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Optimizing vaccination of pigs against *Salmonella* Typhimurium

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List of abbreviations

APC	Antigen Presenting Cell
BGA	Brilliant Green Agar
BPW	Buffered Peptone Water
CD14	Cluster of Differentiation
CFU	Colony Forming Units
DCs	Dendritic Cells
DGZ	DierenGezondheidsZorg
DIVA	Differentiating Infected from Vaccinated Animals
DNA	DesoxyriboNucleicAcid
DT	Definitive Phage Type
EC	European Community
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked Immunosorbent Assay
EU	European Union
FASFC	Federal Agency for the Safety of the Food Chain
FCA	Freund's Complete Adjuvant
FIA	Freund's Incomplete Adjuvant
GEF	Guanine-nucleotide Exchange Factor
HBSS	Hank Buffered Salt Solution
IFN- ɣ	InterFeroN-gamma
Ig	Immunoglobulin
IL	InterLeukin
IPEC-J2	Intestinal Porcine Epithelial Cell
KDO	3-Keto-2-DeoxyOctonate
LAL	Limulus Amebocyte Lysate
LB	Luria-Bertani
LBP	Lipopolysaccharide Binding Protein
LPS	LipoPolySaccharide
LT	Lilleengen Type

M	Microfold
MAP	Mitogen Activated Protein
MDa	MilliDalton
MHC	Major Histocompatibility Complex
MOI	Multiplicity Of Infection
NAL	NALidixic acid
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NF-ĸB	Necrosis Factor Kappa-B
NK	Natural Killer cell
NOD	Nucleotide-binding Oligomerization Domain
Nramp1	Natural resistance associated macrophage protein
OD	Optical Density
PAMPs	Pathogen-Associated Molecular Patterns
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEEC	Pathogen Elicited Epithelial Chemo-attractant
PMN	PolyMorphoNulear
PP	Peyer's patches
RNI	Reactive Nitrogen Intermediates
ROS	Reactive Oxygen Species
SAP	*Salmonella* Actie Plan
SCV	*Salmonella* Containing Vacuole
SDS	Sodium Dodecyl Sulfate
SPF	Specific Pathogen Free
SPI	*Salmonella* Pathogenicity Island
T3SS	Type Three Secretion System
TCR	T-Cell Receptor
TLR	Toll-Like Receptor
TNF-α	Tumor Necrosis Factor alpha
TTSS	Type Three Secretion System
UV	UltraViolet

Chapter 1: General introduction

1.1 The genus *Salmonella*

Salmonella, a facultative intracellular gram negative rod, belongs to the family of the Enterobacteriaceae. This genus, which is estimated to have diverged from *Escherichia coli* approximately 100–150 million years ago, is genetically diverse and has adapted to colonize many different niches and hosts (Dougan *et al*., 2011). *Salmonella* is mostly associated with acute, non-systemic gastroenteritis, but some serovars (e.g. *Salmonella* Typhi, *Salmonella* Paratyphi) are historically important as causative agents of the human systemic infection, typhoid fever, which is still a common illness in many developing countries (Dougan *et al*., 2011). The genus *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori* (Guibourdenche *et al.*, 2010).

Salmonella enterica is further divided in six subspecies based on biochemical and antigenic characteristics as well as genome phylogeny: *Salmonella enterica* subsp. *enterica*, *Salmonella enterica* subsp. *salamae*, *Salmonella enterica* subsp. *arizonae*, *Salmonella enterica* subsp. *diarizonae*, *Salmonella enterica* subsp. *houtenae* and *Salmonella enterica* subsp. *indica*. Most *Salmonella* serovars belong to the subspecies *Salmonella enterica* subsp. *enterica* (Guibourdenche *et al*., 2010; Grimont *et al*., 2007).

Salmonella enterica comprises more than 2500 different serovars, distinguished according to extremely variable and highly immunogenic surface antigens such as lipopolysaccharide (LPS) O-antigens (Nagy *et al.*, 2008b). *Salmonella enterica* subsp. *enterica* serovar Typhimurium will be abbreviated as *Salmonella* Typhimurium and *Salmonella enterica* subsp. *enterica* serovar Enteritidis will be abbreviated as *Salmonella* Enteritidis. LPS of *Salmonella* is a virulence determinant and essential for functions such as: (1) swarming motility, (2) intestinal colonization, (3) serum resistance, (4) invasion/intracellular replication, and (5) resistance to killing by macrophages; all of which are important for successful infection (Kong *et al.*, 2011). LPS is the most abundant component of the Gram negative cell wall and consists of three components: (1) O-antigen or somatic antigen, which is a long-chain polysaccharide consisting of repeating units containing one to seven monosaccharides; (2) core-oligosaccharides, composed of approximately ten monosaccharides, including 3-keto-2-deoxyoctonate (KDO), heptose, glucose, galactose, and N'-acetylglucosamine, which are added sequentially by unique glycosyl transferases; and (3) lipid A, a unique lipid backbone consisting of di-glucosamine in β1-6 linkage with ester-, amide-, and di-ester-

linked fatty acids and with phosphate, 4-amino-arabinose substituents both on the reducing and non-reducing sugars (Hitchcock *et al*., 1986; Nagy *et al.*, 2008a) (Figure 1).

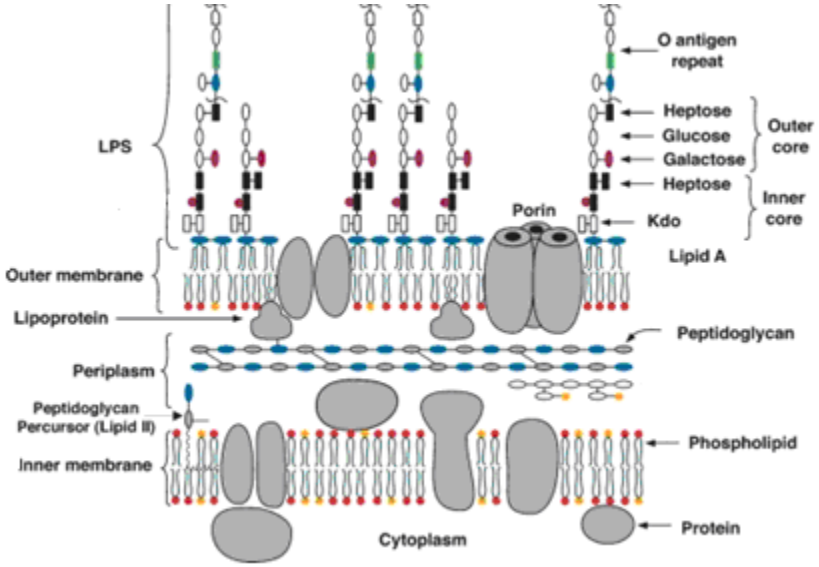

Figure 1: Schematic representation of the Gram negative cell envelope: Localization and structure of LPS. LPS is the most outer component of the Gram negative cell wall and consists of three components: (1) O-antigen, (2) core-oligosaccharides, including 3-keto-2-deoxyoctonate (KDO); and (3) lipid A. (Adopted and modified from Raetz *et al.*, 2002).

Lipid A, also called endotoxin, is anchored in the outer leaflet of the outer membrane and is responsible for most of the biological activities of the LPS molecule, i.e. for the majority of clinical syndromes elicited upon generalized Gram-negative infections, even though it is poorly immunogenic (Nagy *et al.*, 2008a/b). The inner core and outer core of the LPS structure is relatively conserved within the family of the Enterobacteriaceae (Nagy *et al.*, 2008a) and provides an attachment site for O-polysaccharides (O-antigen) (Raetz *et al.*, 2008).

Genes involved in the synthesis of LPS are clustered on the *Salmonella* chromosome (Raetz *et al.*, 2002). The chromosomal *waa* region (*rfa* region) contains three core oligosaccharide assembly operons: the *gmhD*-, the *waaQ*- and the *waaA*-operon (Figure 2) (Raetz *et al.*, 2002; Kong *et al.*, 2011). In the *Salmonella gmhD*-operon, the *rfaF* gene encodes a heptosyltransferase, which catalyzes the transfer of the second L-glycero-D-manno-heptose residue to the core oligosaccharide moiety of LPS, and a mutation of *rfaF* results in a

truncated inner core (Oldfield *et al.*, 2002). The long central *waaQ* operon contains genes involved in the biosynthesis of the outher core. Products of the *rfaJ*, *rfaI*, and *rfaG* genes encode the enzymes glucosyltransferase and galactosyltransferase and a core biosynthesis protein. The *rfaL* gene has not been shown to play a role in core synthesis but encodes an O-antigen ligase, necessary to link O-polysaccharide to the complete core (Klena *et al.*, 1992; Raetz *et al.*, 2002). Lipopolysaccharide biosynthesis genes involved in the synthesis and assembly of sugars of the O-antigenic side chain are located in the *rfb* gene cluster and are responsible for part of the biosynthetic pathway for dTDP-L-rhamnose, CDP-abequose and GDP-mannose (Jiang *et al.*, 1991; Mitchison *et al.*, 1997). The *rfbA* gene encodes a TDP-glucose pyrophosphorylase, an enzyme needed in group B salmonellae for the biosynthesis of dTDP-L-rhamnose, a donor of sugar residues in the O-antigenic side chain (Lew *et al.*, 1986).

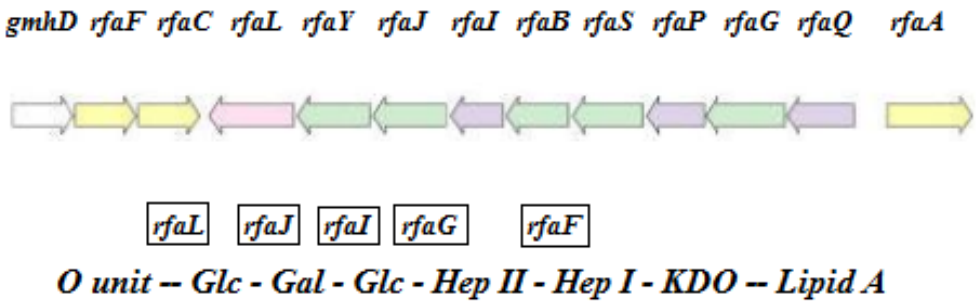

Figure 2: Organization of the *rfa* region of *Salmonella* Typhimurium indicating the *gmhD*-, *rfaQ*- (*waaQ*-) and the *rfaA*- (*waaA*-) operon. The *rfaL*, *rfaJ*, *rfaI*, *rfaG* and *rfaF* genes encode an O-antigen ligase, a glucosyltransferase, a galactosyltransferase, a core biosynthesis protein and a heptosyltransferase. (Adopted and modified from Pradel *et al.*, 1992)

Salmonella enterica, preceded by *Campylobacter spp.*, are the second most common and widely distributed foodborne pathogens in the European Union, with *Salmonella* Typhimurium and *Salmonella* Enteritidis the most frequently reported serovars in humans, followed by *Salmonella* Virchow (Pires *et al*., 2011). In Northen, Eastern and most parts of Western Europe, eggs are the most important source of human salmonellosis, with between 30% and 57.6% of the reported cases; while in Southern Europe pigs are the major source of salmonellosis, with 43.6% of all reported salmonellosis cases in humans (Pires *et al*., 2011). In the European Union, a total of 9.2% of all salmonellosis cases were reported as being travel-related and 3.6% of cases were reported as being part of outbreaks with unknown source (Pires *et al*., 2011). *Salmonella* Enteritidis cases are most commonly associated with consumption of contaminated eggs and poultry meat (Gantois *et al*., 2009), while *Salmonella*

Typhimurium cases are mostly associated with consumption of contaminated pork (Hald *et al*., 2003; EFSA, 2010; Pires *et al*., 2011). Before 2005, human *Salmonella* infections in Belgium were mostly (63%) attributed to *Salmonella* Enteritidis and only 25% were linked to *Salmonella* Typhimurium (Bertrand *et al*., 2004). In 2010, 74% of all human salmonellosis cases were mainly caused by *Salmonella* Typhimurium originating from contaminated pork (EFSA, 2010). This decrease of human *Salmonella* Enteritidis cases in Belgium is explained by the successful implementation of a vaccination programme in layer flocks, diminishing the number of contaminated hens (Collard *et al*., 2008). These findings suggest that vaccination of pigs might also be a strategy to focus on to reduce the number of human salmonellosis cases.

In pigs, *Salmonella* Typhimurium is by far the most frequently isolated serotype in the European Union, followed by *Salmonella* Derby (EFSA, 2010). In Belgian pig herds, 67.5% of the isolates were identified as *Salmonella* Typhimurium and 7.3% as *Salmonella* Derby (CODA report, 2010), as shown in Figure 3. A distinct strain of *Salmonella* Typhimurium, known as definitive type 104 (DT104) and resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline has become an emerging phage type in pigs and is known to be very widespread in Europe. In Belgium it is present in live stocks since 1993 (Imberechts *et al*., 1998; Glynn *et al*., 1998). This multidrug-resistant strain of *Salmonella* Typhimurium has become a major cause of illness in both animals and humans in Europe (Glynn *et al*., 1998). Korsak *et al.* (2003) showed that 58% of the *Salmonella* Typhimurium isolated in his study were characterized as *Salmonella* Typhimurium DT104 or as being closely related to *Salmonella* Typhimurium DT104.

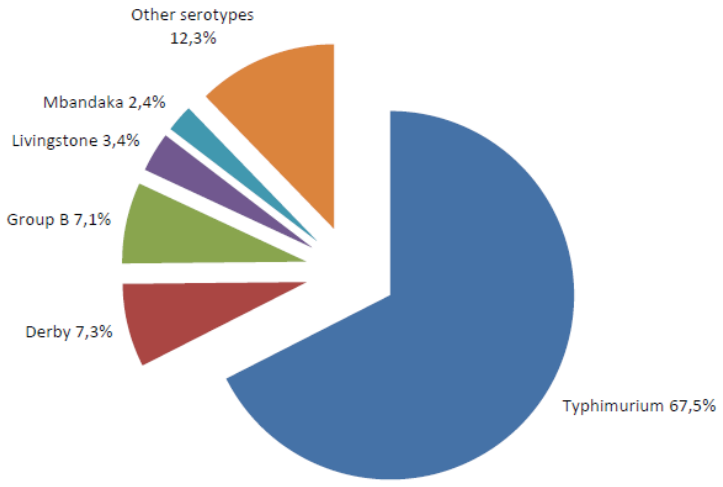

Figure 3: Principal *Salmonella* serotypes isolated from pigs in 2010 (CODA report, 2010)

1.2 Interactions of *Salmonella* Typhimurium with its porcine host

The first step in the pathogenesis of a *Salmonella* infection in pigs is the intestinal phase of infection and is characterized by invasion and colonization of the gut (Boyen *et al.*, 2006a). Secondly, *Salmonella* is spread throughout the body during the systemic phase of infection (Boyen, 2008c).

a. Intestinal phase of infection

The most commonly accepted mode of *Salmonella* transmission is faecal-oral transmission, both from pig-to-pig contact or from a contaminated environment. Besides, nose-to nose contact, aerosol and dust transmission are possible routes of *Salmonella* entry (Oliveira *et al*., 2007; Binter *et al*., 2011). To successfully colonize a host, *Salmonella* has to overcome several host defence mechanisms: (1) porcine epithelial beta-defensin 1 expressed in the dorsal tongue (Shi *et al.*, 1999); (2) the low pH: 1- 4 of the stomach (Bearson *et al.*, 2006); (3) intestinal peristalsis; (4) the normal intestinal microbiota (i.e. colonization resistance); (5) the cationic antimicrobial peptides present on epithelial cells (Viala *et al*., 2011; Alvarez-Ordóñez *et al*., 2011); and (6) the effect of bile that causes DNA damage of bacteria as a result of an increased frequency of nucleotide substitutions, frameshifts and chromosomal rearrangements (Prieto *et al.*; 2004; Merritt *et al.*, 2009).

After reaching the intestines, *Salmonella* Typhimurium attaches to the intestinal mucosa of the gut using adhesins, such as the type 1 fimbriae. Type 1 fimbriae are mannose-sensitive agglutination factors that mediate bacterial adhesion to a broad range of eukaryotic cells by interaction with mannosylated glycoproteins (Althouse *et al.*, 2003; Baek *et al.*, 2011). Following adhesion to the intestinal mucosa, *Salmonella* penetrates the mucosa and invades the intestinal epithelium. *Salmonella* invades microfold (M) cells or membranous epithelial cells, which are specialized epithelial cells that sample the antigenic content of the gut and are overlying the subepithelial lymphoid follicles of the Peyer's patches (PP) in the gastrointestinal tract (Wolf *et al.*, 1984). These sub-epithelial dome regions (SED) harbour large populations of lymphoid (T and B cells) and non-lymphoid (macrophages, DCs) immune cells (Scharek *et al.*, 2007). Furthermore, *Salmonella* can invade porcine adsorptive enterocytes, goblet cells (Fedorka-Cray *et al*., 1995) and dendritic cells (DCs) present in the follicle-associated epithelium (Iwasaki *et al.*, 2001) and in the lamina propria (Figure 4).

After the bacteria reach the lamina propria they can access the mesenteric lymph nodes (MLN) within migrating DCs and reach the bloodstream in phagocytes (Tam *et al.*, 2008).

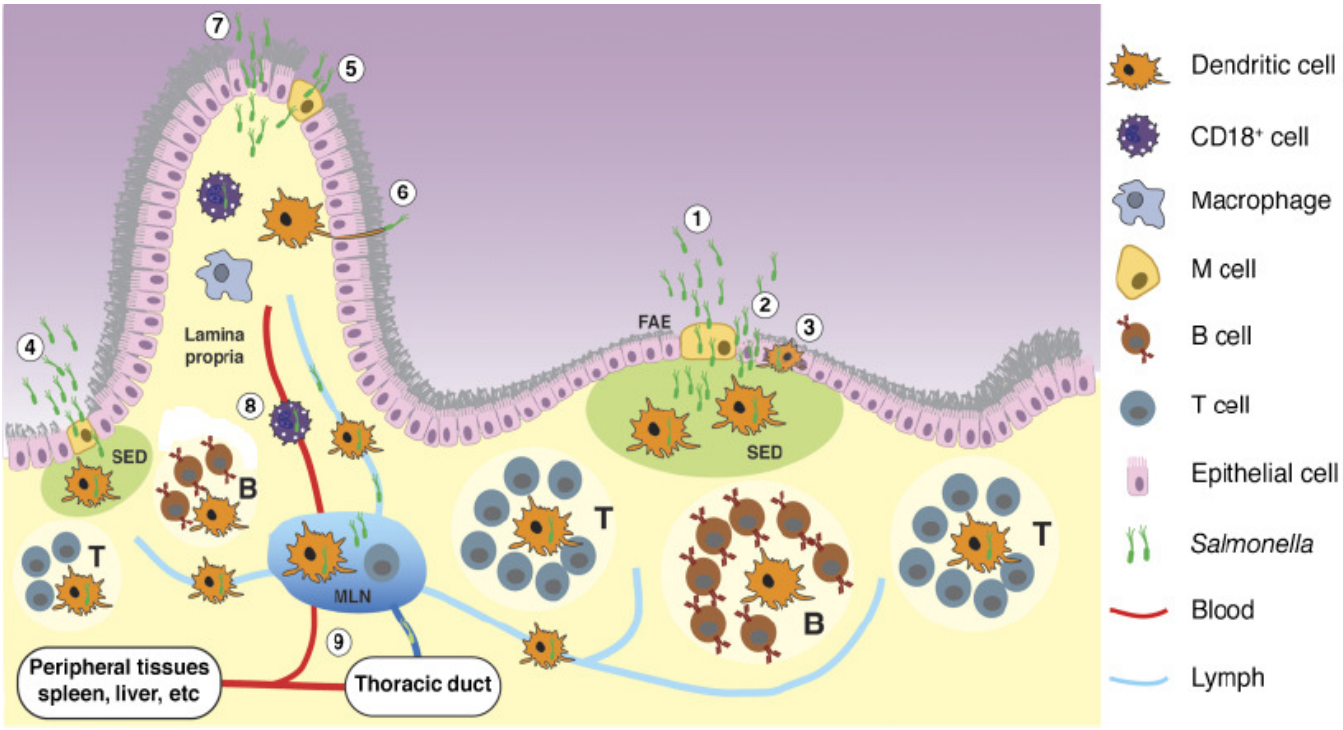

Figure 4: Different entry sites of orally acquired *Salmonella* bacteria. *Salmonella* can cross the intestinal barrier trough: (1) M cells within the follicle-associated epithelium (FAE), (2) epithelial cells forming the FAE, (3) DC's, (4) solitary intestinal lymphoid tissue, (5) M cells, (6) capturing by DCs extending dendrites, (7) or between epithelial cells (Figure adopted from Tam *et al.*, 2008).

The intestinal and the systemic phase of infection are both regulated by genes on pathogenicity islands. *Salmonella* pathogenicity islands (SPI) are large clusters of genes within the chromosome required for a particular virulence phenotype (Srikanth *et al.*, 2011). A total of 17 *Salmonella* pathogenicity islands have been identified (Schmidt *et al.*, 2004). *Salmonella* Pathogenicity Island 1 (SPI-1) is described as being primarily required for bacterial penetration of the epithelial cells of the intestine, an important step in the intestinal phase of infection (Boyen *et al.*, 2006a; Lara-Tejero *et al.*, 2009). SPI-1 genes encode a bacterial type III secretion system (TTSS) that is activated when bacteria migrate through the gastrointestinal mucus layer and a drop of bile concentration occurs (Prouty *et al.*, 2000; Boyen *et al.*, 2006). Furthermore, TTSS produces several effector proteins that contribute to pathogenesis through interaction with eukaryotic proteins. The TTSS is a multi-protein complex that builds a continuous channel across both the bacterial and epithelial cell membranes, resulting in efficient translocation of bacterial SPI-1 effectors (SipA, SipC,

SopB, SopD, SopE and SopE2: reviewed by Boyen, 2008c) directly through a needle-like channel into the cytosol of epithelial host cells. SopB, SopE and SopE2 activate the host's Rho-family of small GTPases, Cdc42 and Rac1, which are key regulators of the actin skeleton and they stimulate the Mitogen Activated Protein (MAP) kinase pathway. Together with SopD, these effectors cause membrane deformation and rearrange the actin cytoskeleton of the host cell (i.e. membrane ruffling) resulting in internalization of the bacterium in enterocytes (Donnenberg, 2000). After the invasion has occurred, *Salmonella* actively reverses the changes it has induced, returning the cytoskeleton to its original state (Schlumberger *et al.*, 2005).

As a result of invasion, the porcine gut will react by producing several pro-inflammatory cytokines, of which interleukin 8 (IL-8) is the most studied one (Cho *et al*., 2003). Secretion of IL-8 is induced via activation of the MAP kinase pathway and activation of the transcription factor Nuclear Factor Kappa-B (NF-ΚB) and is followed by attraction of neutrophils to the lamina propria and eventually the lumen of the gut (Hobbie *et al.*, 1997). Neutrophils are the first line of defence against a *Salmonella* infection and presence of large amounts of these cells might prevent successful salmonellosis (Hyland *et al*., 2006).

b. Systemic phase of infection

In the lamina propria, *Salmonella* is phagocytised by macrophages and then spread throughout the body via the blood stream and lymph into organs such as Peyer's patches and gastric, hepatic, jejunal and ileocecal lymph nodes. *Salmonella* Typhimurium can be isolated from mesenteric lymph nodes at two hours after oral inoculation (Boyen *et al.*, 2008a). Genes located in S*almonella* pathogenicity island-2 (SPI-2) are required for systemic disease (Boyen *et al.*, 2008a) and are expressed when bacteria are inside macrophages (Donnenberg, 2000). Specifically, SPI-2 genes encoding TTSS2 effectors, but also SPI-1 encoded TTSS1 effectors, promote survival of the bacterium in the *Salmonella*-containing vacuole (SCV) inside host cells (Bakowski *et al.*, 2008). The SCV forms a protected niche where *Salmonella* can replicate in a safe environment. A study has shown a critical role for the SPI-1 effector SopB in 'directing traffic' at early stages of infection, allowing the bacteria to control SCV maturation by modulating its interaction with the endocytic system (Bakowski *et al.*, 2008). At later stages of infection, the SCV establishes a 'nest' near the Golgi where optimal bacterial growth takes place (Bakowski *et al.*, 2008).

Since *Salmonella* Typhimurium mainly colonizes the gastrointestinal tract in pigs, while infections in BALB/c mice result in systemic spread, the requirement for SPI-2 genes may differ between swine and mice (Slc11a1$^{-/-}$) (Bearson *et al.*, 2011). BALB/c mice are often used as model of systemic disease and pathogenesis of *Salmonella* Typhimurium infection. Bearson (2011) and Boyen (2008c), illustrated that the SPI-2 encoded SsrAB two-component system, required for systemic disease in BALB\c mice, seems unimportant in gastrointestinal colonization of pigs. Genetic resistance to systemic disease caused by *Salmonella* Typhimurium in mice is linked to factors including: (1) the natural resistance associated macrophage protein (Nramp1, now termed Slc11a1), involved in the control of the exponential growth of *Salmonella* in the reticuloendothelial organs during the early phase of infection (Wigley *et al.*, 2004); (2) the major histocompatibility complex (MHC) haplotype (Hormaeche *et al.*,1985); and (3) Toll-like receptor 4 (TLR4) (Wigley *et al.*, 2004). The H2^{b} haplotype presents the lowest bacterial clearance in mice (Caron *et al.*, 2002). Macrophages that are deficient in Slc11a1 are extremely susceptible to *Salmonella* infection and cannot control replication as efficiently as wild type macrophages (Dougan *et al.,* 2011).

1. 3 Immune responses against *Salmonella* Typhimurium infection

The immune system comprises a variety of components that defend a host against infectious agents. These components can be divided in innate immune defence mechanisms, independent of previous exposure to antigens, and acquired or adaptive immune defence mechanisms, which require contact with an antigen prior to optimal functional capacity (Tizard, 2009). A key difference between the innate and the acquired immune system lies in their use of receptors to recognize foreign invaders.

The innate immune system uses pre-existing receptors, recognizing pathogen-associated - molecular patterns (PAMPs), such as bacterial LPS (Tizard, 2009). In contrast, the cells of the acquired immune system generate enormous numbers of structurally unique receptors. The major components of the innate immune system are phagocytic cells (macrophages, neutrophils and eosinophils), natural killer cells, and interferons. The body also uses enzymes that are triggered by the presence of invaders to cause microbial destruction, known as the complement system. These components are mostly important in controlling an infection during the first days. The innate immune system lacks any form of memory, and each infection is treated similarly (Tizard, 2009).

