cytotoxic concentrations of T-2 toxin do not affect the intracellular proliferation of *Salmonella* Typhimurium in porcine macrophages and enterocytes. This might imply that the effect of stress would possibly dominate, with a recrudescence of the infection as result. On the other hand, the effect of both stress and T-2 toxin on the cytoskeleton of host cells should also be taken into account.

Indeed, we demonstrated that cortisol causes an increased intracellular replication of *Salmonella* Typhimurium in porcine macrophages, which is both actin and microtubule dependent. By the use of iTRAQ labeling, we showed that a low T-2 toxin concentration (5 ng/mL) induces alterations in the expression of proteins that are involved in the cytoskeleton

formation of differentiated porcine intestinal epithelial cells. Vandenbroucke et al. (2009) demonstrated that deoxynivalenol modulates the cytoskeleton of porcine macrophages by activation of extracellular signal-regulated kinase 1/2. This is a specific MAPK family member, which plays a role in the invasion and the intracellular proliferation of *Salmonella* Typhimurium in macrophages (Procyk et al., 1999; Uchiya and Kinai, 2004; Zhou et al., 2005). Since both T-2 toxin and cortisol influence the cytoskeleton of host cells, they might act synergistically, resulting in increased intracellular proliferation of *Salmonella* Typhimurium. Further research is however needed to confirm or reject this hypothesis.

### ***Salmonella* as a never ending story: future perspectives.**

It is clear that the interplay between *Salmonella*, its host and environmental factors is very complicated and still a lot of work has to be done to unravel the interactions and their outcome. *Salmonella* still has to show us many aspects of its lifestyle within the *Salmonella* containing vacuole. Investigations are needed to clarify whether cortisol influences the composition of the microenvironment within the *Salmonella* containing vacuole. Furthermore, it must be examined whether and how cortisol causes a transmigration of *Salmonella* bacteria or *Salmonella* infected macrophages from the lamina propria over the intestinal epithelium towards the intestinal lumen. Alternatively, is *Salmonella* able to survive and is it excreted in bile fluids following a stress response? Possibly, catecholamines and glucocorticoids work together to cause the recrudescence of a *Salmonella* infection. The answers to these questions would help us to understand the stress induced shedding of *Salmonella* Typhimurium in pigs. Next to these future directions, it would be interesting to investigate the effect of T-2 toxin on the serological response of pigs after an experimental infection with *Salmonella* Typhimurium. Finally it would be interesting to examine the effect of both stress and T-2 toxin on the colonization and persistency of *Salmonella* Typhimurium in pigs.

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# **Summary**





Worldwide, *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is the predominant serovar isolated from slaughter pigs and one of the major causes of foodborne salmonellosis in humans. Infections of pigs with *Salmonella* Typhimurium often result in the development of carriers that excrete *Salmonella* in very low numbers. The pathogenesis of a *Salmonella* Typhimurium infection in pigs can however be influenced by environmental and host factors. In this thesis, we investigated the effects of host stress, T-2 toxin and a commercially available modified glucomannan feed additive on host-pathogen interactions of *Salmonella* Typhimurium.

Periods of stress, such as transport to the slaughterhouse, may induce recrudescence of *Salmonella*. This results in increased cross-contamination during transport and lairage and as a consequence in a higher level of pig carcass contamination. This implies that stress induced recrudescence plays a key role in contamination of the human food chain with *Salmonella*. During the past decade, microbial endocrinology was introduced as a new research area where microbiology and neurophysiology intersect. Recent work from this field shows that bacteria can exploit the neuroendocrine alteration due to a stress reaction as a signal for growth and pathogenic processes. However, until now, the mechanism of stress related recrudescence of *Salmonella* Typhimurium by pigs remains poorly understood.

When we started our studies, most research in microbial endocrinology was limited to the influence of catecholamines. However, in the **first chapter** of this thesis, we established that cortisol plays an important role in the stress related recrudescence of *Salmonella* Typhimurium in pigs. We mimicked the feed withdrawal period before slaughter by submitting *Salmonella* Typhimurium carrier pigs to a 24 hour feed withdrawal. This “starvation stress” raised the serum cortisol levels and increased the intestinal *Salmonella* Typhimurium load in these pigs. In addition, we revealed that a short-term treatment of carrier pigs with dexamethasone, a synthetic cortisol derivative, resulted in the recrudescence of *Salmonella* Typhimurium. In persistently infected pigs, *Salmonella* resides mainly as an intracellular pathogen inside macrophages. After invasion of macrophages, *Salmonella* remains within *Salmonella* containing vacuoles that serve as unique intracellular compartments where the bacterium eventually replicates. We showed that cortisol, but not catecholamines, promotes intracellular proliferation of *Salmonella* Typhimurium in primary porcine alveolar macrophages. By the use of a microarray based transcriptomic analysis, we revealed that cortisol did not directly affect the growth, nor the gene expression of *Salmonella*