In the acquired immune system, T and B lymphocytes and their products are the major components (Tizard, 2009). The first branch of the acquired immune system is sometimes called the humoral immune response since antibodies are found in the body fluids and mostly promote destruction of extracellular invaders. The other branch of the acquired immune system is called the cell-mediated immune response and is mainly directed against intracellular invaders. Stimulation of the immune system e.g. in response to vaccination, results in a potent mechanism for protection against infectious agents, such as *Salmonella* (Tizard, 2009).

a. Innate immunity to *Salmonella*

To initiate an adequate immune response against *Salmonella*, recognition of invading microorganisms by "sentinel cells" such as antigen presenting cells (APC) i.e. DCs, macrophages and B cells, is crucial (Tizard, 2009). APC express pattern recognition receptors, such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-containing proteins, which bind conserved PAMPs (Tizard, 2009). TLRs recognize PAMPs at an extracellular level, whereas NOD receptors recognize PAMPs intracellularly (Chiarella *et al*., 2007; Tizard, 2009).

During the early phases of a *Salmonella* infection, components of the bacterial cell wall such as LPS (detailed immunity to LPS see 9/e), DNA motifs, flagella, and certain lipoproteins, activate TLRs on host cells and induce the production of a number of cytokines and soluble factors (e.g. IL-8, IFN-ɣ, TNF-α, IL-12, IL-18) as well as the activation of phagocytic cells like macrophages, dendritic cells and neutrophils (Dougan *et al.*, 2011). IL-8, the best studied pro-inflammatory cytokine, is released as a result of activation of the NF-KB signalling pathway and is followed by the attraction and migration of neutrophils from the bloodstream into the intestinal lumen (Foster *et al.*, 2003; Wick, 2004). In contrast to swine, mice do not produce IL-8 (Scharek *et al.*, 2007). Instead chemokine KC and macrophage inflammatory protein 2 act as homologues (Scharek *et al.*, 2007).

Salmonella has developed an array of tools to manipulate the host cell and to establish an intracellular niche by forming a *Salmonella*-containing vacuole (SCV), consequently successfully establishing as a facultative intracellular pathogen (Malik-Kale *et al*., 2011). Therefore, release of interferon-gamma (IFN-ɣ), stimulating the antibacterial activity of

macrophages, is extremely important in innate immunity to *Salmonella* (Dougan *et al.*, 2011). Natural killer (NK) cells, NK T cells, T cell receptor alpha beta T cells, macrophages and neutrophils are sources of early IFN-ɣ production (Wick, 2004). In mice, activated macrophages (and other phagocytic cells) control the growth of *Salmonella* by alteration of the intracellular production of defensins, bactericidal peptides, reactive oxygen species [(ROS) generated by NAPDH oxidase] and reactive nitrogen intermediates [(RNI) generated by inducible nitric oxide sythase] (Mastroeni *et al.*, 2001). These activities are necessary for bacterial killing and bacterial digesting resulting in antigen presentation to T cells (see acquired immunity to *Salmonella*) (Scharek *et al.*, 2007).

Tumor necrosis factor alpha (TNF- α) produced by macrophages is central to early resistance to salmonellae and it helps to enhance the host's survival by suppression of exponential bacterial growth in the reticuloendothelial organs towards the end of the first week after infection (Mastroeni *et al.*, 1991). Later, TNF- α will also facilitate acquired immunity by enhancing antigen presentation and T cell stimulation (Tizard, 2009). IL-12 and IL-18 both activate macrophages (Dougan *et al.*, 2011). Furthermore, IL-12 has a role in maintaining IFN-ɣ production by T cells and stimulates polarization of T-helper cells towards the Th1 response, which helps in the transition from innate to acquired immunity (Dougan *et al.*, 2011).

b. Acquired immunity to *Salmonella*

While the innate immune response is successful in controlling the initial growth of *Salmonella*, it is insufficient to bring about full protection to ensure resistance to a *Salmonella* infection (Dougan *et al.*, 2011). The initial step of triggering the acquired immune system is the formation of the immunological synapse between the APC and the T lymphocyte. Each T cell has about 30000 T-cell receptors (TCR) on its surface (Tizard, 2009). TCR (CD4+ or CD8+) sensitisation occurs as a consequence of interaction with the MHC-peptide complex of the APC, which is followed by T-cell activation and cytokine production leading to an orientation towards the Th1 or the Th2 specific functional pathway (Chiarella *et al.*, 2007; Tizard, 2009).

Th1 cells promote cell mediated immune responses such as activation of macrophages and cytotoxic T-lymphocytes, which is very important to generate immunity to the facultative

intracellular bacterium, *Salmonella*. While macrophages from unimmunised animals are normally incapable to destroy facultative intracellular bacteria, this capacity is increased after activation in vaccinated animals (Haesebrouck *et al*., 2004). In the absence of IL-12, T-helper cells switch from Th1 to Th2 and promote B cells and secretion of immunoglobulins (IgG and IgA) (Tizard, 2009; Dougan *et al.*, 2011).

Cytotoxic T-lymphocytes destroy *Salmonella*-containing-host-cells, resulting in release of the bacteria in the extracellular space where they can be processed by antibodies (Haesebrouck *et al*., 2004). Dougan *et al.* (2011) have shown that in vaccinated mice antibodies are not only important in this late protection against *Salmonella*, but also contribute to control in very early stages of infection, namely by enhancing bacterial killing before *Salmonella* has entered its safe intracellular niche.

Once *Salmonella* has entered the *Salmonella*-containing vacuole (SCV), it has been suggested that the SPI-2 effector SifA plays a role in maintaining the integrity of the SCV by gaining membrane for the SCV (Mitchell *et al*., 2004). This was explained by recruiting vesicles that transport MHCII molecules to the cell membrane of human Mel JuSo cells (Mitchell *et al*., 2004). Recently, Van Parys *et al.* (2012) have shown that *Salmonella* is able to interfere with the MHCII presentation pathway in porcine macrophages. Consequently, *Salmonella* can circumvent the pigs' antibody response. We can conclude that the success of *Salmonella*, as an intracellular pathogen, lies in its ability to resist and circumvent certain innate and adaptive host immune responses.

1.4 *Salmonella* induced diarrhoea

Bacterial invasion is not correlated with diarrhoea, but an inflammatory response with influx of polymorphonuclear leucocytes (PMN) into the lumen of the gut is needed to induce intestinal electrolyte- and fluid secretion (Grøndahl *et al*., 1998). A streptomycin mouse model for *Salmonella* diarrhoea has shown that the TTSS-1 effector proteins, SipA, SopE and to a lesser extent SopE2, are necessary for driving gut inflammation (Kaiser *et al.*, 2012). The *sipA* gene regulates secretion of the pathogen-elicited epithelial chemo-attractant (PEEC) or hepoxilin A_3 ($hepA_3$) at the apical side of the epithelial cells, which mediates trans-epithelial migration of PMN into the lumen of the host's gut (McCormick *et al.*, 1998; Lee *et al.*, 2000; Mrsny *et al.*, 2004). SopE is a potent guanine-nucleotide exchange factor (GEF), activating

Rho GTPases such as Rac1 and Cdc42 which trigger a signalling cascade that activates caspase-1 and thereby the maturation and release of potent cytokines of the IL-1 family, namely IL-1 and IL-18 (Kaiser *et al.*, 2012).

AvrA, another TTSS-1 effector protein that modulates pathogen-host interactions, inhibits NF-ΚB signalling which indicates that *Salmonella* Typhimurium simultaneously produces pro- (e.g. SipA, SopE and SopE2) and anti-inflammatory effector proteins (e.g. AvrA) (Kaiser *et al.*, 2012). The inhibitory activity of AvrA might prolong the survival of the infected host cell and thereby enhance pathogen survival (Kaiser *et al.*, 2012). Furthermore, the development of diarrhoea is also triggered by SopB- modulated closure of chloride channels on the intestinal epithelial cells (Feng *et al.,* 2001).

1.5 *Salmonella* Typhimurium persistence

Salmonella Typhimurium might persist asymptomatically for months in the tonsils, gut and gut-associated lymphoid tissue, colon and caecum of pigs, resulting in so called carriers (Letellier *et al.*, 2000). Bacterial genes involved in *Salmonella* survival in the tonsils differ from those essential for persistence in for example the lymph nodes or the ileum of pigs (Van Parys *et al.*, 2011). The complete subset of genes, important in prolonged fecal shedding by persistently infected pigs, has not been identified. The CS54 Island and more specifically the *shdA* and *misL* genes, encoding fibronectin binding autotransporter proteins, were identified as important loci for persistence of *Salmonella* Typhimurium in mice (Bogomolnaya *et al.*, 2008), but not in pigs (Boyen *et al.*, 2006b). Furthermore, the outer membrane protein RatB, encoded just upstream of *shdA*, is also involved in intestinal persistence in resistant mice (Bogomolnaya *et al.*, 2008). In swine and not in mice type I fimbriae are important for intestinal persistence (Althouse *et al.*, 2003; Bogomolnaya *et al.*, 2008). Van Parys *et al.* (2011) found that *efp* and *rpoZ* were specifically expressed in porcine lymph nodes during *Salmonella* persistence in pigs and comparable sets of *Salmonella* genes are involved in colonization of the ileum. Furthermore, *dnaK* and *aroK* were found to play a general role in *Salmonella* persistence in pigs (Van Parys *et al*., 2011).

Extracellular persistence in porcine tonsils has recently been shown to be independent of biofilm genes *csgA*, *csgD* and *adrA* (Van Parys *et al.*, 2010). As mentioned before, the inhibitory activity of AvrA might prolong the survival of the infected host cell and therefore

promotes persistence of *Salmonella* Typhimurium in both pigs and mice (Kaiser *et al.*, 2012). Recently, Van Parys (2012), showed that several *Salmonella* serovars are able to downregulate MHC II expression on porcine macrophages *in vitro* and indicated a correlation between early onset of seroconversion, reduced faecal shedding and reduced persistence capacity and *vice versa*. Circumvention of the pig's antibody response induced by *Salmonella* might therefore contribute to long-term persistence in pigs. *Salmonella* clearly uses different host specific tools to persistently infect its host.

1.6 Stress related recrudescence of *Salmonella* Typhimurium in pigs

Porcine carcass contamination with *Salmonella* Typhimurium can be linked to persistently infected pigs, which intermittently shed low numbers of *Salmonella* bacteria (Letellier *et al.*, 2000). However, during periods of stress recrudescence of *Salmonella* may occur (Letellier *et al.*, 2000) and re-excretion of the bacterium significantly contributes to porcine carcass contamination in turn resulting in a higher number of human infections (Verbrugghe *et al.*, 2011a).

It has been repeatedly illustrated that stress associated with transportation of pigs (Morrow *et al.*, 2002; Rostagno *et al.*, 2005; Scherer *et al.*, 2008) and feed withdrawal stress result in increased shedding of *Salmonella* Typhimurium (Isaacson *et al.*, 1999; Martin-Pelaez *et al.*, 2009; Verbrugghe *et al.*, 2011b). In addition, it was established that due to stress, sows become more susceptible to primary *Salmonella* infections and carrier sows are more likely to start shedding the pathogen (Nollet *et al.*, 2005).

Recently, Verbrugghe *et al.* (2011b) pointed out that a 24 hour feed withdrawal period increased the intestinal *Salmonella* Typhimurium load in pigs and that this is correlated with increased serum cortisol, a glucocorticoid produced by the adrenal gland and released in response to stress levels. They also showed that re-excretion of *Salmonella* Typhimurium in pigs can be reproduced by an intramuscular injection of dexamethasone, a potent synthetic member of the glucocorticoid class of steroid drugs. Furthermore, cortisol promotes intracellular proliferation of *Salmonella* Typhimurium in porcine alveolar macrophages and *scsA* was indicated as the major driver (manuscript in preparation). The *scs* locus includes two operons, one operon contains a single *scsA* gene and another operon contains the *scsB*, *scsC* and *scsD* genes, encoding proteins that may mediate copper tolerance indirectly by

catalyzing the correct folding of periplasmic copper-binding target proteins via a disulfide isomerise-like activity (Gupta *et al.*, 1997). Consequently, *scsA*, a negative regulator for the *scsBCD* operon, might be extremely important in stress related recrudescence of *Salmonella* Typhimurium in pigs. Stress related re-excretion of the bacterium, resulting in porcine carcass contamination and human infections, emphasizes the importance of *Salmonella* control measures at farm level.

1.7 Measures to control *Salmonella* Typhimurium in pig farms

Food safety has emerged as a top consumer concern. Therefore, *Salmonella* control measures have been implemented at three levels: the pre-harvest level (on farm), the harvest level (transport to and procedures in the slaughterhouse) and post-harvest (cutting, processing, retail and food preparation at home) (Fosse *et al.*, 2009). We will focus on pre-harvest control measures and monitoring programmes that might help to reduce the number of non-typhoidal *Salmonella* infections in humans from contaminated pork (Wales *et al.*, 2005; Fosse *et al.*, 2009).

a. Hygienic and bio-security measures

The EU integrated legislations assure a high level of food safety through consistent measures and suitable monitoring. The Hygiene Package (Regulation EC-854/2004) (and Regulation EC-2160/2003; Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs and Regulation (EC) No 1441/2007) demands information from farm to slaughterhouse to augment European consumers protection in a 'farm to fork' approach and concerns food-borne zoonotic hazards, such as *Salmonella enterica* (Fosse *et al*., 2009). It points out the importance of good hygiene and bio-security practices in pig herds (Fosse *et al.*, 2009). The farmer's attitude to adopt these on-farm bio-security measures is, however, very important to actually improve food safety (Boyen *et al.*, 2008b; Fraser *et al*., 2010). Three types of *Salmonella* bio-security measures are extremely meaningful: (1) efficient rodent/insect control (Vico *et al*., 2011; Wang *et al.*, 2011), (2) hygienic measures and (3) adequate management practices (Fosse *et al*., 2011).

Barder *et al.* (2002) showed that, on *Salmonella* infected pig farms, between 2% and 10% of all rodents carry *Salmonella*. Rodents and insects can act as reservoirs of *Salmonella*

Typhimurium and are potential sources of *Salmonella* infection. Therefore, efficient rodent/insect control is extremely important and is generally well applied in Belgian pig farms (Ribbens *et al.*, 2008).

Hygienic measures such as cleaning followed by disinfection of pens between batches of pigs has shown to reduce *Salmonella* contamination at herd level (Mannion *et al.*, 2007). Attention must also be paid to: (1) informing visitors on hygiene and handwashing, (2) provision of handwashing facilities, and (3) a footwear cleaning facility. Mostly, hygienic measures seem easy to adopt by farmers, but need to be persevered (Fosse *et al*., 2011). Bio-security measures, such as limiting the mixing of batches (Fosse *et al.*, 2009) based on an "all in/all out (AIAO) management", seem less easy to adopt and more time consuming for farmers because leaving accommodation empty for a longer time could confer significant extra costs (Fraser *et al*., 2010). Vacancy for at least six weeks might reduce the microbial population with 20% but is not obligatory when a good level of decontamination is ensured (Maes D., 2011).

In professional pig herds in Belgium, bio-security and hygienic measures are generally acceptable and help to reduce *Salmonella* infection in pigs (Ribbens *et al.*, 2008). A number of improvements are, however, still possible e.g. entrance control with a changing room, herd clothing and disinfection baths. Ribbens *et al.* (2008) mention that small and non-commercial herds often lack bio-security and management practices, which makes them a possible threat for professional herds.

b. The influence of feed additives, feed characteristics and drinking water treatment

Various interventions to reduce or eliminate *Salmonella* infection in pigs are available and in this chapter we highlight the role of treatments in feed and water. Because cereal and vegetable ingredients for animal feedstuffs may come in contact with wildlife excreta and agricultural effluents, during growth and processing, *Salmonella* contamination often occurs (Wales *et al.*, 2010). As a consequence, feed manufacturers and users of animal feeds often add antimicrobial feed additives to feed, aiming at a lower number of animal infections, probably through a reduction of bacterial numbers in the feed and the animal itself.

Supplementation of feed with organic acids (formic-, propionic-, lactic- and coated butyric acid), their salts, derivatives (e.g. potassium diformate), membrane-disrupting compounds and other chemical treatments (e.g. essential oils, chlorate salts), may provide adequate protection against new *Salmonella* challenges in feed and can act synergistically to reduce endemic *Salmonella* problems (Davies *et al.*, 1997; Wales *et al.*, 2010). Treatment of feedstuffs may be done before or after compounding into finished feed (Wales *et al.,* 2010). Preparations may comprise straight acids applied via nozzles or adsorbed onto inert powder carriers, powdered blends of acids and acid salts (Davies *et al.*, 1997). Organic acids may achieve the goal of *Salmonella* reduction in two ways: (1) toxicity *ex vivo* associated with relatively high concentrations in feed (lowering pH) or (2) toxicity exerted *in vivo* after ingestion (Boyen *et al*., 2008b). In general, medium-chain fatty acids are more effective than short chain fatty acids. Certain short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) decrease expression of virulence genes inhibiting invasion in porcine intestinal epithelial cells (Boyen *et al.*, 2008b; Wales *et al.,* 2010) and survival inside macrophages (Davies *et al.*, 2010). Boyen *et al.* (2008b) have shown that the intestinal *Salmonella* load and bacterial shedding in pigs was decreased using supplemented coated butyric acid compared to uncoated butyric acid. This is explained by the protection of coated butyric acid from the intestinal environment, where SCFA are usually rapidly metabolized and absorbed.

Feed characteristics including form, texture, moisture content, and pH might influence *Salmonella* infection of pigs as well (Nollet *et al.*, 2004). Liquid feeding assists to practical control of *Salmonella* on pig farms, particularly when the diet has been fermented (O' Connor *et al.*, 2008). Dry feed enhanced the risk of *Salmonella* shedding compared to wet feed and this is explained by the acidification of the intestinal content due to wet feed (Fosse *et al.*, 2009). Davies *et al.* (1997) showed that pelleted feed was associated with a higher prevalence of *Salmonella* compared to meal feed. The effect of a feed with a very small particle size, such as pelleted feed, is that the stomach content is very fluid and the emptying time is relatively rapid (Straw *et al.*, 2006). On the other hand, feeding pigs a coarsely ground meal feed gives the gastric content a porridge-like consistency, resulting in slow emptying of the stomach and a prolonged retention time in the acidic environment of the fundus and the pylorus (pH 2), resulting in a reduced number of Enterobacteriaceae, including *Salmonella* (Mikkelsen *et al.*, 2004). In distal parts of the gastrointestinal tract, feed with a larger particle size results in higher starch concentrations. Starch can be fermented by lactobaccilli to short chain fatty acids, such as acetate, propionate and butyrate,

limiting the number of *Salmonella* bacteria (Taube *et al.*, 2009). Furthermore, Rajic *et al.* (2007) mention that *Salmonella* adheres less to the ileal tissue of pigs fed non-pelleted feed. They suggest that pigs fed pelleted diets secrete mucins that are capable of binding *Salmonella*, consequently allowing higher colonization.

Because pigs with full stomachs at slaughter are prone to intestinal rupture, gut spill and fecal leakage, withdrawal of feed from pigs for 16-18h prior to slaughter is a commonly used practice (www.belgianmeat.com). Therefore, drinking water treatment has the advantage that it can be applied even when feed withdrawal occurs. Drinking water administration of experimental chlorate preparations effectively reduced ceacal *Salmonella* concentrations and reduced rectal *Salmonella* concentrations in weaned pigs (Tzortzis *et al*., 2011). The antibacterial success of organic acids added to drinking water is based on the fact that the pH is lowered to a level of 4.2 or lower at which Enterobacteriacea cannot multiply (Ostling *et al.*, 1993). Moreover, organic acids have the ability to enter bacteria in their non-dissociated form affecting protein and DNA synthesis (De Busser *et al.*, 2009). The same study showed that short term application of organic acids, during only 14 days prior to slaughter, is not able to affect shedding of *Salmonella* which suggest that long-term acidification of drinking water is necessary to influence the *Salmonella* prevalence at the slaughterhouse (De Busser *et al.*, 2009).

c. The use of pro- and prebiotics

Promoting normal gut micro-flora and targeting intestinal pathogens by use of pro- and prebiotics might be an important tool to target well-being of pigs. The adherence to the intestinal mucosa is indispensable for the success of probiotics and might prevent colonization of pathogens through competition for binding sites and nutrients (Kim *et al.*, 2007). Lactobacilli have been demonstrated to effectively antagonise *Salmonella* invasion in both spleen and liver of BALB/c mice (Tsai *et al.*, 2005) and have potential for use as probiotic additives in pig feed (De Angelis *et al.*, 2006). *Lactobacillus salivarius*, *L. plantarum*, *L. reuteri* (Yun *et al.*, 2009) and a mixture consisting of four strains of *Lactobacillus spp.* and *Pediococcus pentosaceous*, were able to reduce the growth of *Salmonella* Typhimurium and the clinical signs during a *Salmonella* infection in pigs (Casey *et al.*, 2007).

Prebiotics are non-digestible food ingredients that encourage proliferation of selected groups of the colonic microflora, thereby altering the composition toward a more beneficial community of microorganisms. The advantage of prebiotics is that it overcomes the survivability problems of some probiotic microorganisms after ingestion (Collins *et al.*, 1999). Tzortizis *et al.* (2005) showed that an oligosaccharide mixture, derived from *Bifidobacterium bifidum*, inhibited attachment of *Salmonella* Typhimurium in pigs.

1.8 *Salmonella* surveillance and eradication programs

In EU regulations 53 2003/99/EC and 2160/2003, the European Commission has set deadlines for its member states to implement *Salmonella* surveillance programs for foodborne infections in different livestock species (Anonymous, 2003a/b; Cortinas Abrahantes *et al*., 2009). To fulfill these obligations, many EU countries have already applied national control programmes for *Salmonella* in pigs (Mousing *et al.*, 1997; Wahlström *et al.*, 1997; Benschop *et al.*, 2008; Snary *et al.*, 2010; Merle *et al.*, 2011; Belsué *et al.*, 2011).

In 2005, the Belgian Federal Agency for the Safety of the Food Chain (FASFC), implemented a national *Salmonella* surveillance and control program for pigs, namely the *Salmonella* Action Plan (SAP) (Brossé *et al*., 2011). The SAP became compulsory by means of a Royal act in July 2007 (Brossé *et al*., 2011). The ultimate goal of this guideline was to control *Salmonella* at the pre-harvest stage, objecting a lower number of *Salmonella*-positive pigs transported to the slaughterhouse, subsequently reducing the degree of carcass contamination. Since July 2007, all Belgian pig farms with a capacity of over 31 pigs are compelled to enforce the SAP (Vangroenweghe, 2010). The program is organized as follows. Farms are assigned a *Salmonella* status, based on a 4-month interval serological analysis of blood samples collected from twelve fattening pigs (Meeusen *et al*., 2007).

For serological detection of *Salmonella* Typhimurium infections in swine, four indirect *Salmonella* enzyme-linked immunosorbent assay (ELISA) tests have been discribed (Farzan *et al*., 2007; Szabó *et al.*, 2008). These tests are based on detection of LPS-antibodies (Salmotype® Pig screen, Enterisol® Salmonellen-Diagnostikum, HerdChek® Swine *Salmonella* Antibody) or antibodies directed against a *Salmonella* Typhimurium whole-cell-lysate (Salmotype® Pig STM-WCE). Indirect ELISA tests utilize unlabeled primary antibodies in conjunction with labeled secondary antibodies (Szabó *et al.*, 2008).

Samples taken for *Salmonella* monitoring in the SAP are analysed using the indirect LPS based *Salmonella* HerdChek® ELISA (IDEXX Laboratories). Coating antigens in this ELISA include LPS of serogroups B, C1 and D (O-antigens 1, 4, 5, 6, 7 and 12) (Farzan *et al.*, 2007). Interpretation of test results is done based on measurement of optical densities, using a spectrophotometer. Results are expressed in optical density percentages (OD %) or as sample to positive ratios (S/P) calculated as follows:

$$OD\% = \frac{\text{sample OD} - \text{OD negative control}}{\text{OD positive control} - \text{OD negative control}}$$

$$S/P = \frac{OD\% \times 2.5}{100\ \%}$$

Different critical values are used to determine a sample as positive for *Salmonella* antibodies depending on the test kit used. For the IDEXX ELISA kit (HerdChek *Salmonella*; IDEXX Laboratories), which is used in the current Belgian monitoring program, an S/P value of 0.25 is considered as the scientific cut-off value to classify pigs as serologically negative (S/P < 0.25) or serologically positive (S/P ≥ 0.25) for *Salmonella*. Control programs can take other cut-off values into consideration, in most cases for practical reasons. Generally, an average cut-off value of S/P = 1.0 has been considered as appropriate for large scale screening (Mousing *et al.*, 1997; Osterkorn *et al.*, 2001), although some countries have decreased the cut-off value to S/P = 0.5, such as Denmark (Alban *et al.*, 2002), or S/P = 0.6, such as Belgium (Brossé *et al.*, 2011), to enable a more stringent screening of pig herds.

Belgian pig farms are considered as risk farms if the mean S/P ratio, from twelve fattening pigs, is equal to or higher than 0.6 (or an optical density (OD) % ≥ 24%) for 3 successive sampling events (Meeusen *et al*., 2007; Brossé *et al.*, 2011). Risk farms are obliged to take reasonable care to improve their *Salmonella* status within twelve months, assisted by the responsible veterinarian. In case they are not able to fulfill the requirements they are accompanied by a veterinarian of Dierengezondheidszorg (DGZ) Vlaanderen (Vangroenweghe, 2010). Farms that fulfill the requirements are not considered as *Salmonella* problem farms. Currently, the Belgian SAP is under revision (Anonymous, 2012).

1.9 Vaccination as a measure to control *Salmonella* Typhimurium in pigs

Vaccination aims to mimic the development of naturally acquired immunity by inoculation of non-pathogenic but still immunogenic components of the pathogen in question, or closely related organisms which confer cross protection. One of the major goals of vaccination in pigs is to prevent animal-to-human transmission by reducing the number of infectious animals (Haesebrouck *et al*., 2004) and therefore passive and active immunization strategies have been developed.

1.9.1. Passive immunization

Passive immunization requires that antibodies are produced in a donor animal (e.g. the sow) by active immunization and that these antibodies are transferred to susceptible animals (e.g. to suckling piglets through colostrum) to confer immediate protection (Tizard, 2009).