Typhimurium in a rich medium. This implies that the increase in intracellular proliferation is triggered by cortisol induced changes in the host cell and not by a direct interaction of the bacterium with cortisol. Since until now, most of the research concerning this topic has been limited to the effects of catecholamines, our results highlight the fact that the influence of cortisol and glucocorticoids in general, on pathogenic infections, is severely underrated.

Because the gene expression of *Salmonella* Typhimurium was not affected by physiological stress cortisol concentrations, it was hypothesized **in chapter 2** that cortisol exerts its effect on intracellular *Salmonella* bacteria through modulation of the *Salmonella* containing vacuole. Intracellular replication of *Salmonella* Typhimurium is accompanied by a complex series of cytoskeletal changes, such as F-actin rearrangements and the formation of continuous tubular aggregates. We showed that the cortisol induced increased survival of *Salmonella* Typhimurium in primary porcine macrophages was both actin and microtubule dependent. In addition to this, proteomic analysis of *Salmonella* Typhimurium infected primary porcine macrophages revealed that cortisol caused an increased expression of cytoskeleton associated proteins, including a constituent of the *Salmonella* containing vacuole, a component of microtubules and proteins that regulate the polymerization of actin. Using *in vivo* expression technology, we established that the intracellular gene expression of *Salmonella* Typhimurium was altered during cortisol treatment of *Salmonella* Typhimurium infected primary porcine macrophages and we identified *scsA* as a major driver for the increased intracellular survival of *Salmonella* Typhimurium during cortisol exposure to these cells. We thus conclude that cortisol affects the host cell, and by doing so, indirectly influences the bacterial gene expression, resulting in an increased survival within this harmful intracellular environment.

Besides *Salmonella* infections, mycotoxin contamination of cereals and other food types for human and animal consumption is a major problem. T-2 toxin is an emerging mycotoxin classified as a type A trichothecene, produced by various *Fusarium* spp. and it is considered the most acutely toxic trichothecene, especially for pigs which are one of the most sensitive species to T-2 toxin. The effects of moderate to high amounts of T-2 toxin in pigs are well characterized and they range from immunosuppression, feed refusal, vomiting, weight loss, reduced growth to skin lesions. However, when we started our studies, most of the T-2 toxin related research was limited to the effects of high and sometimes irrelevant concentrations of T-2 toxin. Since T-2 toxin may affect intestinal epithelial cells and

macrophages, which are two cell types that play an important role in the pathogenesis of a *Salmonella* Typhimurium infection, we aimed at investigating the effects of low and relevant concentrations of T-2 toxin on a *Salmonella* Typhimurium infection in pigs.

In the **third chapter** of this thesis, we showed that the addition of 15 and 83  $\mu\text{g}$  T-2 toxin per kg feed reduced the number of *Salmonella* Typhimurium bacteria present in the cecal contents of experimentally infected pigs. We furthermore showed that a low concentration of 83  $\mu\text{g}$  T-2 toxin per kg feed reduced the weight gain in uninfected pigs. These data indicate that even low concentrations of T-2 toxin are detrimental for pig growth and that they interfere with the pathogenesis of a *Salmonella* Typhimurium infection in pigs. In order to elucidate the mechanism of the reduced colonization of *Salmonella* Typhimurium in pigs, we tried to explain the effects of T-2 toxin on host cells, the bacterium itself and host-pathogen interactions of *Salmonella* Typhimurium with these cells. The studies discussed in the third chapter, showed that exposure of primary porcine macrophages and porcine intestinal epithelial cells to T-2 toxin, led to an increased invasion and transepithelial passage of the bacterium. Proteomic analysis demonstrated that T-2 toxin induces alterations in the expression of proteins that are involved in the cytoskeleton formation of differentiated porcine intestinal epithelial cells. Possibly T-2 toxin and *Salmonella* Typhimurium act synergistically, inducing cytoskeletal reorganizations which increase the invasion of the bacterium. Extrapolating these results to the *in vivo* situation, one would expect an increased colonization of *Salmonella* in pigs. However, microarray analysis showed that T-2 toxin causes an intoxication of *Salmonella* Typhimurium, represented by a reduced motility and a downregulation of metabolic and *Salmonella* Pathogenicity Island 1 genes. This demonstrates that although T-2 toxin increases bacterial invasion and translocation, it also affects the gene expression and motility of *Salmonella* Typhimurium resulting in a reduced colonization of the bacteria in pigs. These data are of particular importance to understand how feed-associated mycotoxins and pathogenic bacteria interact.