Vaccination of pregnant sows with: (1) an attenuated Δ*cpxR*Δ*lon Salmonella* Typhimurium strain (Hur *et al.*, 2010) or (2) an inactivated herd-specific *Salmonella* Typhimurium DT104 vaccine (Roesler *et al.*, 2006) strongly enhanced serum IgG and IgA antibody levels in piglets and diminished *Salmonella* infections in suckling piglets. New borne BALB/c mice placed with a foster mother, immunized with a Δ*aro Salmonella* Typhimurium mutant, were better protected against oral challenge with a virulent *Salmonella* Typhimurium strain compared to pups suckled by a non-immunized foster mother (Shope *et al.*, 1991).

Furthermore, prophylactic administration of a *Salmonella* Enteritidis immune lymphokine (SEIL), derived from the T cells of *Salmonella* Enteritidis immunized pigs, protected weaned pigs against a *Salmonella* Choleraesuis infection (Genovese *et al.*, 1999). Oral administration of egg yolk containing anti-*Salmonella* immunoglobulinY (IgY) derived from chickens challenged with *Salmonella* Typhimurium, however, did not effectively protect piglets against *Salmonella* Typhimurium (Letellier *et al.*, 2000; Mathew *et al.*, 2009). This was explained by alteration of immunoglobulins from eggs by the swine digestive tract resulting in a failure to recognize *Salmonella* (Letellier *et al.*, 2000).

We conclude that passive immunization of sows might be useful to protect piglets against a *Salmonella* Typhimurium infection early in life.

1.9.2. Active immunization

Active immunization can be achieved by using live attenuated micro-organisms, inactivated exotoxins (toxoid vaccines), inactivated whole bacteria (bacterins), purified bacterial components (subunit vaccines) or DNA (Haesebrouck *et al*., 2004).

a. Live and inactivated vaccines

Salmonella, as a facultative intracellular pathogen, requires both cell-mediated and humoral immune responses for protection (Mastroeni *et al*., 2001). As a pathogen often present in the intestinal tract, induction of a strong mucosal immune response (IgA production) is also of critical importance (Roesler *et al*., 2004).

Killed *Salmonella* vaccines may induce efficient antibody responses, but generally speaking insufficiently trigger Th1-cell and mucosal responses (Mastroeni *et al*., 2001). Bearing this in mind, the use of live attenuated vaccines appears a more promising tool to minimize *Salmonella* infections (Meeusen *et al.*, 2007).

Live attenuated vaccines must have the ability to reduce undesirable disease symptoms, should be sufficiently invasive and persistent to stimulate strong and lasting immune responses and the attenuation should be an inherent quality of the bacterial vaccine and not be reversible by diet or by the host-resident microbial flora (Curtiss, 2002). Traditional methods for attenuation were empirical and usually involved adapting organisms to unusual conditions (Tizard, 2009). The first live vaccines were spontaneous mutants or strains derived by ultraviolet (UV) mutagenesis or chemical treatment (multiple passages) (Meeusen *et al.*, 2007) or the isolation of temperature-sensitive mutants (Mastroeni *et al.*, 2001). Today, bacterial attenuation is often the result of genetic manipulation (Meeusen *et al.*, 2007) e.g. (1) mutants deficient in the biosynthesis of aromatic amino acids (e.g. *aroA*, *aroC* mutants); (2) mutants deficient in purines (e.g. *purA* and *purE* mutants); (3) mutants altered in the utilization or synthesis of carbohydrates (e.g. *galE* mutants); (4) mutants altered in production of adenylate cyclase (*cya*) or of the cyclic AMP receptor protein (*crp*); (5) mutants with an affected global regulatory system (*phoP*); (6) mutants with metabolic drift mutations (e.g. gyrAcpxA-rpoB); (7) mutants in 'house-keeping' genes such as those involved in the synthesis of bacterial structural components (e.g. LPS); (8) mutants in stress response genes

htrA and *lon* (Mastroeni *et al.*, 2001; Matsui *et al.*, 2003; Roesler *et al.*, 2004; Kodama *et al.*, 2005; Karasova *et al*., 2009; Lowe *et al.*, 2009; Kong *et al*., 2011).

Lon is a primary ATP-dependent quality control protease required to resist multiple stressors such as e.g. ultraviolet light (UV) (Downs *et al.*, 1986). Furthermore, disruption of the *lon* gene might influence pathogenicity of *Salmonella* Typhimurium and influence elimination of the strain from its host as a consequence of up-regulation of SPI-1 invasion gene expression and down-regulation of SPI-2 related genes (Takaya *et al*., 2002; Takaya *et al.*, 2003).

Generally, live attenuated *Salmonella* strains must neither be under-attenuated, consequently harboring residual virulence of the strain, or be over-attenuated, resulting in ineffective vaccines. For example *purA* mutants of *Salmonella* Typhimurium are highly attenuated in mice (Mastroeni *et al.*, 2001) and pigs (Van Parys *et al.*, 2011) and might not be able to induce a cell mediated- and an antibody immune response (Mastroeni *et al.*, 2001).

Some used live vaccines are still the result of non–directed mutagenesis processes and have therefore non-characterized defects. Nowadays, guidelines for licensing of live vaccines are very rigid and claim well defined and irreversible mutations, therefore, development of new vaccines is a very time consuming process (Cheminay *et al.*, 2007; Sheridan *et al.*, 2010). Currently, only one licensed live attenuated *Salmonella* Typhimurium vaccine for pigs is commercially available in Europe, namely *Salmoporc*® (Springer *et al.*, 2001). *Salmoporc*® is manufactured by IDT Biologika (Germany) and can be introduced in Belgian pig farms, through the cascade system. It is formulated based on a double attenuated *Salmonella* Typhimurium mutant strain, phage type DT 9, and generated by chemical mutagenesis (Lindner *et al.*, 2007). The vaccine strain is auxotrophic for both adenine and histidine and can be differentiated within 24-48 hours from field strains of the same serotype, using an IDT *Salmonella* Diagnostic Kit (Eddicks *et al.*, 2009; www.idt-biologika.de). Furthermore, molecular biological methods, using plasmid analysis, ribotyping and macro-restriction analysis can also be used to differentiate the vaccine strain from field strains (Selke, 2006). *Salmoporc*® has proven to diminish both colonization of the intestinal tract and shedding of *Salmonella* Typhimurium in pigs (Selke *et al.*, 2007; Lindner *et al.*, 2007; De Ridder *et al.*, unpublished results). Furthermore, this vaccine is well tolerated when given orally in three day old suckling piglets and vaccination results in a significant anti-*Salmonella* seroconversion compared to non-immunized control piglets (Eddicks *et al.*, 2009).

Not only *Salmoporc*® is able to reduce *Salmonella* prevalance. Maes *et al.* (2001) illustrated that a live modified *Salmonella* Choleraesuis vaccine (Argus SC™) can provide cross-protection against infections with other *Salmonella* serotypes, such as *Salmonella* Typhimurium, and might be a useful tool to lower the prevalence of *Salmonella* in swine herds. Serological discrimination of infected pigs from *Salmoporc*® or Argus SC™ vaccinated pigs, however, is not possible (Maes *et al*., 2001; Selke, 2006).

b. Adjuvants for vaccines as immunomodulators

Despite their sub-optimal capacity to protect against *Salmonella*, killed vaccines have been widely used (Mastroeni *et al.*, 2000) and their protective capacity is mostly enhanced by the addition of an adjuvant. Adjuvants are compounds that enhance the immune response against co-inoculated antigens. The word adjuvant stems from the latin word *adjuvare*, which means to help or to enhance (Aguilar *et al.*, 2007). Adjuvants can be used for various purposes: (1) to enhance immunogenicity of highly purified or recombinant antigens; (2) to reduce the amount of antigen or the number of immunizations needed for protective immunity; (3) to improve the efficacy of vaccines in newborns, the elderly or the immuno-compromised; (4) as an antigen delivery system for the uptake of antigens by the mucosa (5) to direct towards a Th1/Th2 response (Aguilar *et al.*, 2007).

Killed whole vaccines or subunit vaccines generally require the addition of an adjuvant to be effective (Aguilar *et al.*, 2007) and an adjuvant is designed to target signals of the innate immune response and the adaptive immune response, e.g. enhancement of signals of the immunological synapse between APCs such as DCs, macrophages, B cells and the T lymphocytes (Chiarella *et al.*, 2007).

Many adjuvants have been developed (e.g. water-in-oil formulations, LPS and derivatives, bacterial CpG-DNA, mineral salt adjuvants) (Petrovsky *et al.*, 2004; Chiarella *et al.*, 2007). In 1936, Freund developed an emulsion of water and mineral oil containing killed mycobacteria, thereby creating one of the most potent known adjuvants, i.e. Freund's complete adjuvant (FCA) (Petrovsky *et al.*, 2004). This FCA, however, causes severe local reactions (e.g. pain, inflammation, swelling, injection site necrosis, lymphadenopathy, granulomas, ulcers and the generation of sterile abscesses) and is considered too toxic for human use (Petrovsky *et al.*, 2004). The oil emulsion without added mycobacteria is know as Freund's incomplete

adjuvant (FIA) and, being less toxic, has been used in human and veterinary vaccines (Petrovsky *et al.*, 2004). The mechanism of action of FIA includes the formation of a depot at the injection site, enabling the slow release of antigen and the stimulation of antibody producing plasma cells (Petrovsky *et al.*, 2004).

c. Lipopolysaccharide based vaccines

As mentioned before, bacterial LPS are PAMPS and potent inducers of innate immunity. In serum, LPS binds to LPS-binding protein (LBP) which transfers LPS molecules to a protein called the phosphatidylinositol glycan-linked surface protein (CD14) and to the soluble accessory protein MD2 located on the surface of macrophages (Freudenberg *et al.*, 2001; Raetz *et al*., 2002; Tizard, 2009). Next CD14 binds to TLR4, the most important pattern-recognition receptors located on macrophages, resulting in the formation of the CD14/TLR4 complex which stimulates the cytoplasmic NF-KB to enter the nucleus triggering different cytokine genes, inducing release of e.g. TNF-α a, IL1-β and co-stimulatory molecules required for the adaptive immune response (Raetz *et al*., 2002; Tizard, 2009) (Figure 5). Next, LPS subsequently dissociates from CD14 and binds to lipoproteins and its toxic activities are lost (Freudenberg *et al.*, 2001; Heumann *et al.*, 2002; Tizard, 2009).

Intestinal macrophages in pigs do not express CD14 and colonic macrophages only express the LPS receptor in low levels (Scharek *et al.*, 2007). Consequently, intestinal macrophages are unable to respond to LPS and production of inflammatory cytokines, in response to IFN-ɣ or bacterial antigens, is a result of recruitment of $CD14^+$ blood monocytes (Smith *et al*., 2005). Because intestinal macrophages are derived from blood monocytes, loss of CD14 is explained by exposure of monocytes to stromal cell products [e.g. transforming growth factor-β (TGF-β)], resulting in cells with the intestinal macrophages phenotype and cytokine profile (Smith *et al*., 2005).

Furthermore, LPS can trigger the complement cascade which is part of the innate immune response and also binds TLR-4 on B lymphocytes to trigger an adaptive, humoral response without the need of T-helper cells; thus, LPS is known as a T-independent antigen (Takeuchi *et al.*, 2001; Nagy *et al.*, 2008a; Tizard, 2009). Consequently, disruption the *tlr4* gene results in abolishment of LPS responsiveness of macrophages and B cells (Takeuchi *et al.*, 2001). Recognition of LPS by complement and production of antibodies results in lysis and

opsonization, in turn, activating phagocytes such as monocytes, macrophages and polymorphonuclear leukocytes. An LPS-activated macrophage not only produces several cytokines but becomes metabolically more active and produces intracellular stores of oxygen free radicals (e.g. H_2O_2 and O^{2-}) and other antimicrobial agents (e.g. nitric oxide, lysozyme, cationic proteins, acid hydrolases and lactoferrin) responsible for killing phagocytised microorganisms (Van Amersfoort *et al.*, 2003).

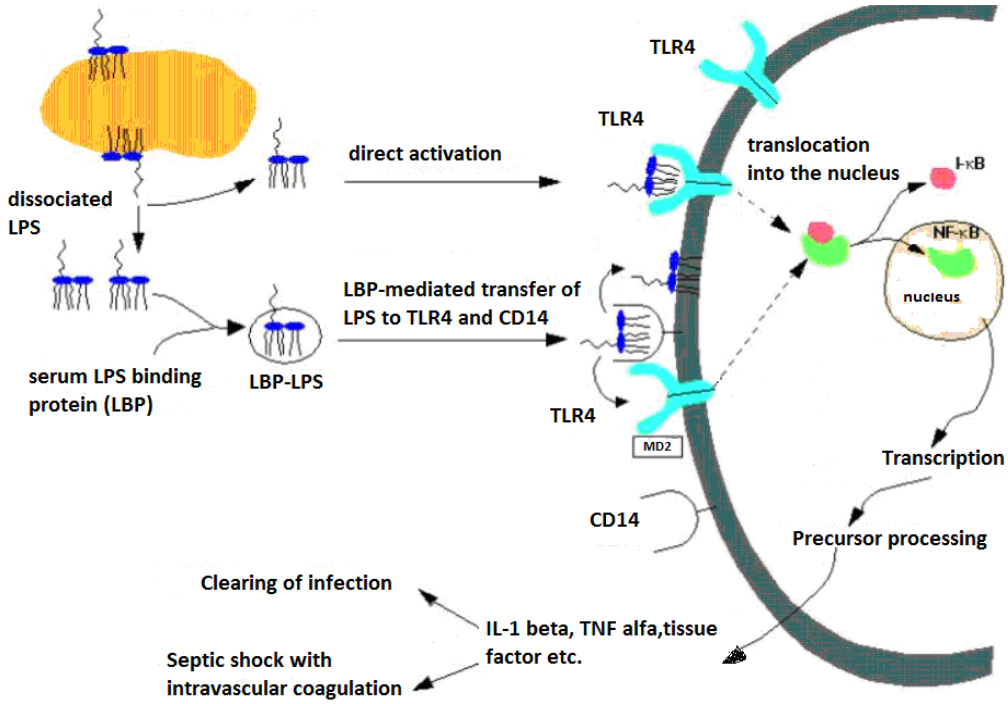

Figure 5: Detection of LPS by the TLR4 innate immunity receptor of animal cells (Adopted and modified from Raetz *et al*., 2002)

Notwithstanding bacterial LPS is an indispensable component of Gram-negative bacteria and drastic truncation of the LPS chain [e.g. deep rough or heptoseless mutants (Hitchcock *et al.*, 1986)] might not be a viable option for development of live *Salmonella* vaccines, many LPS mutants have been experimentally studied in relation to vaccine development (Nagy *et al.*, 2008a/b; Kong *et al.*, 2011) e.g. (1) The *wzy* (*rfc*) mutant of *Salmonella* Typhimurium, which retains one O-antigen, is able to establish an optimal balance between sufficient attenuation and retained immunogenicity in BALB/c mice (Kong *et al.*, 2011); (2) Unlike piglets immunized with a deep-rough mutant of *Salmonella* Minnesota, animals immunized with a rough *Salmonella* Typhimurium strain (SF1591) were protected against subsequent challenge with a *Salmonella* Typhimurium LT2 strain (Dlabac *et al.*, 1997); (3) A 'Gently rough' *rfaH*

mutant of *Salmonella* Typhimurium, is able to invade but not proliferate in antigen-presenting cells. Therefore, Δ*rfaH* may be an attenuated, safe and immunogenic vaccine strain with potential comparable to that of Δ*aroA*. Moreover, rough strains might generate cross-protection against other enterobacterial pathogens which is explained by better accessibility of less immune-potent molecules, such as lipid A and core antigens compared to wild type strain (Nagy *et al.*, 2006 and Nagy *et al.*, 2008a).

d. DIVA vaccines

The term DIVA is generally used as an acronym for 'differentiating infected from vaccinated animals' (Pasick, 2004) and is based on the absence of at least one immunogenic protein in the vaccine strain which is present in the wild-type parent (Selke, 2006). DIVA vaccines and their companion diagnostics have been successfully applied for eradication of infectious bovine rhinotracheitis, pseudorabies and have been proposed for erradication of classical swine fever and for use in foot-and-mouth disease eradication campaigns (Meeusen *et al.*, 2007).

When we started our studies, only one DIVA strain was available in connection with *Salmonella* Typhimurium in pigs, namely SalmoporcΔ*ompD* (Selke *et al.*, 2007). The protein OmpD is one of the most abundant proteins in the outer membrane of *Salmonella enterica* and is not found in other gram-negative bacteria, which makes it a suitable marker protein (Santiviago *et al.*, 2003). Selke *et al.* (2007) proved that oral immunization of pigs with *Salmoporc*Δ*ompD*, consequently challenged with a multi-resistant *Salmonella* Typhimurium isolate, resulted in a significant reduction of clinical symptoms, colonization of lymph nodes and the intestinal tract of pigs and allowed differentiation of infected and vaccinated animals, using an OmpD based ELISA. Unfortunately, this DIVA-vaccine is not broadly applicable because European *Salmonella* serosurveillance programmes are mostly based on the detection of antibodies against the LPS of *Salmonella* (Cortiñas *et al.*, 2009). Moreover, Gil-Cruz *et al.* (2009) showed that the *Salmonella* Typhimurium protein, OmpD, is important in mediating a protective B cell antibody response.

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Aims of the study

Vaccination of pigs with live attenuated vaccines reduces the colonization rate of *Salmonella* Typhimurium in the porcine intestinal tract and might thus help pig farms to achieve a low *Salmonella* status. Due to the LPS-based serological method of sample analysis in current *Salmonella* monitoring programs, the existing DIVA vaccination strategy is, however, not applicable within current European *Salmonella* serosurveillance systems. Furthermore, live vaccines are potentially excreted by the vaccinated animals, especially when pigs are stressed, and this may result in environmental contamination and might pose a public health hazard.

The general aim of this thesis was optimization of live *Salmonella* Typhimurium vaccine strains for use in pigs. Preferably, vaccination should not only result in a reduction of the colonization rate of *Salmonella* during the acute phase of the infection, but also induce protection against *Salmonella* re-excretion by carrier pigs during periods of stress. It should also allow discriminating infected from vaccinated animals and it should not only be safe for the vaccinated pig, but also for the consumer and the environment.

The specific aims of this dissertation were therefore:

1) Evaluation of the ability of a commercial live *Salmonella* Typhimurium vaccine to protect against stress related wild type *Salmonella* recrudescence in carrier pigs.

2) Development and characterization of a vaccine marker strain of *Salmonella* Typhimurium that allows discrimination between vaccinated and infected pigs in currently used *Salmonella* surveillance programs.

3) Construction of a vaccine strain in *Salmonella* Typhimurium that does not recrudesce in stressed pigs and is hence safer for the consumer.

4) Construction of a DIVA vaccine that is more susceptible to environmental stress.

Chapter 2: Experimental studies

2.1 Vaccination of pigs reduces *Salmonella* Typhimurium numbers in a model mimicking pre-slaughter stress

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2.1.1 Abstract

In pigs infection with *Salmonella* Typhimurium often results in the development of carriers that re-excrete *Salmonella* during periods of stress. Previous studies have shown that cortisol plays a significant role in the recrudescence of *Salmonella* Typhimurium and that re-excretion of *Salmonella* can be induced by injections of dexamethasone. This study evaluated whether a commercially available *Salmonella* Typhimurium vaccine was able to reduce *Salmonella* excretion in a model mimicking pre-slaughter stress. Pigs were randomly assigned to either a vaccination or a control group and, 5 weeks later, infected with *Salmonella* Typhimurium. Twenty-three days post infection, pigs were injected with dexamethasone to induce recrudescence of *Salmonella* and *Salmonella* Typhimurium numbers were determined. *Salmonella* loads were significantly lower in the ileum and colon and in the contents of the ileum and caecum in vaccinated pigs than in non-vaccinated pigs. In addition, significantly more *Salmonella* positive tonsil and colon samples were found in non-vaccinated pigs. Vaccination with an attenuated vaccine reduced but did not eliminate *Salmonella* Typhimurium in pigs in conditions mimicking pre-slaughter stress.

2.1.2 Introduction

Salmonella enterica subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) is an important cause of human bacterial gastroenteritis and pigs are a major reservoir for this serotype (Boyen *et al*., 2008). In pigs infection with *Salmonella* Typhimurium often results in the development of carriers that intermittently excrete *Salmonella* in very low numbers (Boyen *et al.*, 2008). During periods of stress, such as transport to the slaughterhouse, recrudescence of *Salmonella* may occur and result in carcass contamination (Mannion *et al.*, 2008; Verbrugghe *et al.*, 2011). If a slaughterhouse produces contaminated carcasses, all post-harvest stages may be contaminated (Delhalle *et al.*, 2009). Cortisol plays an important role in stimulating *Salmonella* re-excretion by carrier pigs (Verbrugghe *et al.*, 2011). A licensed live vaccine (*Salmoporc®*; IDT Biologika), against *Salmonella* Typhimurium is commercially available for use in pigs in Europe, and has been shown to reduce both shedding and colonization of host tissues (Selke *et al.*, 2007; Delhalle *et al.*, 2009). However, it has not been shown whether vaccination can reduce shedding during periods of stress. The aim of this study was to evaluate whether vaccination with *Salmoporc®* reduced *Salmonella* Typhimurium numbers in a model mimicking pre-slaughter stress in pigs (i.e. after injection with dexamethasone).

2.1.3 Materials and methods

All animal use was approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2010/143). Twenty-two, three-week-old piglets, obtained from a serologically negative breeding herd and negative for *Salmonella* at faecal sampling, were randomly assigned to a vaccinated (V) (n = 11) and a non-vaccinated (NV) (n = 11) group. Prior to the onset of the study, all piglets were tested for antibodies to *Salmonella* Typhimurium using Swine *Salmonella* Ab Test (IDEXX Laboratories). Coating antigens in this ELISA include LPS of serogroups B, C1 and D (O-antigens 1, 4, 5, 6, 7 and 12) (Farzan *et al.*, 2007). One week later, pigs in group V were vaccinated orally with 1 mL of the vaccine (*Salmoporc®*), and pigs in the NV group were given 1 mL of a saline solution. This was repeated 3 weeks later. Five weeks after the first vaccination all animals were again tested for antibodies to *Salmonella* Typhimurium and then orally inoculated with 2 x 10^7 colony forming units (CFU) of *Salmonella* Typhimurium strain 112910a (resistant to 20 µg/mL nalidixic acid). To mimic pre-slaughter stress conditions, all animals received an IM injection

of 2 mg dexamethasone (Kela Laboratoria) per kg body weight 23 days after challenge. This dose has been previously shown to induce recrudescence of *Salmonella* in pigs without causing immunosuppression (Delhalle *et al.*, 2009; Verbrugghe *et al.*, 2011). Twenty-four h later, all animals were humanely euthanased, organ samples collected for bacteriological analysis and blood samples collected for serological examination.

2.1.4 Results and discussion

Organ and faecal samples were examined for the presence of the challenge strain by plating tenfold dilutions on brilliant green agar (Oxoid) supplemented with 20 µg/mL nalidixic acid. If negative at direct plating, samples were pre-enriched in buffered peptone water, enriched in tetrathionate broth and plated on brilliant green agar supplemented with 20 µg/mL nalidixic acid. Samples that were negative after direct plating but positive after enrichment were presumed to contain 83 CFU/g (detection limit for direct plating). Samples that remained negative were presumed to contain 0 CFU/g. All statistical analyses were performed with an independent-Samples T test, using the SPSS Statistics 19.0 software (SPSS Inc.). A *P*-value of < 0.05 was considered significant.

Serological examination indicated that all animals were serologically negative for *Salmonella* before the start of the experiment. One 1 h before challenge, all vaccinated animals were seropositive for *Salmonella,* while non-vaccinated pigs were serologically negative. All pigs were serologically positive at euthanasia (Figure 1).

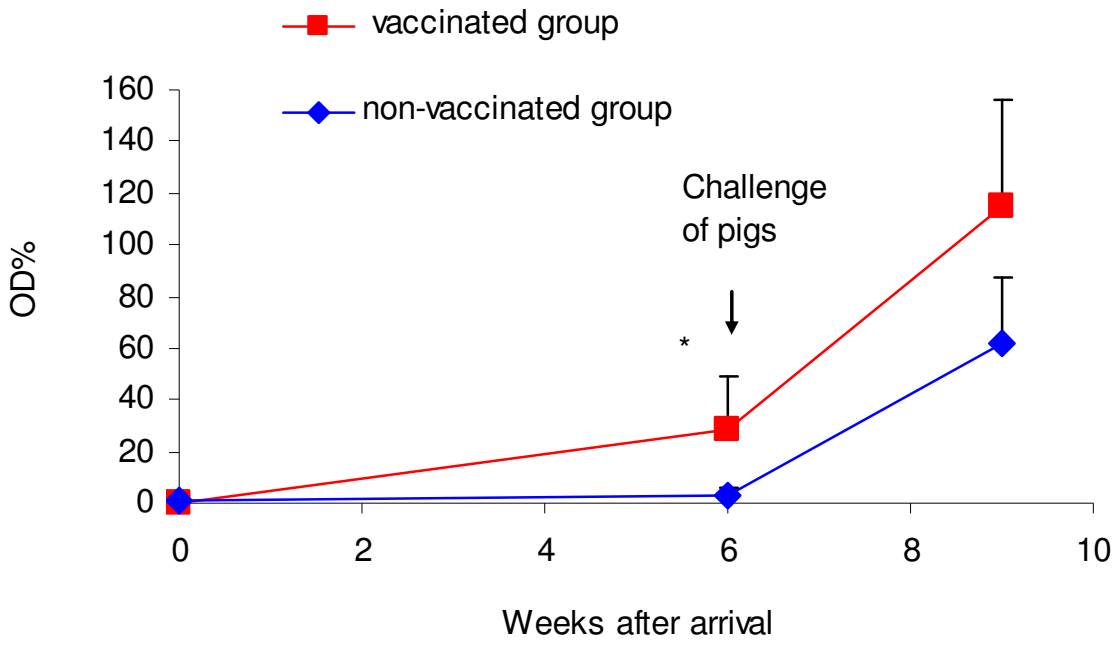

Figure 1: Serological analyses of vaccinated and non-vaccinated pigs. Blood samples were analysed prior to first vaccination, 1 h before challenge with *Salmonella* Typhimurium strain 112910aNal_{20} (5 weeks after first vaccination) and prior to euthanasia (9 weeks after arrival). An asterisk refers to a significant difference ($P < 0.05$) between vaccinated and non-vaccinated pigs.