One strategy for reducing the exposure of pigs to mycotoxins is to decrease the bioavailability of mycotoxins through the use of mycotoxin binders in the feed. Modified glucomannan binders are mycotoxin-adsorbing agents that theoretically counteract the effects of mycotoxins. These binding agents are derived from the cell wall of *Saccharomyces cerevisiae*, and consist of  $\alpha$ -D-mannans and  $\beta$ -D-glucans. Both are polysaccharides of which their protective role against *Salmonella* infections has been described. Therefore, the aim of

the **fourth study** was to investigate the effect of a commercially available modified glucomannan feed additive on the course of a *Salmonella* Typhimurium infection in T-2 toxin exposed and unexposed pigs. We showed that the addition of the modified glucomannan feed additive to feed contaminated with 83  $\mu\text{g}$  T-2 toxin per kg feed neutralized the reduced weight gain seen in pigs that were fed 83  $\mu\text{g}$  T-2 toxin. As demonstrated in experimental study 3, supplementation of pig feed with 83  $\mu\text{g}$  T-2 toxin per kg feed reduced the intestinal *Salmonella* load. We now showed that the addition of the feed additive to T-2 toxin contaminated feed, also significantly reduced the amount of *Salmonella* Typhimurium bacteria present in the cecum and cecal contents, in comparison to control pigs. Moreover, an overall reduced intestinal colonization was observed after addition of the modified glucomannan to blank feed, however not significant. It has been described that  $\alpha$ -D-mannans bind with mannose-specific lectin-type receptors, such as type 1 fimbriae of *Salmonella*. We showed that after 4 hours, the modified glucomannan feed additive significantly reduced the number of *Salmonella* bacteria in a minimal medium in comparison to the start of the incubation period, possibly by binding to the bacteria. Thus, we highlighted the protective role of the modified glucomannan feed additive tested here, against the weight loss in pigs caused by T-2 toxin and we provided evidence that it may also reduce the intestinal colonization of *Salmonella* Typhimurium.

The results presented in this thesis clearly demonstrate that stress, T-2 toxin and the modified glucomannan feed additive can modulate host-pathogen interactions of *Salmonella* Typhimurium in pigs at various stages. Our studies present new insights in the interactions of the stress hormone cortisol with host-pathogen interactions. We showed that the glucocorticoid cortisol is involved in a stress induced recrudescence of *Salmonella* Typhimurium in carrier pigs. This stress hormone promotes the intracellular proliferation of *Salmonella* Typhimurium in porcine macrophages, via an indirect effect through the cell. We furthermore showed that the cortisol induced host-cell alterations are associated with the alteration of the intracellular gene expression of *Salmonella* Typhimurium resulting in a *scsA* dependent intracellular proliferation of *Salmonella* Typhimurium in pig macrophages. As shown by the T-2 toxin research, we pointed out that although T-2 toxin increases bacterial invasion and translocation, it also affects the gene expression and motility of *Salmonella* Typhimurium resulting in a reduced colonization of the bacteria in pigs.

# **Samenvatting**



Besmet varkensvlees is een belangrijke bron van *Salmonella* infecties bij de mens. Momenteel is *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) het meest geïsoleerde serotype bij varkens. De pathogenese van een *Salmonella* Typhimurium infectie in varkens kan beïnvloed worden door gastheer- en omgevingsfactoren. In deze thesis werden de effecten nagegaan van stress, het T2-toxine en een gewijzigd glucomannaan voederadditief op de interactie van *Salmonella* Typhimurium met het varken.