All piglets were positive for the *Salmonella* Typhimurium challenge strain in at least one sample (Table 1). All samples from pigs in group NV, except for one colon sample, were positive for the challenge strain in contrast the mean number of positive samples in pigs in group V was 7/9. Pigs in group NV had higher numbers of *Salmonella* Typhimurium in their gut tissues and contents in comparison to carrier pigs from the V group. This increase was significant in the ileum ($P = 0.04$), ileum contents ($P = 0.003$), colon ($P = 0.007$) and caecum contents ($P = 0.006$) (Figure 2). These data show that vaccination with a live vaccine reduced the numbers of *Salmonella* Typhimurium, even in conditions mimicking pre-slaughter stress. However as *Salmonella* Typhimurium was not eliminated by vaccination, further research is required to establish the impact of vaccination on the risk of contamination at slaughter.

Table 1: The effect of vaccination with a live *Salmonella* Typhimurium vaccine on the proportion of samples positive for the challenge strain (*Salmonella* Typhimurium 112910aNal_{20}), 24 days post challenge. An asterisk refers to the proportion of the positive sample lower then in non-vaccinated group ($P < 0.05$).

Site	Vaccinated (n = 11)	Non vaccinated (n = 11)
Tonsil	6*	**11**
Ileum	10	**11**
Ileal contents	8	**11**
Ileocaecal lymph nodes	11	**11**
Caecum	9	**11**
Caecal contents	10	**11**
Colon	4*	**10**
Colon contents	10	**11**
Faeces	9	**11**
Total	**77/99**	**98/99**

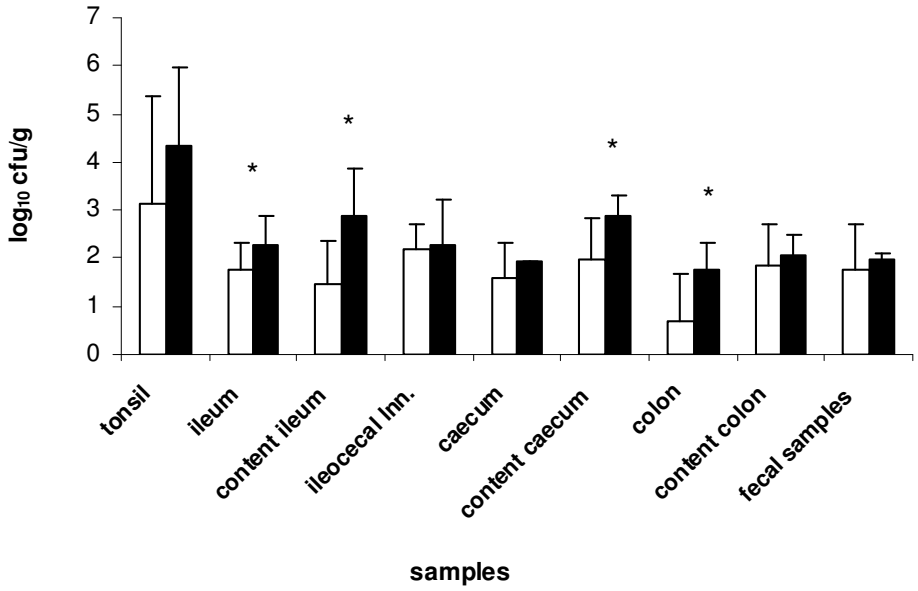

Figure 2: Recovery of *Salmonella* Typhimurium strain 112910aNal_{20} from various organs 24 days post challenge. White bars represent vaccinated animals and black bars represent non-vaccinated animals. The mean log_{10} value of CFU per gram sample and the standard deviations are given. An asterisk refers to a significant difference ($P < 0.05$) between vaccinated and non-vaccinated pigs.

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2.2 *Salmonella* Typhimurium LPS mutations for use in vaccines allowing differentiation of infected and vaccinated pigs

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2.2.1 Abstract

Contaminated pork is a major source of human salmonellosis and the serovar most frequently isolated from pigs is *Salmonella* Typhimurium. Vaccination could contribute greatly to controlling *Salmonella* infections in pigs. However, pigs vaccinated with the current vaccines cannot be discriminated from infected pigs with the LPS-based serological tests used in European *Salmonella* serosurveillance programmes. We therefore examined which LPS encoding genes of *Salmonella* Typhimurium can be deleted to allow differentiation of infected and vaccinated animals, without affecting the vaccine strain's protective capacity. For this purpose, deletion mutants in *Salmonella* strain 112910a, used as vaccine strain, were constructed in the LPS encoding genes: Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG* and Δ*rfaF*. Primary inoculation of BALB/c mice with the parent strain, Δ*rfaL,* Δ*rfbA* or Δ*rfaJ* strain but not the Δ*rfaG*, Δ*rfaF* or Δ*rfaI* strain protected significantly against subsequent infection with the virulent *Salmonella* Typhimurium strain NCTC12023. Immunization of piglets with the Δ*rfaJ* or Δ*rfaL* mutants resulted in the induction of a serological response lacking detectable antibodies against LPS. This allowed a clear differentiation between sera from pigs immunized with the Δ*rfaJ* or Δ*rfaL* strains and sera from pigs infected with their isogenic wild type strain. In conclusion, applying deletions in the *rfaJ* or the *rfaL* gene in *Salmonella* Typhimurium strain 112910a allows differentiation of infected and vaccinated pigs in an LPS based ELISA without reducing the strain's protective capacities in mice.

2.2.2 Introduction

Salmonellosis is one of the most important bacterial zoonotic diseases in humans (Majowicz *et al*., 2010) and *Salmonella* infections are often linked with the consumption of contaminated pork (Boyen *et al.*, 2008). The serovar most frequently isolated from pigs is *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium), which is also the most prevalent serovar in humans (Boyen *et al.*, 2008). In order to reduce human *Salmonella* Typhimurium infections, minimization of the *Salmonella* intake into the food chain is very important and efforts to reduce transmission of *Salmonellae* by food should be implemented on a global scale (Majowicz *et al*., 2010). A combined approach using hygienic measures, the use of feed additives and different protection measures, such as vaccination, has been proposed to reduce the contamination on farms (Boyen *et al.*, 2008; Boyen *et al.*, 2009; Van Immerseel *et al.*, 2005). Vaccination has already proven to be efficient in laying hens, reducing faecal shedding and internal egg contamination of *Salmonella*, resulting in reduction of the number of human salmonellosis cases (Collard *et al.*, 2008; Gantois *et al.*, 2006). Currently, one licensed *Salmonella* Typhimurium live vaccine for pigs is commercially available in Europe (Lindner *et al.*, 2007) and has shown to reduce both shedding and colonization of host tissues (Selke *et al.*, 2007) and to induce a substantial *Salmonella* antibody response seven days after the second immunization (Eddicks *et al.*, 2009). These antibodies are, however, not distinguishable from those induced after a wild type *Salmonella* Typhimurium infection. An isogenic mutant of the *Salmonella* vaccine strain was developed, which lacks the *ompD* gene. This allowed differentiation of infected and vaccinated animals, using an OmpD based ELISA (Selke *et al.*, 2007). Unfortunately, this DIVA-vaccine is not broadly applicable, despite its ability to reduce colonization (Selke *et al*., 2007), because European *Salmonella* serosurveillance programmes are mostly based on the detection of antibodies against the lipopolysaccharides (LPS) of *Salmonella* (Cortinas Abrahantes *et al.*, 2009). It was therefore the aim of this study to develop and characterize LPS mutations that might be used as DIVA markers with application in the currently used monitoring programmes in the EU. Since a marker should not affect the vaccine strain's protective capacity, we first compared the protective capacity of LPS mutants and their parent strain in a standardized mouse virulent assay. Secondly, the LPS deletion mutants were tested for their capability to elicit a DIVA antibody response in pigs.

2.2.3 Materials and methods

All *in vivo* experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2009/124, EC 2009/131, EC 2010/080 and EC 2010/108).

Bacterial strains

Salmonella Typhimurium strain 112910a, phage type 120, isolated from a pig stool sample and characterized previously (Boyen *et al.*, 2009), was used as the wild type background to construct several isogenic LPS knock-out mutants: Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG*, Δ*rfaF*. These strains were used for immunization of mice and pigs. The bacterial strains and primers used in this study are shown in table 1A/B. The knock-out mutants were constructed as described before (Boyen *et al.*, 2006). Briefly, the genes of interest were first substituted by a PCR adjusted antibiotic resistance cassette (kanamycin) using the helper plasmid pKD46. This plasmid encodes the phage λ Red system, which promotes recombination between the native gene and the PCR adjusted antibiotic resistance cassette. Recombinant clones were selected by plating on Luria-Bertani agar (LB; Sigma Aldrich Chemie Gmbh, Steinheim, Germany) containing 100 µg/ml kanamycin. The substitution was confirmed by PCR. In the last step, the antibiotic resistance cassettes were eliminated using the helper plasmid pCP20. The targeted genes were completely deleted from the start codon through the stop codon, as confirmed by sequencing and gene disruptions were nonpolar. *Salmonella* challenge strains comprised, spontaneous mutants resistant to 20 µg/ml nalidixic acid (Nal^{20}) in a NCTC12023 *Salmonella* Typhimurium strain highly virulent in BALB/c mice, in *Salmonella* Enteritidis strain SE147, in *Salmonella* Heidelberg strain 704Sa06 and in *Salmonella* Typhimurium strain 112910a. All bacteria were routinely grown in LB broth or on brilliant green agar (BGA) at 37 °C, unless stated otherwise.

Table 1A: Strains used in this study

Strain	Genotype and O-antigens	Product of the deleted gene	Source or reference
WT	*Salmonella* Typhimurium 112910a (O: 1, 4, 12)	no deletions	(Boyen *et al.*, 2008)
NCTC12023Nal^{20}	*Salmonella* Typhimurium NCTC 12023 Nal^{20} (O: 1. 4, 12)	no deletions	(Hensel *et al.*, 1995)
Δ*rfaL*	*Salmonella* Typhimurium 112910a Δ*rfaL*	*Salmonella* Typhimurium O-antigen ligase	This study
Δ*rfaJ*	*Salmonella* Typhimurium 112910a Δ*rfaJ*	LPS 1,2-glucosyltransferase	This study
Δ*rfaI*	*Salmonella* Typhimurium 112910a Δ*rfaI*	LPS 1,3-galactosyltransferase	This study
Δ*rfaG*	*Salmonella* Typhimurium 112910a Δ*rfaG*	LPS core biosynthesis protein	This study
Δ*rfaF*	*Salmonella* Typhimurium 112910a Δ*rfaF*	LPS heptosyltransferase II	This study
***Salmonella* Enteritidis**	*Salmonella* Enteritidis Nal^{20} (O: 1, 9, 12)	no deletions	This study
***Salmonella* Heidelberg**	*Salmonella* Heidelberg Nal^{20} (O: 1, 4, 5, 12)	no deletions	This study
Δ*rfbA*	*Salmonella* Typhimurium 112910a Δ*rfbA*	glucose-1-phosphate thymidylyltransferase	This study
WT Nal^{20}	*Salmonella* Typhimurium 112910a Nal^{20} (O: 1, 4, 12)	no deletions	(Van Immerseel *et al.*, 2005)

Table 1B: Primers used in this study

Primers	Sequences
***rfbA* forward**	5'- TAATAAATTTAAATGCCCATCAGGGCATTTTCTATGAATGAGAAATGGAATGTGTAGGCTGGAGCTGCTTC - 3'
***rfbA* reverse**	5'- GGCTCTAAGATCAAGACATCTGGTATTGCTGTTTTAATCACAATCACCATATGAATATCCTCCTTAG -3'
***rfaL* forward**	5'- CATTAAAGAGACTCTGTCTCATCCCAAACCTATTGTGGAGAAAAGTGTGTAGGCTGGAGCTGCTTC - 3'
***rfaL* reverse**	5' - TTGAGTCCTGATGATGGAAAACGCGCTGATACCGTCATATGAATATCCTCCTTAG - 3'
***rfaJ* forward**	5' - ATAGCCTACTTTAAACGTAAACTTCTTGAATAAAACCCATAGGTGATGTATGTGTAGGCTGGAGCTGCTTC - 3'
***rfaJ* reverse**	5' - AGTTTTTAATCTTTTTTTCAATAATCATAATGGAGATTTAGGGAGGGGAACATATGAATATCCTCCTTAG -3'
***rfaI* forward**	5' - TTTAAAAATTTTAATAATGCAATATTCTCGAAATTACAAAAGTGATCACTTGTGTAGGCTGGAGCTGCTTC - 3'
***rfaI* reverse**	5' - TTCAGCTATTTCTATCTCAGGAAATGAATCCATTACATCACCTATGGGTTCATATGAATATCCTCCTTAG - 3'
***rfaG* forward**	5'- GAAAAAATGCTGCCGCATGAGGCACGCACCATAGATTTGGACAGCCTGCTTGTGTAGGCTGGAGCTGCTTC - 3'
***rfaG* reverse**	5' - CCTCAAAAGCATCTTTACCGCGCCATAGTGTGGTTAACGGCGCTTTCAGCCATATGAATATCCTCCTTAG -3'
***rfaF* forward**	5' - GCCGAAGGCGTCACGGAGTATATGGCCTGGCTGAACCGCGACGCGTAAGTTGTGTAGGCTGGAGCTGCTTC - 3'
***rfaF* reverse**	5' – GGTATGTAATACGTCGCCCATCGATGATGTTTTAACGATCAAAACCCGCACATATGAATATCCTCCTTAG – 3'

Characterization of the LPS mutants

Validation of the LPS phenotype occurred by SDS-polyacrylamide gel electrophoresis and fluorescent staining. For this purpose LPS was isolated from *Salmonella* Typhimurium strain 112910a and its isogenic knock-out mutants using a commercially available LPS extraction kit (Intron biotechnology, Gyeonggi-do, Korea). The obtained LPS was quantified using a ToxinSensor^{TM} Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, USA) and was separated by standard SDS-polyacrylamide gel electrophoresis. LPS was stained using a Molecular probes Pro-Q Emerald LPS Gel stain kit (Invitrogen, Oregon, USA), creating a bright green-fluorescent signal, which was visualised with a 300 nm UV-transilluminator. To verify whether LPS mutant strains (Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG*, Δ*rfaF*) were still expressing O-antigens on their surface, an *in vitro* agglutination test (PRO-LAB O_4 and O_{12} antisera, diagnostics, Austin, Texas) was performed, according to the manufacturer's instructions. The smooth phenotype was also tested by checking sensitivity of *Salmonella* Typhimurium and its isogenic knock-out mutants to bacteriophage P22 as described elsewhere (Boyen *et al.*, 2006). As a measure of *in vitro* virulence of the wild type strain and its isogenic mutants, invasiveness of all strains was assessed in porcine epithelial cells (IPEC-J2) using a gentamicin protection assay. The polarized porcine epithelial intestinal cell line IPEC-J2 was derived from jejunal epithelia isolated from a neonatal piglet (Rhoads *et al.*, 1994; Schierack *et al.*, 2006). Cells were seeded in 24 well plates at a density of approximately 10^5 cells per well and were allowed to grow to confluency for at least one day. These wells were inoculated with *Salmonella* Typhimurium strain 112910a or one of its isogenic LPS knock-out mutants at a multiplicity of infection (MOI) of 10:1. To synchronize the infection, the inoculated multiwell plates were centrifuged at 365 x g for 10 minutes. After 30 minutes incubation at 37 °C and 5% CO_2, the wells were rinsed and fresh medium supplemented with 50 µg/ml gentamicin (Gibco, Life Technologies, Paisly, Scotland) was added. After 60 minutes of incubation at 37 °C and 5% CO_2, the wells were rinsed three times. To quantify invasion, the cells were lysed with 0.25% deoxycholate (Sigma, Aldrich, Steinheim, Germany) 100 minutes after inoculation and 10-fold dilutions were plated on BGA plates. The invasion assay was conducted in triplicate with three repeats per experiment.

ELISA procedures

A commercially available enzyme-linked immunosorbent assay (ELISA) (HerdChek *Salmonella*; IDEXX Laboratories, Schiphol-Rijk, Noord-Holland, The Netherlands) for the detection of porcine antibodies against the LPS of *Salmonella* was used as a reference according to the manufacturer's instructions. Coating antigens in this ELISA include LPS of serogroups B, C1 and D (O-antigens 1, 4, 5, 6, 7 and 12) (Farzan *et al.*, 2007). Besides, an in-house *Salmonella* Typhimurium strain 112910a whole cell ELISA, to detect porcine anti *Salmonella* Typhimurium antibodies, was prepared as follows. *Salmonella* Typhimurium strain 112910a was cultured overnight at 37 °C in 500 ml LB broth. Inactivation was achieved by adding 0.18% (v/v) formalin overnight at 37 °C. The bacteria were centrifuged three times (5000 x g for 30 min at room temperature) and the resulting pellet was resuspended in a volume of 250 ml Phosphate Buffered Saline (PBS) with 0.18% formalin and incubated overnight at 37 °C. The inactivated culture was centrifuged again (5000 x g for 10 min at 5 °C) and the pellet was resuspended in a final volume of 250 ml coating buffer (1.08 g $Na_2CO_3.10H_2O$, 0.968 g $NaHCO_3$, 0.25 l aqua ad iniectabilia 100 % w/v). F96 maxisorp Nunc-immuno plates (Nunc; Denmark) were coated with 140 µl formalin-inactivated *Salmonella* strains diluted in coating buffer to an optical density of 660 nm, measured using a spectrophotometer (Ultraspec III®), incubated for 24 h at 4 °C and washed three times with 100 µl wash buffer (0.6 g NaH_2PO_4. $2H_2O$, 5.6 g NaH_2PO_4. $12H_2O$, 0.5 ml Tween 20 (Merck, Germany), 12.5 g NaCl). Plates were stored at 4 °C until used. Before starting the assay, the plates were washed with 100 µl destillated water (AD) + 1% milk powder to prevent non-specific binding. A 1/2000 dilution of sera (100 µl) was added to the wells. The cut-off optical density was calculated as the mean obtained from the sera from a bacteriologically and serologically *Salmonella* free pig (the negative control, determined using the HerdChek ELISA) plus two times the standard deviation. All measurements were performed in triplicate.

Protective capacity of the mutant strains

The protective capacity of the LPS deletion mutants was compared to that of the wild type strain using a mouse model. Five-week-old specified pathogen-free (SPF) BALB/c mice (Bio services, Janvier, France) were housed in filter-topped cages at 25 °C under natural day-night rhythm with *ad libitum* acces to feed and water and enriched with mouse houses and play tunnels. Bacterial inocula used for oral protection assays were prepared as follows. Strains

were grown overnight on a shaker at 37 °C in 100 ml LB broth. The bacteria were washed twice in PBS at 3500 x g for 15 min at room temperature and adjusted in PBS to the appropriate concentration of 2 x 10^7 colony forming units per ml (CFU/ml). The number of viable bacteria was determined by plating tenfold dilutions on BGA.

In a first experiment, we tested whether the LPS mutants affect the protective capacity of *Salmonella* Typhimurium strain 112910a against a subsequent challenge with a highly virulent strain. For that purpose seven groups of ten mice were inoculated first via the orogastric route with 2 x 10^7 CFU/ml of one of the LPS mutant strains (either Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG* or Δ*rfaF*) or with the wild type *Salmonella* Typhimurium strain 112910a. A control group of ten mice was sham-inoculated with sterile PBS. Four weeks after primary inoculation, all mice were challenged with a total of 10^8 CFU/ml of the virulent *Salmonella* Typhimurium strain NCTC12023Nal^{20} by the orogastric route.

In a second experiment, we tested whether truncation of the LPS chain in the Δ*rfaJ* strain promotes cross-immunity against other *Salmonella* serovars. Sixty mice were orally inoculated first with 2 x 10^7 CFU/ml of either the Δ*rfaJ* strain (n = 20) or *Salmonella* Typhimurium strain 112910a (n = 20). A control group of 20 mice was sham-inoculated with sterile PBS (n = 20). Sixteen days after primary inoculation, ten mice of each group were challenged with a total of 10^8 CFU/ml of either *Salmonella* Heidelberg strain 704Sa06 Nal^{20} (n = 10) or *Salmonella* Enteritidis strain SE147 Nal^{20} (n = 10).

In both *in vivo* experiments, mice were euthanized nine days post challenge. Tissue of spleen and liver and tissue and caecal contents were examined quantitatively for the presence of the respective *Salmonella* strain. Samples were weighed and 10% (w/v) suspensions were made in buffered peptone water (BPW; Oxoid, Basingstoke, UK) after which the material was homogenized with a stomacher. The homogenized samples were examined for the presence of *Salmonella* by plating 10-fold dilutions on BGA supplemented with nalidixic acid (BGA^{NAL}). If negative at direct plating, the samples were pre-enriched overnight in BPW at 37 °C, enriched overnight at 37 °C in tetrathionate broth and then plated on BGA^{NAL}. Samples that were negative after direct plating but positive after enrichment were presumed to contain 50 CFU per gram tissue (detection limit for direct plating). Samples that remained negative after enrichment were presumed to contain 0 CFU per gram tissue.

Immunization of piglets

In this study, we examined whether it was possible to discriminate between the serological response induced after immunization of pigs with the wild type and its isogenic Δ*rfaL* and Δ*rfaJ* strains. For this purpose, we immunized pigs with adjuvanted bacterins of either the wild type strain, the Δ*rfaL* strain or the Δ*rfaJ* strain to maximize antibody production (Nichols *et al.*, 2010). Fourteen, 6-week-old, bacteriologically and serologically *Salmonella* negative piglets (commercial closed line based on Landrace) were housed together at 25 °C under natural day-night rhythm with *ad libitum* access to feed and water.

For preparation of antigen suspensions for immunization of pigs, strains were cultured for 9 hours at 37 °C in 400 ml LB broth and were adjusted to 5 x 10^8 CFU/ml. Inactivation was achieved by adding 0.18% (v/v) formalin (VWR international, Fontenay Sous Bois, France) overnight at 37 °C. The formalin-inactivated *Salmonella* strains were washed twice (5000 x g for 30 min at room temperature) and the resulting pellet was resuspended in 11 ml PBS with 0.18% formalin and incubated overnight. Thereafter, this suspension was mixed with 11 ml marcol oil (Esso Belgium nv, Antwerp, Belgium) containing 3.4% sterilized Tween 80 (Sigma Aldrich Chemie Gmbh, Steinheim, Germany) and 6.4% mannide monooleate (Sigma Aldrich Chemie Gmbh, Steinheim, Germany). To check sterility, all suspensions were cultured on Columbia agar plates containing 5% sheep blood (COL; Oxoid, Wesel, Germany) and incubated aerobically and anaerobically overnight at 37 °C.

Piglets were randomly allocated to three vaccinated groups (n = 4) and one sham-vaccinated control group (n = 2). One and three weeks after their arrival, pigs were intramuscularly immunized with one of the formalin-inactivated *Salmonella* strains (either: *Salmonella* Typhimurium strain 112910a, Δ*rfaJ* or Δ*rfaL*) with Freund's incomplete adjuvant to elicit an optimal humoral (antibody-mediated/Th2) response (Nichols *et al.*, 2010). The control group was injected with 1 ml of sterile PBS. Four weeks after the second immunization, the pigs were humanely euthanized and blood samples were taken from the vena jugularis externa, using a Venoject system (Terumo; Roma, Italia). All sera samples were examined for the presence of anti *Salmonella* Typhimurium antibodies using the Herdchek ELISA and the in-house *Salmonella* Typhimurium strain 112910a whole cell ELISA, prepared as described previously.

Experimental infection of piglets with *Salmonella* Typhimurium

To obtain sera from *Salmonella* Typhimurium infected piglets, an experimental infection was performed with 4 week-old bacteriologically and serologically *Salmonella* negative piglets (commercial closed line based on Landrace). Piglets were randomly allocated in one experimental group (n = 3) and one negative control group (n = 3) and both groups were housed in separate isolation units at 25°C under natural day-night rhythm with *ad libitum* access to feed and water. One week after their arrival at the facility, three experimental animals were orally inoculated with approximately 2 x 10^7 CFU/ml of *Salmonella* Typhimurium strain 112910aNal[20] in 2 ml Hank's buffered salt solution (HBSS; Gibco Life Technologies, Paisley, Scotland); the negative control group (n = 3) was sham-inoculated with 2 ml HBSS. Temperature, body weight and occurrence of diarrhoea of the pigs were monitored daily. Six weeks after oral inoculation, pigs were humanely euthanized and blood samples were taken from the vena jugularis externa, using a Venoject system (Terumo; Roma, Italia). All sera samples were examined for the presence of anti *Salmonella* Typhimurium antibodies using the Herdchek ELISA and the in-house *Salmonella* Typhimurium strain 112910a whole cell ELISA, prepared as described previously.

Statistical analysis

In all experiments, statistical analysis was performed using a nonparametric Mann-Whitney-U-test, using the SPSS Statistics 17.0 software (SPSS Inc., Chicago, USA). ELISA results were analysed by a one-way ANOVA and Bonferroni corrections were applied. A *P-value* of < 0.05 was considered significant.

2.2.4 Results

Verification of the LPS mutants

A systematic truncation of the LPS chain occurred as a result of defects in genes coding for glycosyl or phosphoryl transferases (or epimerases) and is shown in Figure 1. LPS patterns obtained by standard SDS-polyacrylamide gel electrophoresis of *Salmonella* Typhimurium strain 112910a, the O-antigen mutant (Δ*rfbA*), the outer core mutants (Δ*rfaL,* Δ*rfaJ,* Δ*rfaI*) and the inner core mutants (Δ*rfaG*, Δ*rfaF*) are presented in Figure 2 and show a visible loss of

O-antigens for core mutants (Δ*rfaL,* Δ*rfaJ,* Δ*rfaI* , *rfaG*, Δ*rfaF*) compared to *Salmonella* Typhimurium strain 112910a. Loss of the *rfbA* gene resulted in the presence of a complete core without covalently bound O-antigen ("semirough" LPS), because the rfb locus is responsible for the biosynthesis of O-antigen (Michael *et al.*, 2008). The complete lack of O-antigens in core mutants was also confirmed by resistance to bacteriophage P22 and appearance of the "rough" phenotype. *Salmonella* Typhimurium strain 112910a showed the "wild-type" LPS structure and is denoted as "smooth" LPS (Hitchcock *et al.*, 1986).

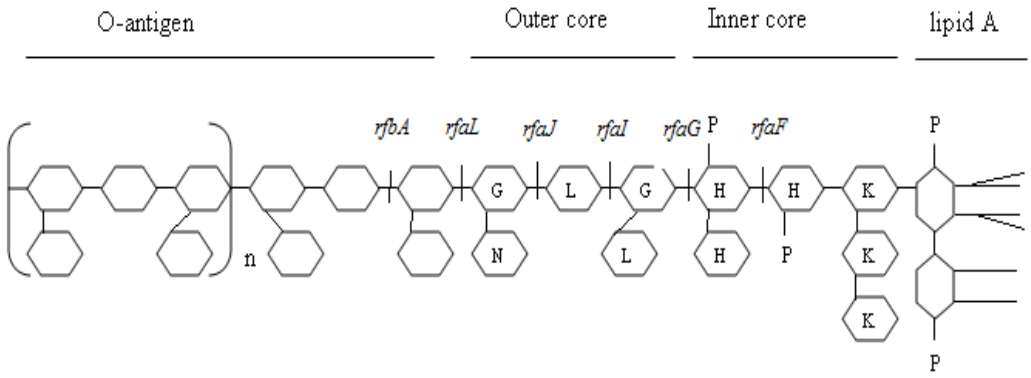

Figure 1: Schematic representation of the structure of lipopolysaccharide (LPS). Truncation of the LPS chain as a consequence of Δ*rfbA,* Δ*rfaL,* Δ*rfaJ,* Δ*rfaI,* Δ*rfaG* and Δ*rfaF* deletions is shown (G: glucose, L: galactose, H: heptose, K: 2-keto 3-deoxy-octulosonate (KDO), N: N-acetylglucosamine).

A slide agglutination test was used to verify expression of O-antigens on the surface of *Salmonella* Typhimurium 112910a and its isogenic knock-out mutants. While *Salmonella* Typhimurium strain 112910a showed a distinct agglutination within 60 seconds, little granular clumping was seen with the *rfbA* mutant strain. No agglutination was observed with Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG* and Δ*rfaF* strains, which confirmed a total loss of O_4 and O_{12} antigens.

Further, invasion of *Salmonella* Typhimurium strain 112910a and its isogenic knock-out strains was compared in an IPEC-J2 cell strain, using a gentamicin protection assay. The Δ*rfbA*, Δ*rfaG* and Δ*rfaF* strains showed a statistically significant decrease ($P < 0.05$) in invasion when compared to the 112910a strain, while the Δ*rfaL*, Δ*rfaJ* and Δ*rfaI* strains were not impaired in invasion. Results are summarized in Figure 3.

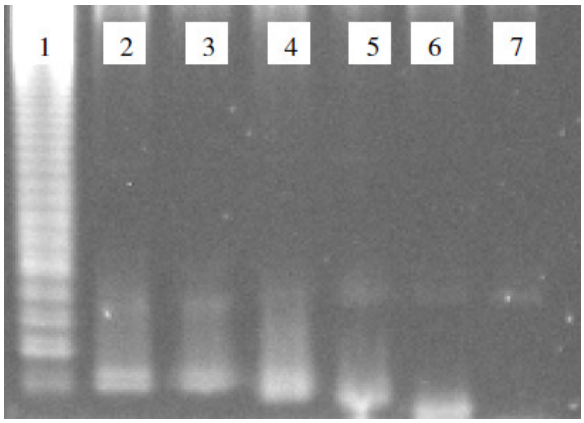

Figure 2: SDS-polyacrylamide gel electrophoresis patterns of LPS of *Salmonella* Typhimurium 112910a (lane 1) and Δ*rfbA* (lane 2), Δ*rfaL* (lane 3), Δ*rfaJ* (lane 4), Δ*rfaI* (lane 5), Δ*rfaG* (lane 6) and Δ*rfaF* (lane 7) mutants are shown. Apart from *Salmonella* Typhimurium strain 112910a (lane 1) all strains show a classical 'rough' type ladder pattern. Staining occurred with fluorescent staining and a ten-fold dilution of 25µg/ml LPS of each strain was loaded.

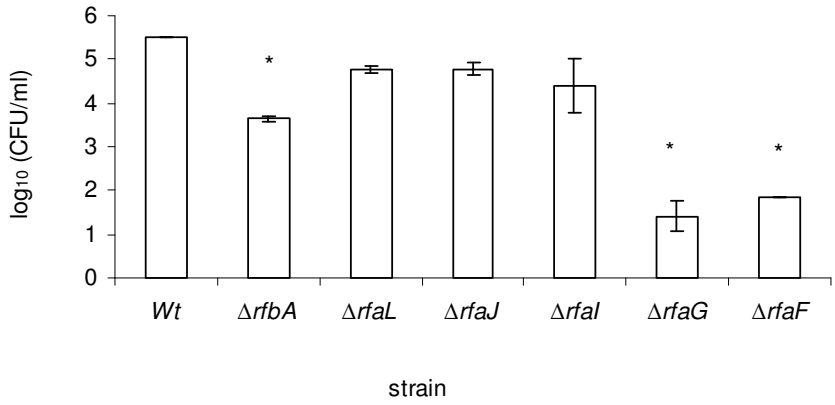

Figure 3: The invasiveness of *Salmonella* Typhimurium and its isogenic knock-out mutants in IPEC-J2 cells. The log values of the number of gentamicin protected bacteria are shown. The results represent the means of three independent experiments conducted in triplicate and standard deviations are given. An asterisk refers to a significantly lower invasion compared to the wild type strain ($P < 0.05$).

Challenge experiments

Oral immunization of mice with *Salmonella* Typhimurium strain 112910a, Δ*rfbA*, Δ*rfaL* or Δ*rfaJ* strains induced a significant ($P < 0.05$) protection against subsequent challenge with NCTC12023Nal[20] in both spleen and liver compared to non immunized control animals. Bacterial counts (wild type, Δ*rfbA*, Δ*rfaL* and Δ*rfaJ*) in caecum samples showed a non

significant ($P > 0.05$) reduction of the numbers of *Salmonella* Typhimurium compared with with control animals. Deletion of *rfaI*, *rfaG* and *rfaF* genes but not *rfbA*, *rfaL* and *rfaJ* genes thus significantly ($P < 0.05$) reduced protection against challenge with *Salmonella* Typhimurium strain NCTC12023Nal[20]. None of the animals immunized with Δ*rfbA*, Δ*rfaG* or Δ*rfaF* died as a result of vaccination, whereas eight mice vaccinated with either strain 112910a (n = 3), Δ*rfaL* (n = 2), Δ*rfaJ* (n = 2) or Δ*rfaI* (n = 1) died as a consequence of vaccination. After challenge, > 60% of the unvaccinated animals or mice vaccinated with either Δ*rfaG* or Δ*rfaF* died opposed to < 40% of mice vaccinated with wild type, Δ*rfaL* or Δ*rfaJ*. Results are illustrated in Figure 4.

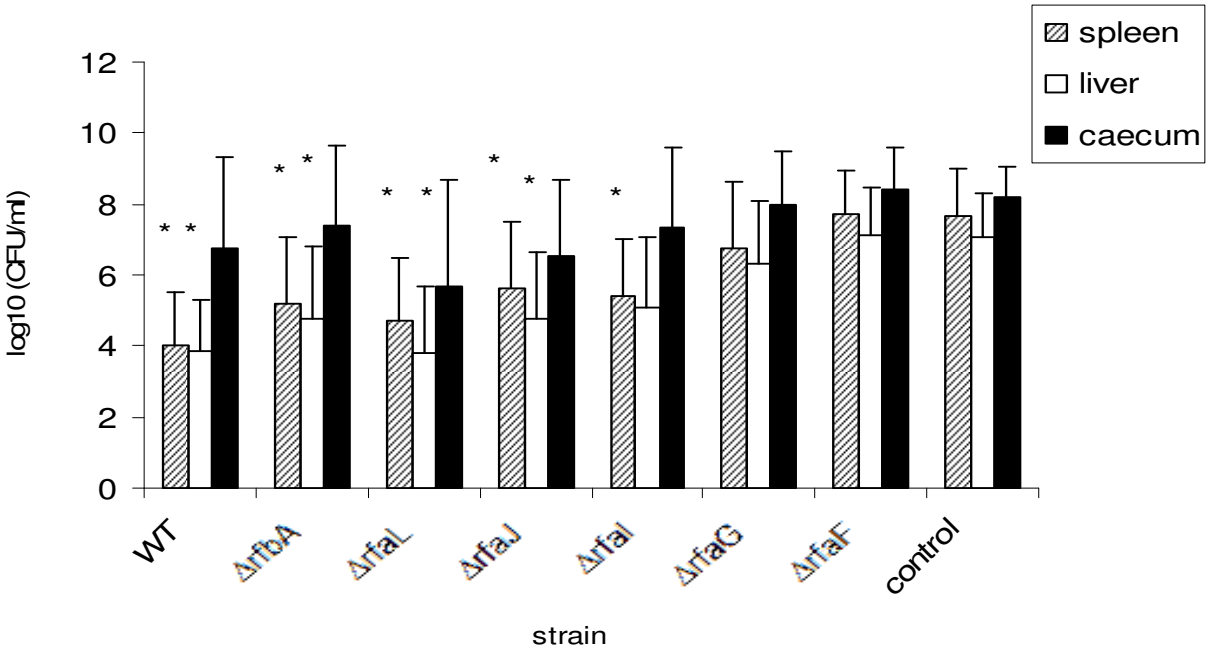

Figure 4: Recovery of *Salmonella* bacteria from various organs of mice immunized with either *Salmonella* Typhimurium, one of its isogenic LPS mutants or non immunized control animals and subsequently challenged with *Salmonella* Typhimurium strain NCTC12023Nal[20]. The $\log_{10}$ value of the ratio of CFU per gram sample and standard deviations are given. An asterisk refers to a significant difference with the control group ($P < 0.05$).

Cross-protection of the Δ*rfaJ* strain

In this experiment we determined to which extent the Δ*rfaJ* strain and *Salmonella* Typhimurium strain 112910a were able to confer cross-protection against *Salmonella* Heidelberg or *Salmonella* Enteritidis. Both strains were equally able to induce a significant ($P < 0.05$) reduction of *Salmonella* Heidelberg in the spleen compared to control animals. In

liver and caecum both strains induced a noticeable, but non significant ($P > 0.05$) reduction of *Salmonella* Heidelberg compared to control animals.

Recovery of *Salmonella* Enteritidis was not significantly different ($P < 0.05$) in spleen, liver and caecum between animals immunized with *Salmonella* Typhimurium strain 112910a and mice immunized with Δ*rfaJ*. Results are shown in Figure 5.

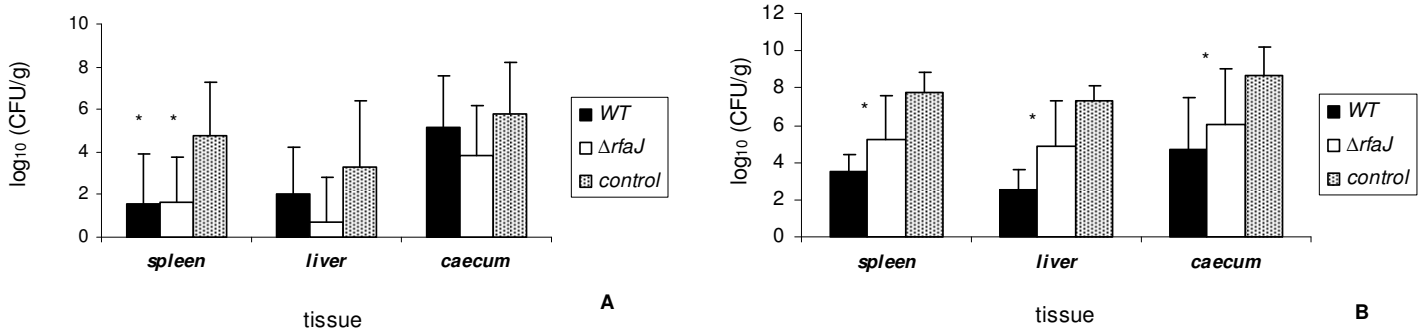

Figure 5: (A) Recovery of *Salmonella* Heidelberg bacteria from various organs of BALB/c mice immunized with *Salmonella* Typhimurium, one of its isogenic LPS mutants or uninfected animals subsequently challenged with *Salmonella* Heidelberg. The $\log_{10}$ average value of the number of CFU per gram sample is given with its standard deviation. An asterisk refers to a significant difference with the control group ($P < 0.05$). (B) Recovery of *Salmonella* Enteritidis bacteria from various organs of BALB/c mice immunized with *Salmonella* Typhimurium, one of its isogenic LPS mutants or uninfected animals subsequently challenged with *Salmonella* Enteritidis. The $\log_{10}$ average value of the number of CFU per gram sample is given with its standard deviation. An asterisk refers to a significant difference with the control group ($P < 0.05$).

Immunological responses in pigs

IDEXX ELISA No significant seroconversion ($P > 0.05$) was noticed in pigs immunized with inactivated Δ*rfaJ* or Δ*rfaL* strains and in control animals (non immunized and non infected animals). Conversely, marked seroconversion occurred in animals immunized or orally infected with the inactivated *Salmonella* Typhimurium strain 112910a. Statistical analysis showed a significant difference ($P < 0.05$) between the antibody response against *Salmonella* Typhimurium LPS in pigs infected with *Salmonella* Typhimurium 112910a and control animals. Results are shown in Figure 6. Results also illustrate a clear differentiation between

sera from piglets immunized with the Δ*rfaJ* strain or Δ*rfaL* strain and sera of pigs infected with their isogenic wild type strain.

Whole-cell ELISA Significant anti-*Salmonella*-antibody titers were detected in the serum of all immunized and infected animals. No significant distinction ($P > 0.05$) regarding *Salmonella*-specific antibody responses could be made between animals that were immunized with the inactivated 112910a strain and those immunized with the inactivated Δ*rfaJ* and Δ*rfaL* strains. Results are shown in Figure 6.

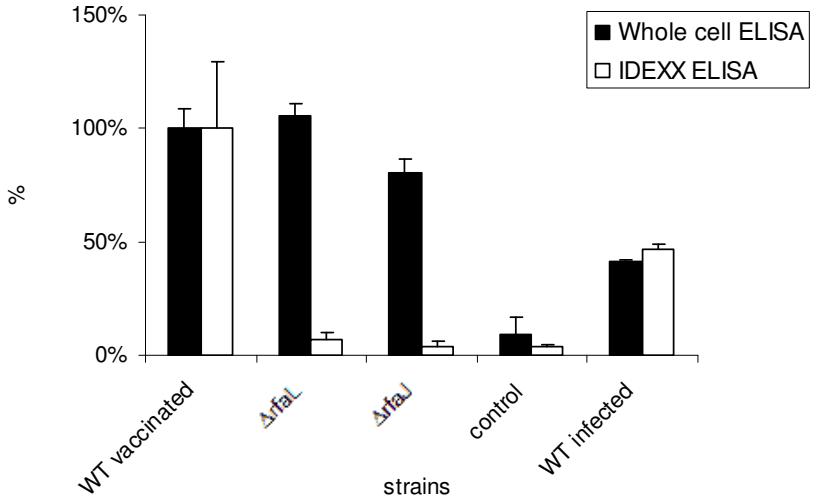

Figure 6: Serological results of pigs immunized with Δ*rfaL*, Δ*rfaJ* or *Salmonella* Typhimurium strain 112910a, control pigs (animals that were not immunized and not infected) and pigs infected with S*almonella* Typhimurium strain 112910a Nal^{20}. Values are represented as a percentage compared to the wild type immunized group.

2.2.5 Discussion

DIVA vaccines are a recent advance in vaccinology enabling distinction between an animal that is seropositive to a particular infectious agent because it has been vaccinated, and one that is seropositive because it has been infected with virulent field organisms (Michael *et al.*, 2008). Because current *Salmonella* serosurveillance programmes are generally based on detection of antibodies against LPS antigens, we selected six LPS genes that might be suitable markers to develop a LPS based DIVA-vaccine. Deletion of LPS genes, however, has some

consequences: LPS represent the main surface antigens of Gram-negative bacteria (O-antigens) and harbour binding-sites for antibodies (Van Amersfoort *et al.*, 2003). Therefore, LPS are important in the recognition and the elimination of bacteria by the host's immune system (Morrison *et al.*, 1992). Truncation of LPS may lead to over-attenuated strains that are not able to fully colonize their host and therefore no longer elicit a sufficient protective immune response (Karasova *et al.*, 2009). Possibly smooth LPS are indispensable for the early steps of the infection process (Nagy *et al.*, 2008) and contribute to invasiveness (Martin *et al.*, 2000). Data on relationship between LPS and invasion are often unclear and sometimes contradictory (Martin *et al.*, 2000). Our results on invasiveness of *Salmonella* Typhimurium strain 112910a and its isogenic LPS mutants, illustrate that the Δ*rfbA*, Δ*rfaG* and Δ*rfaF* strains were less able to invade IPEC-J2 cells, which might indicate that these strain are less able to colonize their host and therefore are no longer able to elicit a protective immune response. In a mouse *in vivo* experiment we showed that the *rfaG* and *rfaF* mutant strains were indeed not able to protect BALB/c mice against a subsequent infection with *Salmonella* Typhimurium NCT12023Nal^{20} and that the Δ*rfaI* strain was only able to significantly reduce bacterial counts in the spleen of mice. Conversely, Δ*rfbA*, Δ*rfaL* and Δ*rfaJ* strains, with less truncated LPS, were able to successfully protect BALB/c mice against a *Salmonella* Typhimurium infection and their protective capacity was not impaired compared to their isogenic wild type strain. These results strongly suggest that a confined truncation of LPS is essential to maintain protection against challenge with the virulent strain *Salmonella* Typhimurium NCTC12023Nal^{20} in mice.

Cross-protection against other enterobacterial pathogens induced by 'rough' mutants is sometimes explained by better accessibility of less immune-potent molecules, such as lipid A and core antigens (Nagy *et al.*, 2006; Nagy *et al.*, 2008a/b). Hence, truncation of LPS might confer enhanced cross protection to other serovars. Therefore, we used the Δ*rfaJ* strain to conduct a cross-protection study. The finding that smooth strains are less capable of inducing a cross-protection against other *Salmonella* serovars could not be confirmed in this study. The wild type and the Δ*rfaJ* deficient strains were equally able to provoke cross-protection against *Salmonella* Heidelberg, whereas only the wild type strain was able to protect against a challenge with *Salmonella* Enteritidis.

The ultimate goal of this study was to verify whether LPS mutant strains were able to elicit a DIVA humoral immune response in pigs. Our results illustrate that both the Δ*rfaL* and the

Δ*rfaJ* strain gave no seroconversion when using a LPS based ELISA, while a clear-cut seroconversion was observed when using an in-house *Salmonella* Typhimurium strain112910a whole cell ELISA. Besides, immunization of piglets with the Δ*rfaJ* or Δ*rfaL* mutants resulted in the induction of a serological response allowing clear differentiation between sera from piglets immunized with the Δ*rfaJ* or Δ*rfaL* strains and sera of pigs infected with their isogenic wild type strain when using a LPS based ELISA. In conclusion, we proved that immunization with *Salmonella* Typhimurium strain 112910a and its isogenic mutant strains: Δ*rfaL* and Δ*rfaJ*, is equally able to provoke protection against a virulent *Salmonella* Typhimurium strain. In addition, deletion of the *rfaL* or the *rfaJ* genes can be used as DIVA markers in current *Salmonella* serosurveillance programmes based on the detection of antibodies against LPS of *Salmonella*.

Acknowledgements

The technical assistance of Nathalie Van Rysselberghe and Rosalie Devloo is greatly appreciated. This work was supported by the Federal Public Service for Health, Food chain safety and Environment (FOD), Brussels, Belgium: project code RT/ 09/5 SALMOSU and the Bijzonder Onderzoeksfonds (BOF): starTT project IOF 09/StarTT/020.

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2.3 *Scs* genes are involved in stress related enhanced replication of *Salmonella* Typhimurium

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Freddy Haesebrouck, Frank Pasmans

2.3.1 Abstract

Salmonella Typhimurium infections in pigs often result in the development of carriers that re-excrete *Salmonella* during periods of stress. A previous study showed that stress related re-excretion of *Salmonella* is linked to increased serum cortisol levels and increased intracellular multiplication in macrophages. *ScsA* was shown to be the major driver for increased intracellular multiplication of *Salmonella* Typhimurium in cortisol-exposed porcine alveolar macrophages. In a first study, pigs were vaccinated with a live *Salmonella* Typhimurium vaccine and injected fourteen days later with dexamethasone, mimicking stress. This resulted in enhanced replication of the vaccine strain. Second, we examined whether deletion of *scsA*, *scsB*, *scsC*, *scsD* or the entire *scs* locus in *Salmonella* Typhimurium strain 112910a would abolish stress-induced multiplication of that strain. For this purpose, we first developed an *in vivo* mouse model, in which dexamethasone-induced enhanced replication of knock-out strains was compared to that of their isogenic wild type strain. We showed that *scsA* and *scsABCD* but not *scsB*, *scsC* or *scsD*, are vital for dexamethasone-induced *Salmonella* replication in organ samples of DBA/2J mice. Consequently, applying a deletion of *scsA* or *scsABCD* might abolish stress-related enhanced replication of live *Salmonella* Typhimurium vaccines.

2.3.2 Introduction

Salmonella Typhimurium is an important cause of human bacterial gastroenteritis and pigs are a major reservoir for this serotype (Boyen *et al.*, 2008). Infections of pigs with *Salmonella* Typhimurium often result in the development of carriers that intermittently excrete *Salmonella* in very low numbers (Boyen *et al.*, 2008). During periods of stress, like transport to the slaughterhouse or feed withdrawal, recrudescence of *Salmonella* may occur and result in carcass contamination (Mannion *et al.*, 2008; Verbrugghe *et al.*, 2011). If a slaughterhouse produces contaminated carcasses, all post-harvest stages may be contaminated (Delhalle *et al.*, 2009).

Recently, Verbrugghe *et al*. (2011) have demonstrated that cortisol plays an important role in *Salmonella* recrudescence by carrier pigs and that re-excretion of *Salmonella* Typhimurium can be reproduced by an intramuscular injection of dexamethasone. Furthermore, *scs* genes were shown to be important in the *in vitro* proliferation of *Salmonella* Typhimurium in cortisol-exposed porcine macrophages (manuscript in preparation). Consequently, the *scs* locus might be important in stress-related enhanced replication of *Salmonella* Typhimurium *in vivo*. The *scs* locus includes two operons, one operon contains a single *scsA* gene and another operon contains the *scsB*, *scsC* and *scsD* genes encoding proteins that may mediate copper tolerance indirectly by catalyzing the correct folding of periplasmic copper-binding target proteins via a disulfide isomerase-like activity (Gupta *et al.*, 1997).

Vaccination of pigs can be an important tool to control *Salmonella* Typhimurium infections in the meat production chain (Wigley *et al.*, 2004). Moreover, live vaccines are considered to confer a better protection against *Salmonella* infections (Mastroeni *et al*., 2001). Among the main issues concerning the use of live vaccines, is safety of the vaccine strains. Ideally, the vaccine strain should colonize the vaccinated animal only transiently. If stress would evoke recrudescence of the *Salmonella* vaccine strain, additional attenuation resulting in loss of recrudescence ability, would be desirable.

It is not known if stressing of vaccinated animals results in enhanced replication of the *Salmonella* vaccine strain. Therefore, the first aim of this study was to verify whether an attenuated *Salmonella* Typhimurium strain present in a commercially available vaccine for

use in pigs, is subject to dexamethasone-induced enhanced replication. Secondly, a mouse model was developed, in which stress-related recrudescence was mimicked. This model was used to verify whether deletion of one or more genes of the *scs* locus could abolish dexamethasone related recrudescence *in vivo*.

2.3.3 Materials and methods

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 Ethical Committee of the Faculty of Veterinary Medicine, Ghent University (EC 2011/099 and EC 2011/116).

Salmonella strains

For oral vaccination of pigs, a commercially available, live, attenuated, *Salmonella* Typhimurium vaccine (*Salmoporc*® IDT, Rodleben) was used. The vaccine consists of a double attenuated *Salmonella* Typhimurium mutant strain (phage type DT 9), unable to synthesize both adenine and histidine (Lindner *et al.*, 2007). The strain is distinguishable from field isolates of the same serotype on the basis of its auxotrophy using a rapid test (IDT *Salmonella* Diagnostic Kit) within 24-48 hours (Eddicks *et al.*, 2009).

Salmonella Typhimurium strain 112910a, phage type 120, isolated from a pig stool sample and characterized previously (Boyen *et al*., 2009), was used as the wild type background to construct the following isogenic knock-out mutants: Δs*csA* (Verbrugghe *et al.*, manuscript in preparation), Δ*scsB*, Δ*scsC*, Δ*scsD*, Δscs*ABCD*. Construction of the knock-out mutants is described in detail in chapter 2.2. Primers used to create nonpolar gene disruptions in this study are shown in Table 1.

For experimental infection of mice, an invasive, spontaneous nalidixic acid resistant *Salmonella* Typhimurium strain 112910aNal^{20}, resistant to 20 µg/ml nalidixic acid, was used.

Table 1: Primers used in this study

Primers	Sequences
***scsB* forward**	5'- CGGTTATTGCTAACGGTTAATTACTCATTCACGGAGAAAAAATTGTGTAGGCTGGAGCTGCTTC -3'
***scsB* reverse**	5'-CGCGATGCTCAGCGTCGAAAACAGCGCCAGCAGTAAAACAATCATGTATTCATATGAATATCCTCCTTAG -3'
***scsC* forward**	5'-GCGATGCGGTATTACAAACGTTGAAAAAAGCGAAAGGAATAACCCAATGATGTGTAGGCTGGAGCTGCTTC -3'
***scsC* reverse**	5'-GCTTCACGCAGCCAACGCCGCAGTTTACCCGCCATT.CATATGAATATCCTCCTTAG -3'
***scsD* forward**	5'-GCCCTGGGATACGCTGGAAGCGGTGGTGAAAGAAAAACTGGCGTCTGCCATGTGTAGGCTGGAGCTGCTTC -3'
***scsD* reverse**	5'-GATTTCGCAAAACGGGGGTTTTTCTTACAGTAAACGCGTTAGCGCCGGGA.CATATGAATATCCTCCTTAG-3'

Effect of dexamethasone on the replication of a live *Salmonella* Typhimurium vaccine strain in pigs

In this *in vivo* experiment we investigated whether an injection of dexamethasone is able to enhance replication of an attenuated *Salmonella* Typhimurium strain, present in a commercial vaccine. For that purpose, twenty, three-week-old, piglets were used. The *Salmonella*-free status of the piglets was tested bacteriologically and serologically using a commercially available enzyme-linked immunosorbent assay (ELISA) (IDEXX Laboratories) according to the manufacturer's instructions. All animals were housed together at 25 °C under natural day-night rhythm with *ad libitum* access to feed and water and were orally vaccinated with 1 ml of the live *Salmonella* Typhimurium vaccine, *Salmoporc*®. Two weeks later, ten animals received an intramuscular injection of 2 mg dexamethasone (Kela laboratoria, Hoogstraten, Belgium) per kg body weight, to mimic stress conditions. This dose was shown to cause recrudescence of *Salmonella* Typhimurium in pigs (Verbrugghe *et al.*, 2011). Ten pigs served as a control group and were intramuscularly injected with 2 ml of Hank's buffered salt solution (HBSS; Gibco Life Technologies, Paisley, Scotland). Twenty-four hours later, all animals were humanely euthanized and organ samples were taken for bacteriological analysis.