Varkens die geïnfecteerd worden met *Salmonella* Typhimurium maken in de regel eerst een acute fase door waarbij de kiem vaak in hoge aantallen wordt uitgescheiden. Sommige dieren blijven daarna vaak nog langdurig drager van de kiem in de tonsillen, het ileum, het colon, het cecum en de ileocaecale lymfeknopen. Dergelijke dragers of “carriers” scheiden slechts minieme en moeilijk detecteerbare aantallen kiemen uit in de mest. Perioden van stress, zoals transport naar het slachthuis, kunnen bij varkens die drager zijn van *Salmonella* de infectie laten opflakkeren, waardoor deze dieren vlak voor het slachten plots massaal *Salmonella* gaan uitscheiden in de mest. Hierdoor worden hun lotgenoten besmet, wat resulteert in een groter aantal positieve varkens voor het slachten en een toename van de karkascontaminatie met *Salmonella*. De kiemuitscheiders spelen dus een sleutelrol in de uiteindelijke contaminatie van varkensskarkassen met *Salmonella* Typhimurium. Het mechanisme van deze stress gerelateerde her-excretie van *Salmonella* in varkens is echter nog niet gekend. Daarom was het eerste doel van deze thesis om meer inzicht te verwerven in het mechanisme dat zorgt voor deze plotse heruitscheiding net voor het slachten.

Stress resulteert in de vrijzetting van stresshormonen zoals catecholamines (adrenaline, noradrenaline en dopamine) en glucocorticoiden (cortisol). In een **eerste hoofdstuk** hebben we aangetoond dat cortisol een belangrijke rol speelt in de stress-gerelateerde heropflakking van *Salmonella* Typhimurium in varkens. In eerste instantie werd het vasten voor het slachten nagebootst door het voeder weg te nemen van drager varkens, 24 uur voor het slachten. Deze stress door vasten resulteerde in verhoogde serum cortisol concentraties en een verhoogd aantal *Salmonella* Typhimurium bacteriën in het darmweefsel en de darminhoud. Bovendien hebben we aangetoond dat één intramusculaire injectie van dexamethasone, een synthetisch cortisol-derivaat, een opflakking veroorzaakt van een *Salmonella* Typhimurium infectie in drager varkens. Er werd beschreven dat bij varkens die langdurig drager zijn van *Salmonella* Typhimurium, de kiem intracellulair

persisteert in macrofagen. Na invasie van macrofagen verblijft *Salmonella* in vacuoles (de zogenaamde “*Salmonella* containing vacuoles”), waarin de kiem zich kan verschuilen en zelfs vermenigvuldigen. We hebben aangetoond dat cortisol de intracellulaire vermeerdering van *Salmonella* Typhimurium in primaire varkensmacrofagen verhoogt. Behandeling van extracellulaire bacteriën in Luria-Bertani broth met cortisol beïnvloedde daarentegen hun groei en genexpressie niet. Deze data impliceren dat de verhoogde intracellulaire proliferatie van *Salmonella* Typhimurium in primaire varkensmacrofagen niet het gevolg is van een direct effect van cortisol op de kiem.

Aangezien cortisol geen directe invloed heeft op de genexpressie van *Salmonella* Typhimurium, werd in **hoofdstuk 2** de hypothese getest of cortisol de proliferatie van de kiem indirect beïnvloedt via de modulatie van de gastheercel. Intracellulaire vermeerdering van *Salmonella* Typhimurium gaat gepaard met cytoskeletale veranderingen, zoals F-actine herschikkingen en de vorming van tubulus-complexen. We hebben inderdaad aangetoond dat de cortisol geïnduceerde verhoogde overleving van *Salmonella* Typhimurium in primaire varkensmacrofagen, zowel actine als microtubulus afhankelijk is. Daarenboven werd via een proteoomanalyse aangetoond dat cortisol een verhoogde expressie veroorzaakt van cytoskeletale eiwitten in met *Salmonella* Typhimurium geïnfekteerde varkensmacrofagen, inclusief een bouwsteen van de “*Salmonella* containing vacuoles”, een component van de microtubuli en eiwitten die betrokken zijn bij de polymerisatie van actine. Dat deze cortisol effecten op de gastheercel een invloed hebben op *Salmonella* Typhimurium blijkt uit een genoom wijde screening via *in vivo* expressie technologie (IVET). Hiermee werd nagegaan welke promotoren van *Salmonella* Typhimurium geactiveerd worden wanneer deze zich intracellulair bevindt in macrofagen die behandeld worden met cortisol. Er werd aangetoond dat de genexpressie van intracellulaire kiemen inderdaad beïnvloed wordt door cortisol behandeling van de gastheercel. Daarenboven werd, via *in vitro* studies met *Salmonella* deletiemutanten, *scsA* geïdentificeerd als een gen dat betrokken is in de proliferatie van *Salmonella* Typhimurium in met cortisol behandelde varkensmacrofagen. Dit werk heeft dus voor de eerste maal aangetoond dat de genexpressie van intracellulaire *Salmonella* Typhimurium bacteriën in primaire varkensmacrofagen beïnvloed wordt door een cortisol geïnduceerd cel-gemedieerd effect.

Naast *Salmonella* Typhimurium infecties, zijn varkens eveneens gevoelig voor de effecten van diverse mycotoxines. T2-toxine wordt geproduceerd door *Fusarium* spp. en het



is een opkomende contaminant van granen en andere gewassen die vaak gebruikt worden in de voeding van mens en dier. Het T2-toxine is ongevoelig voor verhitting en blijft stabiel tijdens de verwerking van de gewassen waardoor het in de voedselketen kan terechtkomen. Varkens zijn één van de gevoeligste diersoorten voor het T2-toxine en de effecten van middelmatige tot hoge concentraties variëren van immunosuppressie, voedselweigeringsbraken, gewichtsverlies, verminderde groei tot beschadiging van de huid. De invloed van opname door het varken van lage, voor de praktijk relevante concentraties van T2-toxine, was bij aanvang van deze thesis echter niet gekend. Aangezien het T2-toxine de darmgezondheid en de immuunstatus van varkens kan beïnvloeden, twee factoren die belangrijk zijn in de pathogenese van een *Salmonella* Typhimurium infectie, was het onze doelstelling om het effect na te gaan van dergelijke voor de praktijk relevante concentraties van T2-toxine op een *Salmonella* Typhimurium infectie in varkens.

In een **derde experimentele proefopzet** werd aangetoond dat de aanwezigheid van zowel 15 als 83 µg T2-toxine per kg voeder, een significante daling veroorzaakt in het aantal *Salmonella* Typhimurium bacteriën in de cecum inhoud van experimenteel besmette biggen. Reeds na een voederperiode van 18 dagen met 83 µg T2-toxine per kg voeder, bleek dat het T2-toxine een nadelige invloed had op de procentuele gewichtstoename van niet besmette biggen. Deze data tonen aan dat zelfs heel lage concentraties van het T2-toxine de gewichtsaanzet van varkens kunnen onderdrukken en dat ze een invloed kunnen hebben op de pathogenese van een *Salmonella* Typhimurium infectie in varkens. Om het mechanisme te achterhalen hoe het T2-toxine de kolonisatie van *Salmonella* Typhimurium in varkens vermindert, werden de effecten bestudeerd van het T2-toxine op gastheercellen, *Salmonella* Typhimurium en de interacties van *Salmonella* Typhimurium met deze gastheercellen. Behandeling van macrofagen en intestinale epitheelcellen met het T2-toxine resulteerde in een verhoogde invasie van de kiem in deze gastheercellen. Daarenboven verhoogde het T2-toxine de translocatie van *Salmonella* Typhimurium over gedifferentieerde intestinale epitheelcellen. Via proteoomanalyse bleek dat het T2-toxine de expressie beïnvloedt van eiwitten betrokken in de formatie van het cytoskelet van gedifferentieerde intestinale epitheelcellen. Mogelijks vertonen het T2-toxine en *Salmonella* Typhimurium een synergistische werking in het induceren van cytoskeletale veranderingen waardoor de bacterie gemakkelijker invadeert in deze gastheercellen. Via microarray analyse werd echter aangetoond dat het T2-toxine ook een intoxicatie van *Salmonella* Typhimurium veroorzaakt. Deze intoxicatie gaat gepaard met een verminderde motiliteit van de kiem en een downregulatie van metabole en *Salmonella*

Pathogeniciteitseiland 1 genen. De studies uit proefopzet 3 tonen aan dat niettegenstaande het T2-toxine *in vitro* een verhoogde invasie en translocatie van de bacterie veroorzaakt, de intoxicatie van kiem leidt tot een verminderde kolonisatie van *Salmonella* Typhimurium in varkens.