Developing a mouse model that mimicks stress-related increased replication of *Salmonella* Typhimurium

In this *in vivo* experiment, we evaluated whether dexamethasone increases the number of *Salmonella* Typhimurium bacteria in the gut of *Salmonella* Typhimurium infected mice, in order to create a mouse model allowing screening of bacterial genes that might be involved in dexamethasone-induced enhanced replication of *Salmonella*. For that purpose, eighteen, four week old DBA/2J mice, intermediately sensitive to *Salmonella* Typhimurium infections

(Sebastiani *et al*., 2002) and eighteen, four week old BALB/c mice, highly susceptible to *Salmonella* Typhimurium infections (Sebastiani *et al.*, 2002), were housed in filter-topped cages at 25 °C under natural day-night rhythm with *ad libitum* access to feed and water and enriched with mouse houses and play tunnels. Five days after arrival, all mice were infected with a total of 1 x 10^6 CFU of *Salmonella* Typhimurium strain 112910aNal^{20} by the orogastric route. At day 7 post inoculation (p.i.) six BALB/c mice were subcutaneously injected once with 100 mg/kg dexamethasone. Simultaneously, six BALB/c mice received a subcutaneous injection of 25 mg/kg dexamethasone, which was repeated after three hours. Fourteen days p.i., six DBA/2J mice were subcutaneously (SC) injected once with 100 mg/kg dexamethasone and simultaneously six DBA/2J mice received a SC injection of 25 mg/kg dexamethasone (repeated after three hours). Six mice of each strain received a SC injection of 200 µl HBSS (24 h before euthanasia) and were used as a control group. Twenty-four hours after the last SC injection of dexamethasone, all animals were humanely euthanized and samples of spleen, liver and cecum were collected for bacteriological analysis.

The role of *scs* genes in a mouse model mimicking stress related enhanced replication of *Salmonella* Typhimurium

A mice model was used to verify whether *scsA, scsB, scsC, scsD* or the entire *scs* locus is important in dexamethasone related multiplication *in vivo*. Therefore, three to four week old DBA/2J mice were used and randomly allocated in six groups of sixteen mice. The animals were housed in filter-topped cages at 25 °C under natural day-night rhythm with *ad libitum* access to feed and water and enriched with mouse houses and play tunnels. Mice were inoculated with a total of 1 x 10^6 CFU of *Salmonella* Typhimurium or its isogenic *scsA*, *scsB*, *scsC*, *scsD* or *scsABCD* knock-out mutants. At day 14 p.i., eight animals of each group were SC injected with 100 mg/kg dexamethasone and eight mice were SC injected with 200 µl HBSS and served as a control group. Twenty-four hours later, all mice were humanely euthanized. Spleen, liver and cecum samples were examined for the number of *Salmonella* Typhimurium bacteria.

Bacteriological analysis

All organ 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 XLD plates (for porcine organ samples and organ samples of the last mouse *in vivo* trial) or on BGA_{Nal20} plates (for samples collected to optimize the mouse model). All plates were incubated for 16 hours at 37 °C. The samples were also 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_{Nal20} or XLD plates.

Samples that were negative after direct plating but positive after enrichment were presumed to contain 83 CFU/gram tissue or contents (detection limit for direct plating). Samples that remained negative after enrichment were presumed to contain 0 CFU/gram tissue or contents and were assigned value '1' prior to log transformation. Subsequently the number of CFU for all samples derived from all animals was converted logarithmically prior to calculation of the average differences between the $\log_{10}$ values of the different groups and prior to statistical analysis.

Statistical analysis

In all experiments, statistical analysis was performed using a one-way ANOVA test (in case of homogeneity of variances), with posthoc Bonferroni corrections or a nonparametric Mann-Whitney-U-test (in case of non-homogeneity of variances), using the SPSS Statistics 19.0 software (SPSS Inc., Chicago, USA). A *P-value* of < 0.05 was considered significant.

2.3.4 Results

Dexamethasone promotes replication of a *Salmonella* Typhimurium live vaccine strain in pigs

In this experiment we determined if treatment of pigs with 2 mg/kg dexamethasone at ten days after vaccination with a commercially available live vaccine (*Salmoporc®*) results in enhanced replication of the vaccine strain. Figure 1 illustrates that recovery of the vaccine strain was higher in organ samples and contents of vaccinated pigs treated with dexamethasone compared to vaccinated pigs that received a saline solution. This elevation was significantly different ($P < 0.05$) for ileocecal lymph nodes, colon contents and cecum contents.

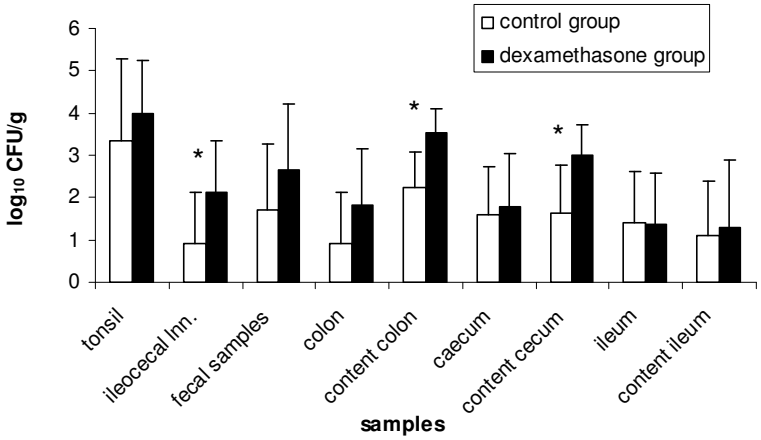

Figure 1: Recovery of *Salmoporc*® from various organs of pigs 14 days post vaccination. Black bars represent vaccinated pigs that received an intramuscular injection of dexamethasone (2 mg/kg) and white bars represent vaccinated animals that received an intramuscular injection of HBSS (control group). The mean $\log_{10}$ values of the number of CFU per gram sample with their standard deviations are given. An asterisk (*) refers to a significant difference ($P < 0.05$) between the control group and the dexamethasone group.

A subcutaneous injection of dexamethasone results in an enhanced replication of *Salmonella* Typhimurium 112910Nal^{20} in DBA/2J mice but not in BALB/c mice

A mouse model was optimized to demonstrate that a subcutaneous injection of 100 mg/kg dexamethasone (or two injections of 25 mg/kg with an interval of three hours) is capable to increase multiplication of *Salmonella* Typhimurium strain 112910aNal^{20} in DBA/2J or BALB/c mice. *Salmonella* infected DBA/2J mice, subsequently injected with dexamethasone

had a significantly ($P < 0.05$) higher number of *Salmonella* Typhimurium bacteria in the spleen, the liver and the cecum, compared to DBA/2J mice that were injected with a saline solution. Results are shown in Figure 2.

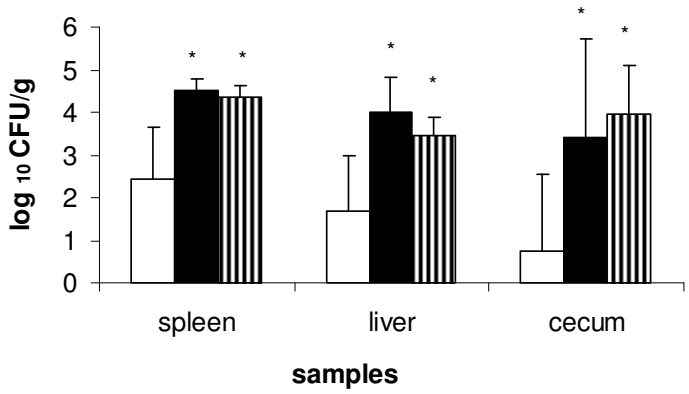

Figure 2: Recovery of *Salmonella* Typhimurium 112910aNal[20] from various organs from DBA/2J mice 14 days post infection. Black bars represent infected DBA/2J mice that received a subcutaneous injection of dexamethasone (100 mg/kg) 24h before euthanasia and white bars represent infected mice that received an subcutaneous injection of HBSS (control group). Striped bars represent DBA/2J mice that received 25 mg/kg dexamethasone 24h and 21h before euthanasia. The mean $\log_{10}$ values of the number of CFU per gram sample with their standard deviations are given. An asterisk (*) refers to a significant difference ($P < 0.05$) between the control group and the dexamethasone group.

Figure 3 shows that the number of *Salmonella* Typhimurium bacteria in organs of infected BALB/c mice, subsequently injected with dexamethasone, was not significantly different ($P > 0.05$) from *Salmonella* numbers isolated from the spleen, the liver and the cecum of infected BALB/c mice that were injected with HBSS 24 h before euthanasia.

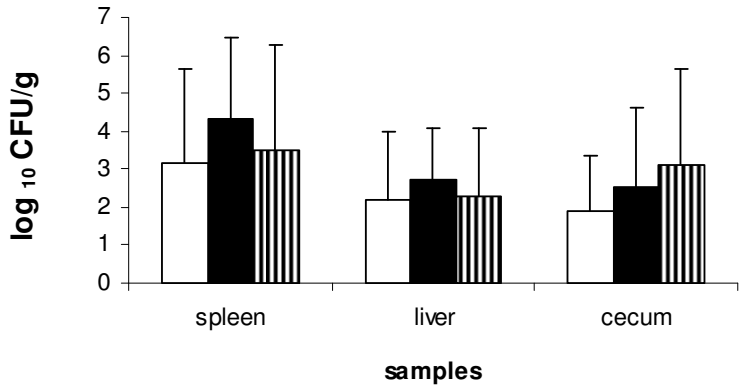

Figure 3: Recovery of *Salmonella* Typhimurium 112910aNal[20] from various organs from BALB/c mice 7 days post infection. Black bars represent infected BALB/c mice that received a subcutaneous injection of

dexamethasone (100 mg/kg) 24h before euthanasia and white bars represent infected mice that received an subcutaneous injection of HBSS (control group). Striped bars represent BALB/c mice that received 25 mg/kg dexamethasone 24 h and 21 h before euthanasia. The mean $\log_{10}$ values of the number of CFU per gram sample with their standard deviations are given.

scsA and *scsABCD* determine dexamethasone induced enhanced replication of *Salmonella* Typhimurium 112910a

Bacterial counts in the spleen ($P > 0.05$), liver ($P > 0.05$) and caecum ($P < 0.05$) of DBA/2J mice infected with Δ*scsA* and subsequently injected with 100 mg/kg dexamethasone, were reduced compared to bacterial numbers in organs of mice infected with its isogenic wild type strain, subsequently injected with dexamethasone. The *Salmonella* Typhimurium load in organ samples of mice, infected with Δ*scsB* or Δ*scsC* and subsequently injected with 100 mg/kg dexamethasone, was not significantly different from that in organs of DBA/2J mice infected with the wild type strain and subsequently injected with dexamethasone. The *Salmonella* Typhimurium load in the liver of mice infected with Δ*scsD* or Δ*scsABCD*, subsequently injected with 100 mg/kg dexamethasone, was significantly different from that in the liver of DBA/2J mice infected with the wild type strain and subsequently injected with dexamethasone. Results are shown in Figure 4.

None of the DBA/2J mice infected with Δ*scsA*, Δ*scsABCD* or their isogenic wild type strain died as a result of the infection, whereas eleven mice infected with either Δ*scsB* (n = 3), Δ*scsC* (n = 4) or Δ*scsD* (n = 4) died as a consequence of challenge.

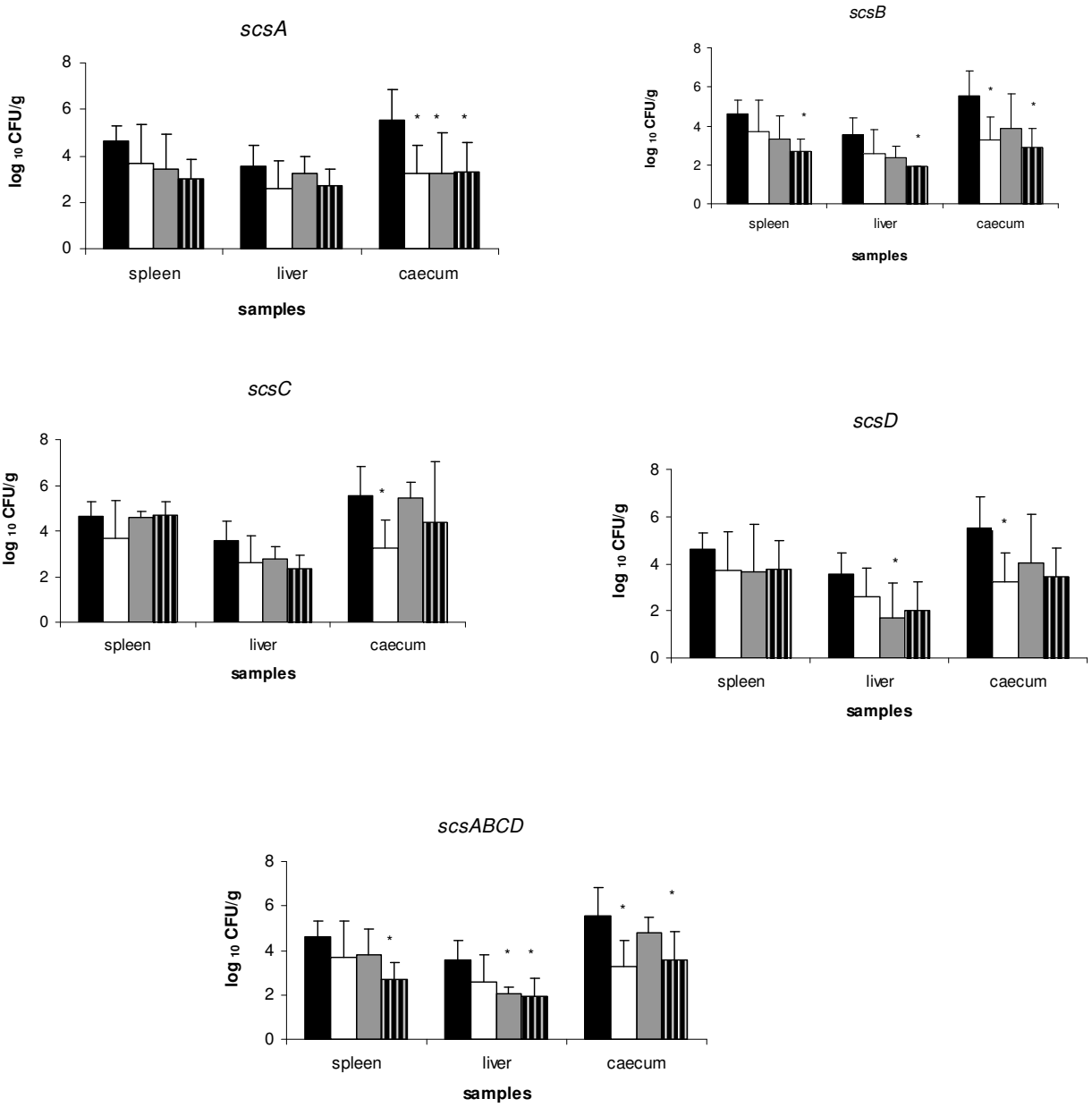

Figure 4: Recovery of *Salmonella* Typhimurium 112910a (WT) and its isogenic *scs* (either *scsA*, *scsB*, *scsC*, *scsD* or *scsABCD*) knock-out mutants from various organs from mice 14 days post infection. Black bars represent WT infected DBA/2J mice that received a subcutaneous injection of dexamethasone (100 mg/kg) 24h before euthanasia. White bars represent WT infected mice that received a subcutaneous injection of HBSS. Gray bars represent either Δ*scsA,* Δ*scsB,* Δ*scsC,* Δ*scsD* or Δ*scsABCD* infected DBA/2J mice that received a subcutaneous injection of dexamethasone (100 mg/kg) 24h before euthanasia. Striped bars represent either: Δ*scsA,* Δ*scsB,* Δ*scsC,* Δ*scsD* or Δ*scsABCD* infected mice that received a subcutaneous injection of HBSS. The mean $\log_{10}$ values of the number of CFU per gram sample with their standard deviations are given. An asterisk (*) refers to a significant difference ($P < 0.05$) with the WT dexamethasone group.

2.3.5 Discussion

Vaccination of pigs reduces shedding and colonization of host tissues (Selke *et al.*, 2007; Delhalle *et al.*, 2009) and diminishes *Salmonella* Typhimurium numbers in a model mimicking pre-slaughter stress (Leyman *et al.*, 2012). Our results, however, indicate that live *Salmonella* Typhimurium vaccine strains can also be subject to stress related enhanced excretion.

We designed a mouse model in which the *in vivo* effect of *scs* genes on stress related replication of *Salmonella* could be investigated. To create our model DBA/2J mice, relatively resistant to *Salmonella* Typhimurium infections, were compared to BALB/c mice, susceptible to *Salmonella* Typhimurium infections (Sebastiani *et al.*, 2002). Genetic resistance to systemic disease caused by *Salmonella* Typhimurium in mice is linked to the natural resistance associated macrophage protein (Nramp1, now termed Slc11a1), involved in the control of the exponential growth of *Salmonella* in the reticuloendothelial organs during the early phase of infection (Wigley *et al.*, 2004). Macrophages that are deficient in Slc11a1 are extremely susceptible to *Salmonella* infection and cannot control replication as efficiently as wild type macrophages (Dougan *et al.,* 2011). Upon phagocytosis, the Slc11a1 protein is recruited to the membrane of maturing phagosomes, containing live pathogens, where its antimicrobial activity is exerted (Fortier et *al.*, 2005). BALB/c mice, which lack the Slc11a1 protein, are extremely susceptible to infection with *Salmonella* Typhimurium 112910a. We illustrated that *Salmonella* Typhimurium 112910a infections in DBA/2J mice are non-lethal.

Bacterial growth in DBA/2J ($Slc11a1^{+/+}$) mice is controlled after several days and reaches a plateau phase that subsequently declines, while in BALB/c ($Slc11a1^{-/-}$) mice the bacterial load in organ samples gradually increases, eventually leading to death (Kita *et al.*, 1992). Furthermore, Kita *et al.* (2008) illustrate that two days after a *Salmonella* infection, DBA/2J Kuppfer cells, and not BALB/c Kuppfer cells, produce membrane associated IL-1 and TNF-α, attracting phagocytic cells to the infection site and inducing acute inflammatory events important in early defence against *Salmonella* (Eckmann *et al.*, 2001). Moreover, only DBA/2J splenocytes are able to produce IFN-ɣ (Kita *et al.*, 1992), a cytokine known to play a central role in *Salmonella* clearance by activating the ability of macrophages to kill intracellular *Salmonella* (Eckmann *et al.*, 2001).

Plant *et al.* (1983) showed that in chronic murine salmonellosis, mortality increased by glucocorticoid treatment. In our DBA/2J mice a *Salmonella* Typhimurium 112910a infection could be reactivated by a subcutaneous injection of dexamethasone, a glucocorticoid analogue. In systemically infected BALB/c mice a subcutaneous dexamethasone injection could not induce a significantly higher replication of the challenge strain compared to mice injected with a salt solution. We postulate that the DBA/2J mice were asymptomatic carriers of *Salmonella* Typhimurium at the moment dexamethasone was injected, and, as a result of stress, they rapidly developed an overwhelming systemic spread of *Salmonella* with 100 – 1000 more *Salmonella* in internal organs compared to the untreated control group. BALB/c mice, that were injected with HBSS 24 h before euthanasia showed already a much higher bacterial load compared to DBA/2J injected with HBSS 24 h before euthanasia. Consequently, the effect of a dexamethasone injection was much more distinct in DBA/2J mice compared to BALB/c mice.

The DBA/2J mice model allowed us to investigate whether *scs* genes are able to reduce dexamethasone induced enhanced replication of *Salmonella in vivo*. Our results indicate that mice infected with Δ*scsA,* Δ*scsD* or Δ*scsABCD* do not show higher numbers of *Salmonella* Typhimurium after a subcutaneous injection with dexamethasone compared to animals injected with HBSS. This does not seem to be the case for mice infected with either Δ*scsB* or Δ*scsC.* Furthermore, deletion of Δ*scsB*, Δ*scsC* or Δ*scsD* increased virulence of *Salmonella* Typhimurium 112910a in DBA/2J mice. Verbrugghe *et al.* (manuscript in preparation) showed that deletion of Δ*scsA* causes an upregulation of the *scsBCD* operon. *ScsA* encodes a suppressor of copper sensitivity protein and Gupta *et al.* (1997) postulated that ScsA prevents formation of free hydroxyl radicals that result from the reaction of copper with hydrogen peroxide. We hypothesize that *scsBCD* might act as a negative regulator for *scsA*. Consequently, disruption of one of the genes of the *scsBCD* operon might result in an up-regulation of the ScsA activity, leading to a reduced number of free hydroxyl radicals. Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). ROS are important in the early defence mechanism that macrophages use to kill engulfed pathogens (Janssen *et al.*, 2003). The hypothesized mechanism has not yet been investigated in *Salmonella* Typhimurium but we postulate that this might explain why deletion of *scsB*, *scsC* or *scsC* increases virulence of *Salmonella* Typhimurium in DBA/2J mice. Another hypothesis might be that *scsB*, *scsC* or *scsD* regulate other virulence genes in *Salmonella* Typhimurium.

In conclusion, our DBA/2J mice model allowed us to prove that *scsA* and *scsABCD* are able to abolish enhanced replication of *Salmonella* Typhimurium induced by a dexamethasone injection, without increasing the virulence of the *Salmonella* Typhimurium strain used. Therefore, we postulate that deletion of *scsA* or the entire *scs* locus in *Salmonella* Typhimurium live vaccines might help to reduce stress related enhanced replication of live vaccine strains.

2.3.6 References

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2.4 Tackling the issue of environmental survival of live *Salmonella* Typhimurium vaccines in pigs: deletion of the *lon* gene

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2.4.1 Abstract

Optimization of live vaccines would contribute greatly to controlling *Salmonella* Typhimurium infections in pigs. A previous study showed that both the Δ*rfaJ* and Δ*rfaL* strains are suitable markers and allow serological differentiation of infected and vaccinated pigs (DIVA). The aim of this study was to verify whether deletion of the *lon* gene in a *Salmonella* Typhimurium Δ*rfaJ* marker strain resulted in decreased environmental survival. Our results indicate that deletion of the *lon* gene in the Δ*rfaJ* strain did not affect invasiveness in IPEC-J2 cells and resulted in an increased susceptibility to UV, disinfectants (such as hydrogen peroxide and tosylchloramide sodium) and citric acid. Immunization of pigs with inactivated Δ*rfaJ* or Δ*lon*Δ*rfaJ* vaccines allowed differentiation of infected and vaccinated pigs. Furthermore, deletion of the *lon* gene did not reduce the protection conferred by live wild type or *ΔrfaJ* vaccines against subsequent challenge with a virulent *Salmonella* Typhimurium strain in BALB/c mice. Based on our results in mice, we conclude that deletion of *lon* in Δ*rfaJ* contributes to environmental safety of the Δ*rfaJ* DIVA strain.

2.4.2 Introduction

Vaccination of pigs is an important measure to control *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) infections in the meat production chain (Boyen *et al.*, 2008a). Live vaccines are considered to confer a better protection against *Salmonella* infections compared to inactivated vaccines, probably due to the more pronounced cellular immune response and the induction of mucosal IgA production (Boyen *et al.*, 2008a). However, the use of live vaccines can only be advocated if 1) vaccinated animals can be differentiated from infected ones; 2) the vaccine strain is easily eliminated from the environment. The first condition has recently been met by the development of DIVA (Differentiation of Infected and Vaccinated Animals) vaccine strains (Leyman *et al*., 2011; Selke *et al.*, 2007). The second condition is of increasing interest in vaccine development since live vaccines are potentially excreted into the environment by the vaccinated animals. In this respect, Lon-deficient *Salmonella* strains that could serve as effective oral vaccines in mammals might decrease survival of live *Salmonella* Typhimurium vaccines in the environment (Matsui *et al.*, 2003; Kodama *et al.*, 2005). Lon, as an evolutionarily conserved stress protein induced by multiple stressors, helps to remove damaged and abnormal proteins during stress and contributes to the regulation of cell division, cell morphology and DNA maintenance (Downs *et al.*, 1986; Takaya *et al.*, 2002, 2003; Kültz *et al.*, 2005; Majdalani *et al.*, 2005; Ngo *et al.*, 2009; Langklotz *et al.*, 2011). However, deletion of the *lon* gene might also severely compromise the protective capacity of a live *Salmonella* Typhimurium vaccine. Therefore, it was the aim of this study to verify whether deletion of the *lon* gene results in a vaccine strain that is more susceptible to environmental stress (e.g. UV, various disinfection agents) without affecting the strain's protective capacity in BALB/c mice on the one hand and without loss of its DIVA quality in pigs on the other hand. To verify whether Δ*lon*Δ*rfaJ* is able to protect mice against a *Salmonella* Typhimurium infection we will use susceptible mice in combination with a highly virulent challenge strain.

2.4.3 Materials and methods

All *in vivo* experiments were approved by the ethical committee of the Faculty of Veterinary Medicine, Ghent University (EC 2008/124 and EC 2009/57).

Bacterial strains

Strains and primers used in this study are summarized in Tables 1 and 2. *Salmonella* Typhimurium strain 112910a, phage type 120, was originally isolated from a pig stool sample on a pig farm with a persistent *Salmonella* problem and was used as the wild type strain (Boyen *et al.*, 2009). This strain was also used for the construction of three deletion mutants (Δ*lon,* Δ*rfaJ* (Leyman *et al.*, 2011) and Δ*lon*Δ*rfaJ*) using the one-step inactivation method described by Datsenko and Wanner (2000). Live and formalin-inactivated strains (WT, Δ*rfaJ*, Δ*lon* and Δ*lon*Δ*rfaJ*) with Freund's incomplete adjuvant, for immunization of mice and pigs were prepared as described previously (Leyman *et al.*, 2011). For challenge of mice, *Salmonella* strain NCTC12023 resistant to nalidixic acid (Nal^{20}, resistant tot 20 µg/ml) was used.