Een strategie om de blootstelling aan mycotoxines te reduceren is de toevoeging van mycotoxine-adsorberende stoffen aan het voeder. In theorie kunnen deze stoffen de mycotoxines binden tijdens het verteringsproces waardoor ze niet meer geabsorbeerd worden in de bloedbaan en ze verwijderd worden uit het lichaam. Gewijzigde glucomannaan voederadditieven worden soms geclaimd als dergelijke mycotoxine-adsorberende stoffen. Deze voederadditieven bestaan uit  $\alpha$ -D-mannanen en  $\beta$ -D-glucanen. Van beide polysacchariden is de beschermende rol tegen *Salmonella* infecties reeds beschreven. In een **vierde reeks** experimenten werd dan ook nagegaan wat de invloed was van een commercieel verkrijgbaar gewijzigd glucomannaan voederadditief op een *Salmonella* Typhimurium infectie in met T2-toxine behandelde en onbehandelde varkens. Uit experimentele studie 3 weten we reeds dat het T2-toxine een verminderde kolonisatie van *Salmonella* Typhimurium in varkens veroorzaakt. In deze studie hebben we nu aangetoond dat de toevoeging van het gewijzigde glucomannaan voederadditief aan met het T2-toxine gecontamineerde (83  $\mu$ g/kg) voeder, eveneens resulteerde in een verminderde kolonisatie van het cecum en de cecum inhoud, bij experimenteel besmette biggen. Daarenboven is het voederadditief zelf in staat om een daling in de intestinale kolonisatie van de kiem te veroorzaken, alhoewel niet significant. Het is bekend dat  $\alpha$ -D-mannanen kunnen binden aan type 1 fimbriae van *Salmonella*. Reeds na 4 uur veroorzaakte het gewijzigde glucomannaan voederadditief een daling in het aantal *Salmonella* bacteriën in een minimaal medium in vergelijking met de start van de incubatieperiode. Het is dus waarschijnlijk dat de verminderde kolonisatie van *Salmonella*, het gevolg is van de binding van *Salmonella* Typhimurium aan dit voederadditief. Daarenboven bleek dat toevoeging van het voederadditief aan het T2-toxine gecontamineerde voeder het negatieve effect van het T2-toxine op de gewichtstoename van varkens neutraliseert. Dit bevestigt de beschermende rol van het gewijzigde glucomannaan voederadditief tegen het gewichtsverlies veroorzaakt door het T2-toxine.

De resultaten van deze thesis tonen aan dat zowel stress, T2-toxine als het gewijzigde glucomannaan voederadditief de kiem-gastheer interacties van *Salmonella* Typhimurium in varkens kunnen beïnvloeden, en dit via verschillende mechanismen. Het stresshormoon

cortisol blijkt betrokken te zijn in de stress-geïnduceerde heropflakking van een *Salmonella* Typhimurium infectie bij varkens. Cortisol heeft een indirecte invloed op intracellulaire *Salmonella* Typhimurium bacteriën, wat resulteert in een verhoogde proliferatie van de kiem in primaire varkensmacrofagen. Daarenboven werd het *scsA* gen geïdentificeerd als een stressgevoelig gen dat een rol speelt in de cortisol geïnduceerde verhoogde proliferatie van *Salmonella* Typhimurium in primaire varkensmacrofagen. Via het T2-toxine onderzoek hebben we aangetoond dat desondanks het T2-toxine *in vitro* een verhoogde invasie en translocatie van de bacterie veroorzaakt, de intoxicatie van kiem leidt tot een verminderde kolonisatie van *Salmonella* Typhimurium in varkens.



# **Curriculum vitae**



Elin Verbrugghe werd geboren op 19 december 1985 in Kortrijk. Na het beëindigen van haar middelbare studies Wetenschappen Wiskunde (6 uur) in het Sint-Aloysiuscollege te Menen, startte ze in 2003 de studies Biomedische Wetenschappen aan de Katholieke Universiteit Leuven Campus Kortrijk, om deze verder te zetten aan de Katholieke Universiteit Leuven. In 2007 studeerde ze af als Licentiaat in de Biomedische Wetenschappen, met onderscheiding.

Onmiddellijk na het behalen van haar diploma werkte ze aan diezelfde universiteit als wetenschappelijk medewerkster aan het Laboratorium voor Moleculaire Oncologie. In maart 2008 vatte ze aan de Faculteit Diergeneeskunde (Universiteit Gent) bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten haar doctoraatsonderzoek aan. Hierin heeft ze het effect van stress en T2-toxine op de pathogenese van *Salmonella* Typhimurium infecties bij het varken onderzocht. Dit onderzoek gebeurde in nauwe samenwerking met het laboratorium Toxicologie van de vakgroep Farmacologie, Toxicologie en Biochemie.

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





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# **Dankwoord**



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Elin