Table 1: Strains used in this study

Strain	**Genotype**	**Product of the deleted gene**	**Source or reference**
WT	*Salmonella* Typhimurium 112910a	no deletions	(Downs *et al*., 1986)
NCTC12023Nal^{20}	*Salmonella* Typhimurium NCTC 12023 Nal^{20}	no deletions	(Boyen et al., 2008b)
Δ*lon*	*Salmonella* Typhimurium 112910a Δ*lon*	DNA-binding ATP-dependent protease La	This study
Δ*rfaJ*	*Salmonella* Typhimurium 112910a Δ*rfaJ*	LPS 1,2-glucosyltransferase	(Leyman *et al.*, 2011)
Δ*lon*Δ*rfaJ*	*Salmonella* Typhimurium 112910a Δ*lon*Δ*rfaJ*	LPS 1,2-glucosyltransferase and DNA-binding ATP-dependent protease La	This study

Table 2: Primers used in this study

primers	**Sequences**
***lon* forward**	5'- CAGCTATACTATCTGATTACCTGGCGGACACTAAACTAAGAGAGAGCTCTTGTGTAGGCTGGAGCTGCTTC - 3'
***lon* reverse**	5' - CGAAATAGCCTGCCAGCCCTGTTTTTATTAGCGCTATTTGCGCGAGGTCACATATGAATATCCTCCTTAG - 3'
***rfaJ* forward**	5' - ATAGCCTACTTTAAACGTAAACTTCTTGAATAAAACCCATAGGTGATGTATGTGTAGGCTGGAGCTGCTTC - 3'
***rfaJ* reverse**	5' - AGTTTTTAATCTTTTTTTCAATAATCATAATGGAGATTTAGGGAGGGGAACATATGAATATCCTCCTTAG -3'

Characterization of bacterial strains

Validation of the lipopolysaccharide (LPS) phenotype of the wild type and the Δ*lon*, Δ*lon*Δ*rfaJ* and Δ*rfaJ* strains occurred by SDS-polyacrylamide gel electrophoresis and fluorescent staining, as described before (Leyman *et al.*, 2011). *In vitro* colony morphology exhibited by the wild type *Salmonella* Typhimurium strain 112910a and its isogenic Δ*rfaJ*, Δ*lon* and Δ*lon*Δ*rfaJ* strains on agar was visualized by plating the strains on Luria-Bertani agar (LB; Sigma-Aldrich Chemie Gmbh, Steinheim, Germany). The plates were then incubated overnight at 37 °C and were visually examined.

As a measure of *in vitro* virulence, invasiveness of Δ*lon*Δ*rfaJ*, Δ*rfaJ*, wild type and Δ*lon*, was assessed in porcine epithelial cells (IPEC-J2) using a gentamicin protection assay. The polarized porcine epithelial intestinal cell line IPEC-J2 was derived from jejunal epithelia isolated from a neonatal piglet (Rhoads *et al.*, 1994; Schierack *et al.*, 2006). Cells were seeded in 24 well plates at a density of approximately 10^5 cells per well and were allowed to grow to confluency for at least one day. These wells were inoculated with *Salmonella* Typhimurium strain 112910a or one of its isogenic knock-out mutants at a multiplicity of infection (MOI) of 10:1. To synchronize the infection, the inoculated multiwell plates were centrifuged at 365 x g for 10 minutes at 37 °C. After 30 minutes incubation at 37 °C and 5% CO_2, the wells were rinsed and fresh medium supplemented with 50 µg/ml gentamicin (Gibco, Life Technologies, Paisly, Scotland) was added. After 60 minutes of incubation at 37 °C and 5% CO_2, the wells were rinsed three times. To quantify invasion, the cells were lysed with 0.25% deoxycholate (Sigma, Aldrich, Steinheim, Germany) 100 minutes after inoculation and 10-fold dilutions were plated on brilliant green agar (BGA) plates. The invasion assay was conducted in triplicate with three repeats per experiment.

Immunization of piglets

We verified whether it was possible to serologically discriminate between (1) pigs immunized with Δ*lon*Δ*rfaJ* and *Salmonella* Typhimurium 112910a on the one hand and (2) pigs immunized with the Δ*lon*Δ*rfaJ* strain and *Salmonella* Typhimurium 112910a infected pigs on the other hand. For this purpose, twenty-one, six-week-old, bacteriologically and serologically *Salmonella* negative piglets (commercial closed line based on Landrace) were used. One and three weeks after their arrival, twelve pigs were intramuscularly immunized with 1 ml of one

of the formalin-inactivated vaccines containing 5 x 10^8 colony forming units per ml (CFU/ml) (either *Salmonella* Typhimurium (n = 3), Δ*rfaJ* (n = 3), Δ*lon* (n = 3) or Δ*lon*Δ*rfaJ* (n = 3)). The control group (n = 3) was injected with 1 ml of sterile phosphate-buffered saline (PBS). Four weeks after arrival, piglets were sacrified by exsanguination under anesthesia and blood samples were taken and centrifuged. To obtain sera from *Salmonella* Typhimurium infected piglets, six piglets were experimentally infected with *Salmonella* Typhimurium strain 112910a, as described previously (Leyman *et al.*, 2011). All sera samples were examined using an LPS based IDEXX enzyme-linked immunosorbent assay (ELISA) kit (HerdChek *Salmonella*; IDEXX Laboratories, Schiphol-Rijk, Noord-Holland, The Netherlands) and an in-house *Salmonella* Typhimurium strain 112910a whole cell ELISA, prepared as described before (Leyman *et al.*, 2011).

Sensitivity of the Δ*lon*Δ*rfaJ* strain to ultraviolet light

The impact of the deletion of the *lon* gene, both in the wild type strain and its isogenic Δ*rfaJ* mutant, on the strain's survival to UV was evaluated. For the UV-resistance test, strains were grown overnight on a shaker at 37 °C and were diluted in PBS to 10^8 CFU/ml. From each suspension, 100 µl was transferred to a 96 well plate and exposed to 0, 30 or 60 seconds of ultraviolet radiation (Sylvania Germicidal lamp, UV-C: 253.7 nm, distance: 15 cm, 15 Watt). The number of viable bacteria was determined by plating 10-fold dilutions on Colombia agar (Sigma Aldrich Chemie Gmbh, Steinheim, Germany) containing 5% sheep blood (aseptically collected, defibrinated sheep blood, biotrading, Mijdrecht, Nederland).

Sensitivity to various disinfection agents and acidic conditions

For resistance to various agents, strains were grown overnight on a shaker at 37 °C in LB broth and were then incubated at room temperature in the presence of the following stressors for the indicated times: (1) 15 mM hydrogen peroxide (H_2O_2) for 75 minutes (2) 20% ethanol for 5 minutes (3) 1 mM tosylchloramidum natricum (chloraminum) (SA Fagron NV, Waregem, Belgium) for 15 minutes (4) citric acid (50mM, pH 3.0) for 50 minutes and 150 minutes (Gruzdev *et al.*, 2001). Following exposure, survival of *Salmonella* was verified by plating 10-fold dilutions on Colombia agar containing 5% sheep blood.

Immunization and challenge of mice

To verify whether deletion of *lon* in Δ*rfaJ* or the wild type strain affects the strain's protective capacity, fifty, five-week-old, specified pathogen-free (SPF) BALB/c mice (Bio services, Janvier, France) were used to compare the clinical protection of Δ*lon*Δ*rfaJ* with Δ*rfaJ* on the one hand and Δ*lon* with the wild type strain on the other hand. Mice were immunized via the orogastric route with 2 x 10^7 CFU/ml of either Δ*lon* (n = 10), Δ*lon*Δ*rfaJ* (n = 10), Δ*rfaJ* (n = 10) or *Salmonella* Typhimurium strain 112910a (n = 10). A control group of ten mice was sham-inoculated with sterile PBS. Four weeks after arrival all mice were challenged with a total of 10^8 CFU/ml of the virulent *Salmonella* Typhimurium strain NCTC12023Nal[20] by the orogastric route. Nine days post-infection, samples of spleen, liver and caecum were examined quantitatively for the presence of the challenge organisms. Samples were weighed and 10% (w/v) suspensions were made in buffered peptone water (BPW; Oxoid, Basingstoke, UK) after which the material was homogenized with a stomacher. The homogenized samples were examined for the presence of *Salmonella* by plating 10-fold dilutions on BGA supplemented with nalidixic acid (BGA^{NAL20}). If negative at direct plating, the samples were pre-enriched overnight in BPW at 37 °C, enriched overnight at 37 °C in tetrathionate broth and then plated on BGA^{NAL}. Samples that were negative after direct plating but positive after enrichment were presumed to contain 50 CFU per gram tissue (detection limit for direct plating). Samples that remained negative were presumed to contain 0 CFU per gram tissue.

Statistical analysis

In all immunization experiments, statistical analysis was performed using a one-way ANOVA test (in case of homogeneity of variances), with posthoc Bonferroni corrections or a nonparametric Mann-Whitney-U-test (in case of non-homogeneity of variances), using the SPSS Statistics 19.0 software (SPSS Inc., Chicago, USA). The gentamicin protection assays were carried out in triplicate with three repeats per experiment. The *in vitro* invasion data and ELISA results were analysed by a one-way ANOVA and Bonferroni corrections were applied. A *P-value of* < 0.05 was considered significant.

2.4.4 Results

Deletion of *lon* results in a mucoid phenotype and a does not affect invasiveness in IPEC-J2 cells

SDS-polyacrylamide gel electrophoresis patterns of LPS of *Salmonella* Typhimurium 112910a, Δ*rfaJ,* Δ*lon* and Δ*lon*Δ*rfaJ* are shown in Figure 1. *Salmonella* Typhimurium strain 112910a and Δ*lon* showed a "wild-type" LPS structure which is denoted as "smooth" LPS (Hitchcock *et al.*, 1986), while Δ*rfaJ* and Δ*lon*Δ*rfaJ* showed a 'rough' LPS phenotype which is explained by the complete lack of O-antigens.

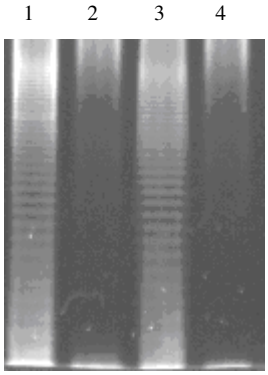

Figure 1: SDS-polyacrylamide gel electrophoresis patterns of LPS of *Salmonella* Typhimurium 112910a (lane 1), Δ*rfaJ* (lane 2), Δ*lon* (lane 3) *and* Δ*lon*Δ*rfaJ* (lane 4) mutants are shown. *Salmonella* Typhimurium strain 112910a (lane 1) and the *lon* deficient strain (lane 3) show a classical 'smooth' type ladder pattern. Δ*rfaJ* (lane 2) and Δ*lon*Δ*rfaJ* (lane 4) show a rough LPS phenotype. A fluorescent staining occurred and a ten-fold dilution of 25µg/ml LPS of each strain was loaded.

On LB agar, *Salmonella* Typhimurium showed smooth colony morphology, while disruption of *rfaJ* resulted in rough colony morphology. The *lon* deficient strains (Δ*lon* and Δ*lon*Δ*rfaJ*) showed mucoid colony morphology. Invasion of all strains was compared in IPEC-J2 cells using a gentamicin protection assay. The Δ*lon* strains were not impaired in invasiveness compared to the wild type strain and the Δ*rfaJ* strain. Although, an elevated invasion was noticed for the *lon* deficient strains, invasion rates of all strains were not significantly different in IPEC-2 cells (Figure 2). Bacterial numbers for the mutant strains recovered in the invasion assay were not affected by the detergent 0.25% deoxycholate (data not shown).

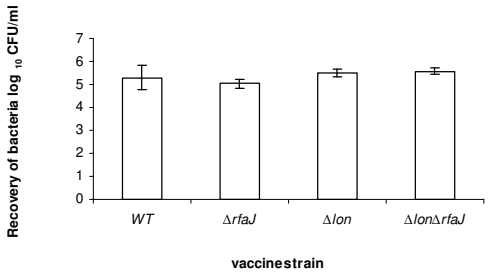

Figure 2: The invasiveness of Δ*lon*Δ*rfaJ*, Δ*lon,* Δ*rfa* and *Salmonella* Typhimurium 112910a in IPEC-J2 cells. The log values of the number of gentamicin protected bacteria and standard deviations are shown. The results represent the means of three independent experiments conducted in triplicate.

Deletion of *lon* does not affect the serological response after immunization of pigs

Serum samples of pigs immunized with *rfaJ* deficient strains (Δ*lon*Δ*rfaJ* (n = 3) or Δ*rfaJ* (n = 3)) and control animals (animals that were not immunized, n = 3) were considered *Salmonella* negative when using a commercially available IDEXX ELISA. Pigs immunized with Δ*lon* (n = 3) or *Salmonella* Typhimurium strain 112910a (n = 3) and *Salmonella* infected animals (n = 4) were considered seropositive for *Salmonella* in the IDEXX ELISA test. Piglets immunized with either: Δ*lon*, Δ*rfaJ*, Δ*lon*Δ*rfaJ* or *Salmonella* Typhimurium 112910a and infected animals were all positive for anti-*Salmonella* antibodies when using a *Salmonella* Typhimurium strain 112910a whole-cell ELISA, while non immunized control animals were seronegative. Results are shown in Figure 3.

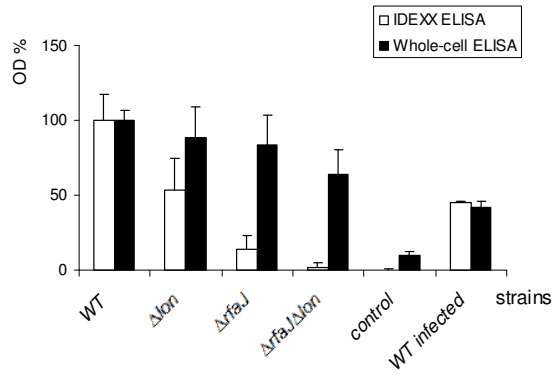

Figure 3: Serological results of pigs immunized with either *Salmonella* Typhimurium strain 112910a, Δ*lon*, Δ*rfaJ*, or Δ*lon*Δ*rfaJ*, control animals (animals that were not immunized) and pigs infected with *Salmonella* Typhimurium strain 112910aNal[20] when using an LPS based IDEXX ELISA (white bars) and a *Salmonella* Typhimurium whole cell ELISA (black bars). Values are represented as a percentage compared to the *Salmonella* Typhimurium immunized group.

Deletion of *lon* increases susceptibility to UV, various disinfection agents and acidic conditions

The *lon* deficient strains were highly susceptible to ultraviolet light compared to the Δ*rfaJ* strain on the one hand and to the wild type strain on the other hand, with a significant reduction ($P < 0.05$) in the number of surviving cells at 60 seconds exposure (Figure 4).

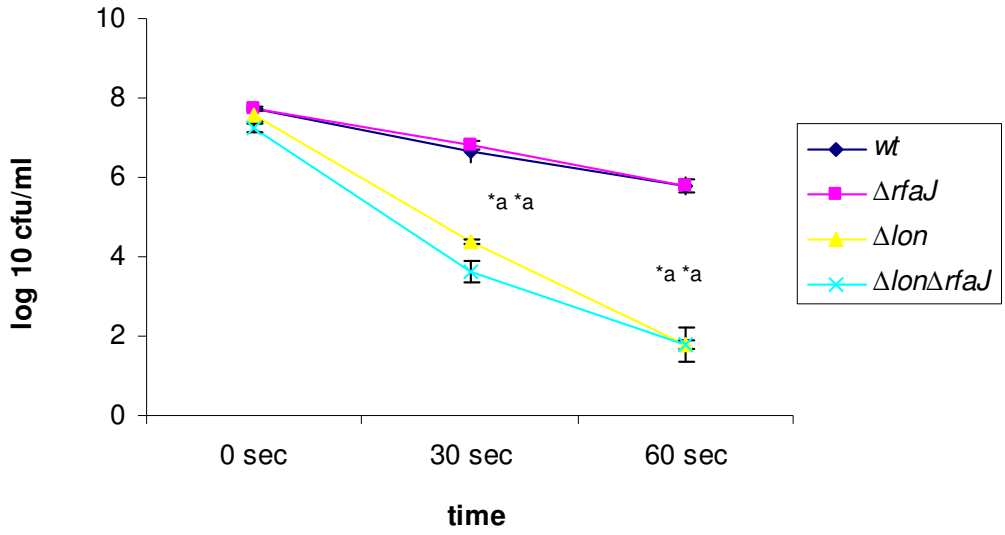

Figure 4: Susceptibility of *Salmonella* Typhimurium strain 112910a and its isogenic knock-out mutants *(*Δ*rfaJ*, Δ*lon* and Δ*lon*Δ*rfaJ*) to UV irradiation. An asterix refers to a significant difference in survival compared with the wild type strain (*P < 0.05*), an 'a' refers to a significant difference ($P < 0.05$) with the Δ*rfaJ* strain.

Furthermore, the Δ*lon* strains (Δ*lon* and Δ*lon*Δ*rfaJ*) were more sensitive to hydrogen peroxide, ethanol, chloraminum and citric acid compared to the Δ*rfaJ* and the wild type strains. The Δ*lon*Δ*rfaJ* strain survived exposure to 15 mM hydrogen peroxide for 75 minutes, with a 0.7 log reduction ($P < 0.05$) compared to the Δ*rfaJ* strain. The Δ*lon* strain survived exposure to 15 mM hydrogen peroxide for 75 minutes, with a 0.8 log reduction ($P < 0.05$) compared to the wild type strain. Results are shown in Figure 5.

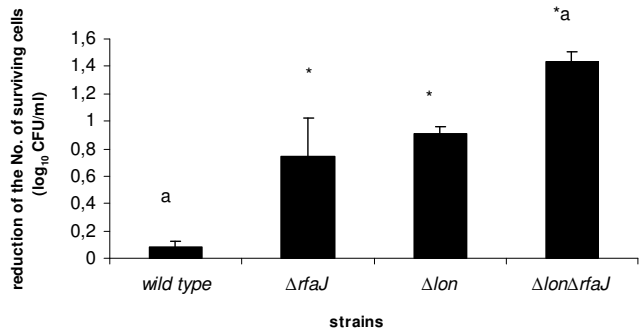

Figure 5: Effect of 15 mM hydrogen peroxide for 75 minutes on the survival of *Salmonella* Typhimurium strain 112910a and its isogenic knock-out mutants *(*Δ*rfaJ*, Δ*lon* and Δ*lon*Δ*rfaJ*). An asterisk refers to a significant difference in survival compared with the wild type strain ($P < 0.05$), an 'a' refers to a significant difference ($P < 0.05$) with the Δ*rfaJ* strain.

All strains were sensitive to exposure to 20 % ethanol, only a 0.2 log reduction ($P > 0.05$) in survival of Δ*lon*Δ*rfaJ* was seen compared to the Δ*rfaJ* strain. Results are not shown. The Δ*lon*Δ*rfaJ* strain survived exposure to chloraminum for 15 minutes, with a 0.4 log reduction ($P < 0.05$) compared to the Δ*rfaJ* strain. The number of surviving cells of Δ*lon* was reduced compared to the wild type strain. However, this reduction was not significant ($P < 0.05$). Results are shown in Figure 6.

The Δ*lon* strains showed a significant reduction ($P < 0.05$) in survival compared to Δ*rfaJ* and the wild type strain when exposed to 50 mM citric acid for 150 minutes (Figure 7).

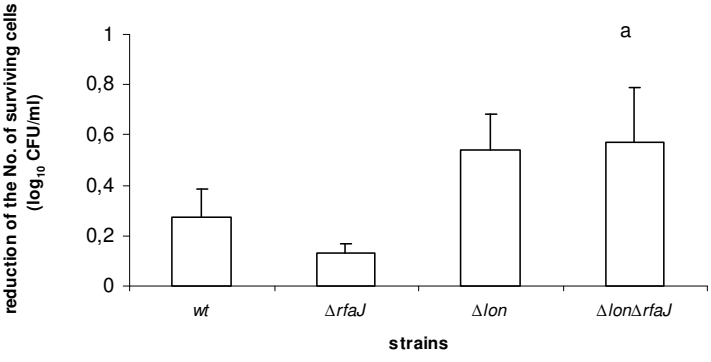

Figure 6: Effect of 1mM chloraminum for 15 minutes on the survival of *Salmonella* Typhimurium strain 112910a and its isogenic knock-out mutants *(*Δ*rfaJ*, Δ*lon* and Δ*lon*Δ*rfaJ*). An 'a' refers to a significant difference ($P < 0.05$) with the Δ*rfaJ* strain.

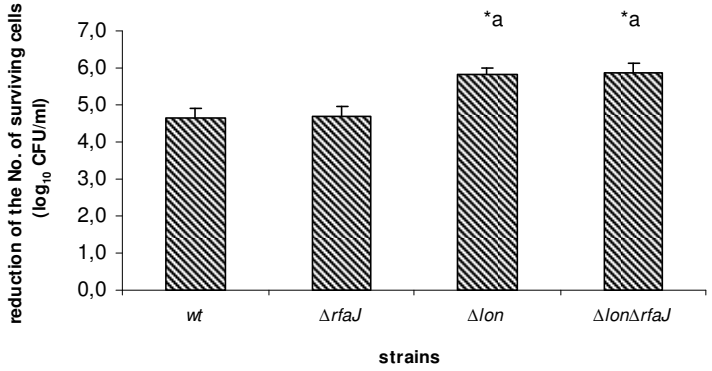

Figure 7: Effect of 50 mM citric acid for 150 minutes on the survival of *Salmonella* Typhimurium strain 112910a and its isogenic knock-out mutants *(*Δ*rfaJ*, Δ*lon* and Δ*lon*Δ*rfaJ*). An asterisk refers to a significant difference in survival compared with the wild type strain ($P < 0.05$), an 'a' refers to a significant difference ($P < 0.05$) with the Δ*rfaJ* strain.

Δ*lon*Δ*rfaJ* and Δ*rfaJ* equally protect mice against *Salmonella* Typhimurium 112910a

All strains were able to significantly ($P < 0.05$) reduce bacterial counts in the spleen and liver of BALB/c mice compared to control animals. The *rfaJ* deficient strains (Δ*lon*Δ*rfaJ* and Δ*rfaJ*) were equally able to protect mice against a *Salmonella* Typhimurium challenge. Only the wild type strain was able to significantly ($P < 0.05$) reduce bacterial counts in the caecum of mice. Results are shown in Figure 8.

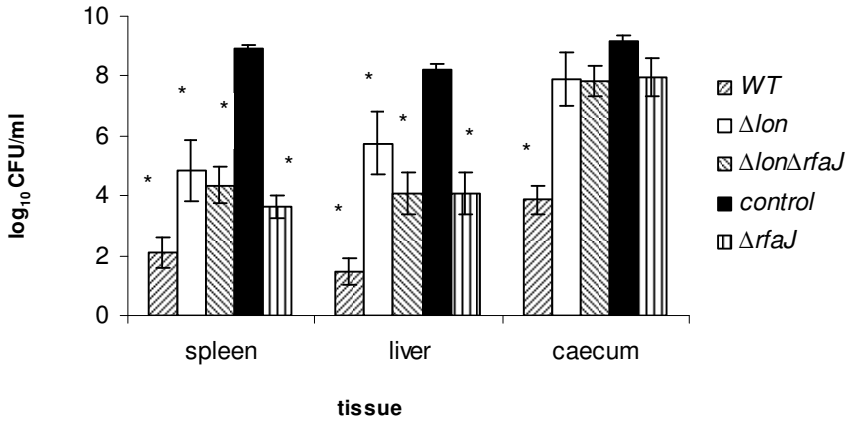

Figure 8: Recovery of *Salmonella* bacteria from various organs of mice vaccinated with either *Salmonella* Typhimurium 112910a, one of its isogenic knock-out mutants (Δ*lon*, Δ*rfaJ*, Δ*lon*Δ*rfaJ*) or non vaccinated animals (control) subsequently challenged with NCTC12023Nal[20]. The log_{10} value of the ratio of CFU per gram sample and standard error (SEM) is given. An asterisk refers to a significant difference with the control (unvaccinated animals) group ($P < 0.05$).

2.4.5 Discussion

Several attenuated live vaccine candidates against *Salmonella* Typhimurium infections have been investigated in the past two decades (Nagy *et al.*, 2006). One major drawback, that vaccinated pigs produce antibodies against the vaccine strain and therefore are no longer distinguishable from field-exposed animals by serological tests, was overcome by using DIVA marker strains, such as Δ*rfaJ* (Leyman *et al.*, 2011) and Δ*lon*Δ*rfaJ*, as proven in this study.

Our study confirmed that deletion of the *lon* gene does not affect invasiveness in IPEC-J2 cells, indicating that *lon* deficient strains might colonize their host cells to that extent that they are able to elicit a protective immune response. The use of susceptible mice in combination with a highly virulent challenge strain, allowed us to confirme that Δ*lon*Δ*rfaJ* successfully protects BALB/c mice against a *Salmonella* Typhimurium infection in both spleen and liver and the strain's protective capacity is not impaired compared to the Δ*rfaJ* DIVA strain. However, it has to be kept in mind that only the wild type strain was able to significantly reduce bacterial counts in the caecum of mice compared to unvaccinated control animals, which highlights that all the deletion mutants used in this study were partially impaired in their ability to protect against a *Salmonella* Typhimurium challenge.

Disruption of the *lon* gene in Δ*rfaJ* increased susceptibility of the Δ*rfaJ* strain to some environmental stresses. Sensitivity of Δ*lon*Δ*rfaJ* and Δ*lon* to ultraviolet light is explained by the overproduction of oxygen species, leading to oxidative damage and the accumulation of naturally unstable proteins, such as SulA, a cell division inhibitor produced after DNA damage (Schoemaker *et al.*, 1984). Furthermore, our study confirmed that Lon, as primary ATP-dependent quality control protease, is required to resist many types of stress, such as disinfection agents and citric acid. Thus, deletion of *lon* could significantly contribute to decreased environmental persistence of live vaccine strains.

Based on our findings, we summarize that deletion of the *lon* gene in the Δ*rfaJ* strain (1) allows differentiation of infected and vaccinated animals (2) does not influence the protective capacity of the Δ*rfaJ* strain in BALB/c mice and (3) results in an increased susceptibility to ultraviolet light, disinfection agents and citric acid. Therefore, we conclude that deletion of

lon in Δ*rfaJ* increases susceptibility of live *Salmonella* Typhimurium vaccines to some environmental stresses.

Acknowledgements

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2.4.6 References

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Chapter 3: General discussion

3.1 Is vaccination the key tool for *Salmonella* Typhimurium control in pigs?

Salmonella contaminated pork is an important source of salmonellosis for humans. Due to an apparent and spectacular drop in *Salmonella* Enteritidis infections in poultry in 2005 and 2006 (Collard *et al.*, 2007), the relative importance of *Salmonella* Typhimurium infections in pigs has increased (EFSA, 2010). Governments of European member states try to mitigate this threat by installing monitoring and control programs. In 2005, the Belgian Federal Agency for the Safety of the Food Chain (FASFC), implemented a national *Salmonella* surveillance and control program for pigs, the *Salmonella* Action Plan (SAP) (Brossé *et al*., 2011). Control actions of the SAP are mainly based on imposing bio-security measures (Brossé *et al.*, 2011). Although these are important in controlling *Salmonella*, it is difficult to predict their impact on the overall prevention of *Salmonella* infections (Miller *et al.*, 2005). Despite ameliorating bio-security on farms with a high risk *Salmonella* status, the overall prevalence of *Salmonella* Typhimurium infections in humans does not seem to have dropped substantially over the last few years (EFSA, 2010; Anonymous, 2012).

Gantois *et al.* (2006) showed that live attenuated *Salmonella* vaccines could protect against colonization in laying hens and could reduce internal egg contamination. Reducing the number of contaminated eggs by vaccination of laying hens has resulted in a significant reduction of the number of *Salmonella* cases in humans (Cogan *et al.*, 2003; Collard *et al.*, 2007). Similarly, vaccination might be a promising tool to decrease the level of *Salmonella* Typhimurium infected pigs and, thus, humans.

Results in this thesis and previous studies indicate that vaccination is able to reduce clinical symptoms and excretion of *Salmonella*, in suckling piglets (Eddicks *et al.*, 2009), in weaned piglets (Roesler *et al.*, 2004) and in piglets from sows vaccinated *ante partum* (Roesler *et al.*, 2006; Hur *et al.*, 2011). Moreover, De Ridder *et al.* (unpublished results) have recently shown that vaccination with a live attenuated *Salmonella* Typhimurium vaccine decreased the transmission of *Salmonella* Typhimurium between swine. Vaccination against *Salmonella* infections in swine not only results in a reduced number of *Salmonella*-infected pigs with positive consequences for public health, but might also benefit the productive performance of pigs. Farzan *et al.* (2010) observed that pigs shedding *Salmonella* experienced a slower growth compared to *Salmonella*-negative pigs. Moreover, De Ridder *et al.* (unpublished results) showed that *Salmonella*-infected but non-vaccinated pigs demonstrated a lower daily

weight gain in comparison with vaccinated pigs subsequently infected with *Salmonella* Typhimurium. In contrast with the generally accepted asymptomatic aspect of *Salmonella* Typhimurium infections in pigs, this lower daily weight gain might be an incentive for farmers to implement control measures, such as vaccination, at herd level.

A combination of vaccination and measures as described in the current SAP might be useful to reduce the number of *Salmonella* Typhimurium infected pigs and, ultimately, the number of infected people consuming pork. At this moment 18.8% of all Belgian breeding farms are positive for *Salmonella* (Anonymous, 2012). Therefore breeding sows are a source of microbial contamination in the food chain. Elimination of the *Salmonella* problem at an early stage of the production chain is highly desirable. We propose to vaccinate breeding sows *ante partum* to interrupt the chain of vertical transmission. Hur *et al.* (2011) showed that *ante partum* vaccination of breeding sows reduces shedding of *Salmonella* and protects neonatal piglets from salmonellosis.

3.2 Use of serology-based monitoring in *Salmonella* control programs: sense or nonsense?

Current serology-based *Salmonella* monitoring programmes in pig herds are under debate because serological screening does not always correlate with bacterial isolation of *Salmonella* from pigs (Laevens *et al.*, 2005; Nollet *et al*., 2005; Belsué *et al*., 2011). Furthermore, Nielsen *et al.* (1995) have shown that not all pigs seroconvert, despite experimental inoculation and subsequent excretion of *Salmonella* in the faeces. Recently, Van Parys *et al.* (2012) observed that some *Salmonella* strains are able to circumvent the pig's humoral response, by down-regulation of MHC II expression on porcine macrophages. Therefore, strains that are able to circumvent their host's humoral immune response, and potentially attribute to long-term *Salmonella* persistence in pigs, might be missed using serological screening systems.

Different serology based monitoring programmes use distinct cut-off values to classify herds as serologically positive which might raise questions concerning the reliability of serological monitoring. To which extent are herds, currently classified as serologically negative (positive) for *Salmonella*, truly negative (positive)? Are there sero-negative pig herds in Belgium? Nollet *et al.* (2005) showed that an average herd prevalence of 83.6% (using a cut-off S/P = 1) to 100% (using a cut-off S/P = 0.25) was found, as measured by serological testing of blood

samples. Figure 1 illustrates the course of mean S/P ratios between 2005 and 2011 in Belgium and shows a downward trend from 2008. However, how these serological data translate to the actual *Salmonella* status of the pig farms is not clear.

In this thesis, all piglets, from a commercial closed line based on Landrace and used as uninfected control animals, stayed bacteriologically negative in their faeces during the entire duration of the experiment and were negative for *Salmonella* in all organ samples at the moment of euthanasia. These animals showed consistently low S/P ratios (S/P < 0.25) for *Salmonella* antibodies during the entire experiment. These results illustrate that serological screening is useful when pigs are bacteriologically negative for *Salmonella*. Moreover, it suggests previous contact with *Salmonella* of all pigs with an S/P value ≥ 0.25.

A top-down approach focussing on breeding sows might reduce the number of contaminated pigs that end up in the slaughterhouse. Only if all pigs at the top levels of the production chain are bacteriologically negative for *Salmonella*, serological classification of pig herds becomes meaningful. We suggest that serological screening could be a reliable and cost-effective tool to screen pig herds (for example breeding herds) once pigs have obtained a bacteriologically *Salmonella* free status.

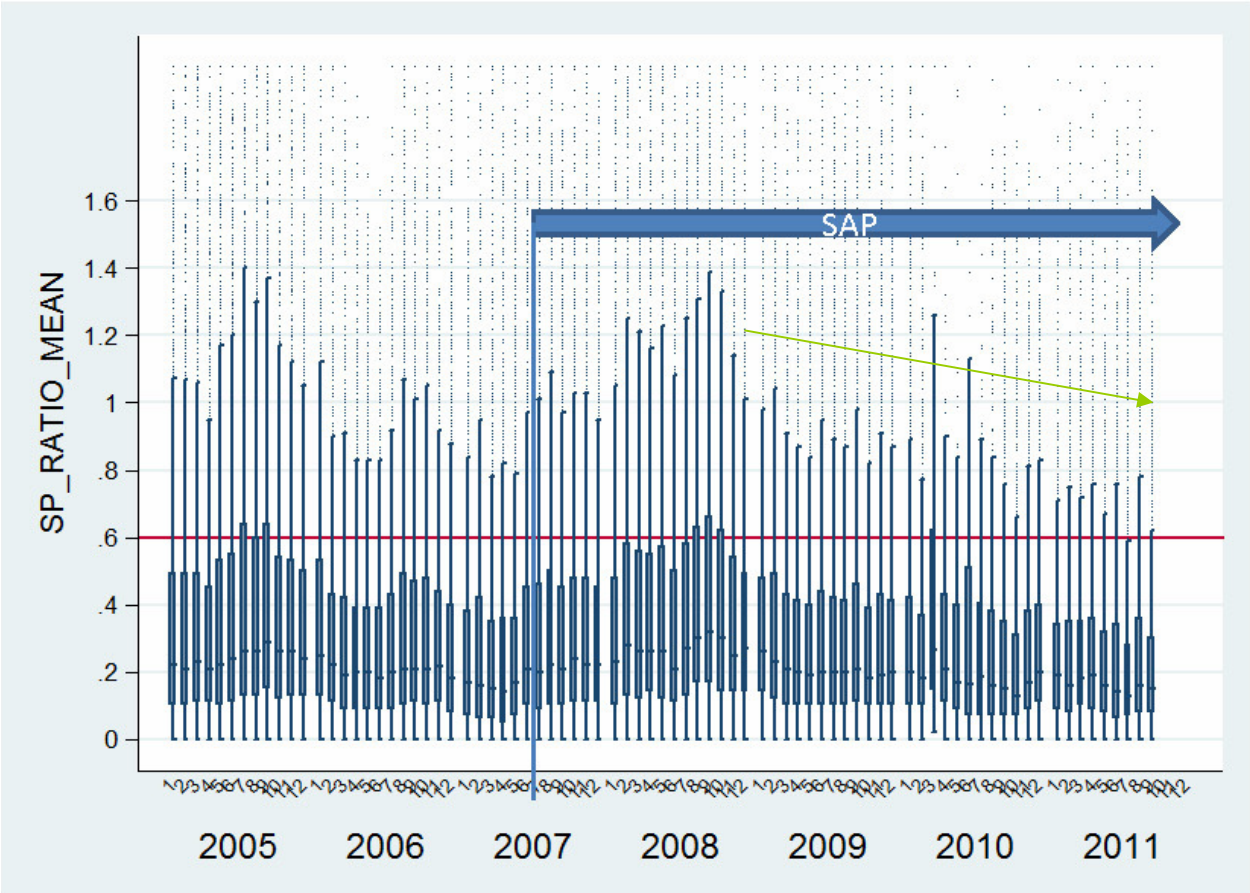

Figure 1: Evolution of the S/P ratios represented as box plots of all Belgian pig farms during the period the SAP came into effect (Anonymous, 2012)

3.3 Is there an opportunity for the use of a DIVA vaccine in pigs?

Currently, all European surveillance programmes, except for the Norwegian and Swedish ones, are based on the serological detection of *Salmonella* antibodies in serum or meat-juice samples of pigs (Wahlström *et al.*, 1997; Sandberg *et al.*, 2002; Benschop *et al.*, 2008; Snary *et al.*, 2010; Merle *et al.*, 2011; Belsué *et al.*, 2011). The first ELISA to detect *Salmonella* antibodies, in serum and meat-juice, was developed in Denmark and was an anti-LPS ELISA using the antigens O: 1, 4, 5 and 12 from *Salmonella* Typhimurium and O: 6 and 7 from *Salmonella* Choleraesuis. Based on this test, many other laboratories created ELISAs using O antigens (Proux *et al.*, 2000; Chow *et al.*, 2004). The use of O-antigen based ELISAs for *Salmonella* monitoring rules out vaccination as a control measure because they cannot differentiate antibodies, induced by a *Salmonella* infection, from antibodies, induced by vaccination. As vaccination might be a key tool to control *Salmonella* Typhimurium in pig herds, development of a DIVA vaccine that allows differentiation between infected and

vaccinated animals is desirable. Until now, one DIVA vaccine strain, SalmoporcΔ*ompD*, has been created (Selke *et al*, 2007). Notwithstanding this negative-marker vaccine allows differentiation of infected from vaccinated animals using an OmpD-specific peptide-based ELISA (Selke *et al.*, 2007), it does not allow distinction between vaccinated and infected animals when using an O-antigen based ELISA. Therefore, the need of a *Salmonella* Typhimurium DIVA vaccine, available for use in the current LPS based screening program, was met in this thesis. We have shown that Δ*rfaJ* and Δ*rfaL* are suitable markers allowing serological differentiation between vaccinated and infected pigs using the ELISA currently employed in the SAP. Both Δ*rfaJ* and Δ*rfaL* have the potential to protect mice from clinical symptoms of a *Salmonella* Typhimurium infection and, most importantly, reduce the *Salmonella* colonization rate in spleen and liver, similar to their parent strain.

Whether or not the DIVA marker strains, constructed in this thesis, will one day be widely implemented in the field will depend on several conditions. First and foremost, is there a suitable live attenuated vaccine strain to introduce our marker in? We postulate that deletion of *rfaJ* or *rfaL* in commercially available *Salmonella* Typhimurium vaccine strains might result in a usable DIVA vaccine for pigs. A combined approach of a vaccine that is able to reduce the colonization rate of *Salmonella* Typhimurium in the gut of pigs (Selke *et al.*, 2007), is able to reduce stress-related recrudescence in carrier pigs (chapter 2.1 of this thesis), combined with the advantages of a DIVA vaccine, might prove a useful tool on the way to reduce the entry of *Salmonella* Typhimurium in the food production chain.

Secondly, will current serology-based *Salmonella* Typhimurium surveillance programmes still exist in the future? This question cannot unambiguously be answered and the answer will depend on decisions made by legislative authorities (e.g. FASFC, EFSA). EFSA (2011) partly discourages serology based *Salmonella* screening of pigs because it does not provide information on *Salmonella* serovars and on *Salmonella* clones with high virulence or resistance towards antimicrobials critically important for treatment of human infections. They suggest bacteriological testing of either faeces, ileal content or carcas swabs as robust sampling methods for *Salmonella* screening (EFSA, 2011). We postulate that notwithstanding disadvantages of the current serology-based screening system serology has many benefits due to easier standardisation, cost-effectiveness and time-effectiveness compared to bacteriological screening (Rajic *et al*., 2007). We conclude that, at this moment, there is an

opportunity for the use of DIVA marker strains in vaccine development, as serology is still the current conventional method for *Salmonella* screening in pigs in Europe.

In third place, we have to keep in mind that the DIVA strains were tested under restricted circumstances. We only used a limited number of pigs to discriminate between the serological response induced after infection of pigs with *Salmonella* Typhimurium and immunization of pigs with the Δ*rfaL* and Δ*rfaJ* marker strains. Consequently, it has not been shown that the DIVA features of the used strains also apply under field conditions, using large groups of animals. Moreover, the antibody response was investigated during a confined time period. Four weeks after the second immunization, the pigs were euthanized and blood samples were examined for the presence of anti-*Salmonella* Typhimurium antibodies using the IDEXX ELISA. In addition it is unclear whether other LPS-based ELISAs are equally specific compared to the IDEXX ELISA to differentiate between the serological response of *Salmonella* infected pigs and animals immunized with either Δ*rfaJ* or Δ*rfaL*. Notwithstanding manufacturers of the different ELISAs claim that the specificity (S/P) of their tests is high for diagnosing *Salmonella* infection, differences could be found in their results when the same field samples were examined (Mejia *et al.*, 2005; Farzan *et al.*, 2007). These discrepancies are linked with test-dependent factors such as the conditions under which the test is performed, the nature of the antigens used for coating (e.g. whole LPS or O polysaccharides), the isotypes of immunoglobulins involved, and the *Salmonella* serotypes targeted by the test (Mejia *et al.*, 2005).

3.4 Improving bio-security of live *Salmonella* Typhimurium vaccine strains for pigs

For their use as a control measure in the field, live attenuated strains have to meet certain requirements. As genetically modified organisms, they might attract attention with regard to their potential risk to animal- and subsequently human health. These issues include: (1) possible reversion to a virulent strain; (2) presence of antimicrobial resistance genes that might be transferred to pathogens or bacteria belonging to the animal's microbiota; and (3) the risk of live vaccine strains to survive in the animal and human environments after excretion by the vaccinated animals.

The first and second issues were met by construction of the DIVA strains according to the one-step inactivation method first described by Datsenko and Wanner (2000), with some modifications (Donné *et al.*, 2005). With this technique, the targeted virulence-associated

genes are completely knocked out from the bacterial genome, making reversion of the mutants to a virulent wild type phenotype extremely unlikely (Haesebrouck *et al.*, 2004). Although in a first step of this method the targeted genes are replaced by a kanamycin resistance cassette, this cassette is afterwards deleted, ruling out its transfer to other bacteria.
The third issue is that live vaccines are potentially excreted into the environment by the vaccinated animals. Consequently, live vaccine strains may be distributed by several vectors including humans and different animal species such as cats, birds, flies and rodents (Letellier *et al*., 1999; Beloeil *et al.*, 2002). Furthermore, wastewaters are potential pollution sources of rivers and groundwater (Cordero *et al.*, 2005) that are natural resources of drinking water and fish and shellfish products (Patchanee *et al.*, 2010). Besides, *Salmonella*-contaminated slurry, disposed on the agricultural soil might pose a health risk for crop consumers, producers and handlers (Baloda *et al.*, 2001; Marranzano *et al.*, 2010). Deletion of *lon* in the Δ*rfaJ* marker strain reduced the environmental survival, as a result of increased susceptibility of the Δ*lon*Δ*rfaJ* strain to environmental stress such as UV and several disinfection agents, like chloraminum and hydrogen peroxide. Moreover, the use of Δ*lon*Δ*rfaJ* as a vaccine strain might aid in the prevention of persistency in water as an UV advanced oxidation (UV/H_2O_2) process is currently used to disinfect human drinking water (Matilainen *et al.*, 2010).

3.5 Abolishment of stress-related recrudescence of a *Salmonella* Typhimurium live vaccine

In the field, stress of transport and feed deprivation are assumed to enhance shedding by carriers as well as the susceptibility of exposed pigs (Verbrugghe *et al.*, 2011). Carrier pigs can start shedding the pathogen within hours after a stress event (Straw *et al.*, 2006). In chapter 2.3 of this thesis we showed that a live vaccine can also be subject to stress-related enhanced replication. The most important mediators in a 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). Straw *et al.* (2006) postulated that catecholamines released in response to stress, result in a decreased gastric acid production and increased intestinal motility. Increased stomach pH stimulates survival of salmonellae and passage through the stomach, consequently enhancing the bacterial load in the intestine and colon. Verbrugghe *et al.*,

(2011b) showed that cortisol promotes intracellular proliferation of *Salmonella* Typhimurium in porcine alveolar macrophages.

In this thesis, animals were artificially stressed by injection of dexamethasone, a synthetic member of the glucocorticoids. Verbrugghe *et al.* (2011b) showed that both natural stress, such as feed deprivation, and artificial stress, such as a dexamethasone injection, result in recrudescence of *Salmonella* Typhimurium in pigs. Artificial stressing with dexamethasone allowed us to highlight the role of *scsA* or the entire *scs* locus in the dexamethasone-induced enhanced replication of a *Salmonella* Typhimurium wild type strain in a DBA /2J mice model. Extrapolation of these findings to pigs vaccinated with an attenuated *Salmonella* Typhimurium vaccine should be done with caution. Further studies are therefore necessary to confirm that deletion of this gene or locus also abolishes enhanced replication of attenuated *Salmonella* Typhimurium strains in pigs undergoing stressful events occurring in the field.

3.6 Conclusion

In this thesis we constructed *Salmonella* Typhimurium marker strains that allow differentiation of infected and vaccinated pigs in currently used LPS-based sero-surveillance programs. We emphasized the role of *scs* genes in the abolishment of stress related enhanced replication of *Salmonella* Typhimurium. Moreover, we constructed a DIVA vaccine that is more susceptible to environmental stress. In this way, we surmounted some issues regarding live *Salmonella* Typhimurium vaccines for pigs, but highlight that the road to optimizing live *Salmonella* Typhimurium vaccines is not yet finished.

Table 1: Genes tested in relation to optimizing vaccination of pigs against *Salmonella* Typhimurium

Strain	**Product of the deleted gene**
Δ***rfaL***	*Salmonella* Typhimurium O-antigen ligase
Δ***rfaJ***	LPS 1,2-glucosyltransferase
Δ***rfaI***	LPS 1,3-galactosyltransferase
Δ***rfaG***	LPS core biosynthesis protein
Δ***rfaF***	LPS heptosyltransferase II
Δ***rfbA***	glucose-1-phosphate thymidylyltransferase
Δ***lon***	DNA-binding ATP-dependent protease La
Δ***scsA***	membrane protein, suppressor for copper-sensitivity A
Δ***scsB***	suppression of copper sensitivity protein B
Δ***scsC***	suppression of copper sensitivity protein C
Δ***scsD***	suppression of copper sensitivity protein D

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Chapter 4: Summary - Samenvatting

4.1 Summary

Contaminated pork is a major source of human salmonellosis and the serovar most frequently isolated from pigs is *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium). Vaccination could contribute greatly to control *Salmonella* infections in pigs. However, a vaccination strategy is not applicable within current European serology-based *Salmonella* surveillance systems because it does not allow differentiation between infected and vaccinated animals. Furthermore, live vaccines are potentially excreted into the environment by the vaccinated animals, especially when pigs are stressed, and this might result in persistent environmental contamination. Therefore, the first aim of this thesis was to construct a DIVA marker strain that allows differentiation between infected and vaccinated pigs on the one hand and is more susceptible to environmental stress on the other hand. A next aim was to abolish stress-related enhanced replication of a *Salmonella* Typhimurium vaccine strain. Finally, we evaluated the ability of a commercial live *Salmonella* Typhimurium vaccine to protect against stress-related *Salmonella* recrudescence in carrier pigs.

We first developed and characterized a vaccine marker strain of *Salmonella* Typhimurium that allows discrimination between infected and vaccinated animals (DIVA) in currently used lipopolysaccharide (LPS) based *Salmonella* surveillance programs. For that purpose, we examined which LPS-encoding genes of *Salmonella* Typhimurium can be deleted to allow differentiation of infected and vaccinated animals in the currently used IDEXX ELISA without affecting the vaccine strain's protective capacity. Deletion mutants in *Salmonella* strain 112910a were constructed in the LPS encoding genes: Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG* and Δ*rfaF* and validation of the LPS phenotype occurred by SDS-polyacrylamide gel electrophoresis. Primary inoculation of BALB/c mice with the parent strain, Δ*rfaL,* Δ*rfbA* or Δ*rfaJ* strain but not the Δ*rfaG*, Δ*rfaF* or Δ*rfaI* strain protected significantly against subsequent infection with the virulent *Salmonella* Typhimurium strain NCTC12023. Immunization of piglets with the Δ*rfaJ* or Δ*rfaL* mutants resulted in the induction of a serological response lacking detectable antibodies against LPS, using the IDEXX ELISA. This allowed a clear differentiation between sera from pigs immunized with the Δ*rfaJ* or Δ*rfaL* strains and sera from pigs infected with their isogenic wild type strain. In conclusion, applying deletions in the *rfaJ* or the *rfaL* gene in *Salmonella* Typhimurium strain 112910a allows differentiation of

infected and vaccinated pigs in an LPS based ELISA without reducing the strain's protective capacities in mice.

Because live vaccines are potentially excreted into the environment by the vaccinated animals, especially when pigs are stressed, this might result in persistent environmental contamination. We evaluated whether deletion of the *lon* gene in the *Salmonella* Typhimurium Δ*rfaJ* marker strain, allowing differentiation of infected and vaccinated animals (DIVA), resulted in decreased environmental survival, without loss of the vaccine's protective capacity and DIVA quality. Deletion of the *lon* gene in the Δ*rfaJ* strain resulted in an increased susceptibility to UV and various disinfectants. Furthermore, immunization of pigs with the inactivated Δ*rfaJ* or Δ*lon*Δ*rfaJ* vaccines allowed differentiation of infected and vaccinated pigs. Deletion of the *lon* gene did not affect protection of live wild type or Δ*rfaJ* vaccine strains against subsequent challenge with a virulent *Salmonella* Typhimurium strain in BALB/c mice. Therefore, deletion of *lon* in Δ*rfaJ* contributes to environmental safety of the Δ*rfaJ* DIVA strain.

A next issue we tackled in this thesis was stress-induced enhanced replication of live *Salmonella* Typhimurium vaccine strains. If stress would evoke recrudescence of the *Salmonella* vaccine strain, additional attenuation resulting in loss of recrudescence ability, would be highly desirable. We first investigated whether increased multiplication of a live *Salmonella* Typhimurium vaccine in pigs can be induced by an injection of dexamethasone, mimicking stress. Our results showed that a live *Salmonella* vaccine strain is indeed subject to stress-related enhanced replication. Second, we examined whether deletion of *scsA*, *scsB*, *scsC*, *scsD* or the entire *scs* locus in *Salmonella* Typhimurium strain 112910a would abolish stress-induced enhanced replication of that strain. A previous study showed that stress related re-excretion of *Salmonella* is linked to increased intracellular multiplication in macrophages. *ScsA* was shown to be the major driver for increased intracellular multiplication of *Salmonella* Typhimurium in cortisol-exposed porcine alveolar macrophages. We first developed an *in vivo* mouse model, in which dexamethasone-induced enhanced replication of knock-out strains was compared to that of their isogenic wild type strain. We showed that *scsA* and *scsABCD* but not *scsB*, *scsC* or *scsD*, are vital for dexamethasone-induced *Salmonella* multiplication in organ samples of DBA/2J mice. We concluded that applying a deletion of *scsA* or *scsABCD* might abolish stress related enhanced replication of live *Salmonella* Typhimurium vaccines.

Infections of pigs with *Salmonella* Typhimurium often result in the development of carriers that re-excrete *Salmonella* during periods of stress. At our laboratory we determined the role of cortisol in the recrudescence of *Salmonella* Typhimurium, which can be reproduced by injection of dexamethasone. In this thesis we evaluated whether a commercially available, attenuated *Salmonella* Typhimurium vaccine (*Salmoporc*®) is able to reduce *Salmonella* excretion after dexamethasone injection, mimicking pre-slaughter stress. For that purpose, pigs were vaccinated and subsequently infected with *Salmonella* Typhimurium. Twenty-three days post infection, pigs were injected with dexamethasone to induce recrudescence of *Salmonella* and subsequently pigs were euthanized and *Salmonella* Typhimurium numbers were determined in organs and organ contents. The *Salmonella* load was significantly lower in ileum, ileum content, caecum content and colon from vaccinated pigs, compared to those from non-vaccinated pigs. Besides, more *Salmonella* positive tonsil and colon samples were detected in non-vaccinated pigs than in vaccinated pigs. We showed that vaccination with *Salmoporc*® is able to reduce but not eliminate *Salmonella* Typhimurium numbers in pigs in conditions mimicking pre-slaughter stress. Further optimization of the capacity to reduce stress induced recrudescence could greatly contribute to the vaccine's efficacy.

In conclusion, we postulate that vaccination with a DIVA marker strain may help to reduce the number of *Salmonella* Typhimurium infected pigs and, ultimately the number of infected people that consume pork. Although serology-based screening methods are under debate, it is still the current conventional method for *Salmonella* screening in many European countries. Therefore, DIVA marker strains might be important to differentiate between infected and vaccinated pigs in the current LPS based screening programs. However, we emphasize that the DIVA strains were tested under restricted circumstances and that more research will be necessary to guarantee the efficacy of these strains in the field.

4.2 Samenvatting

Salmonella infecties zijn één van de belangrijkste bacteriële voedselinfecties bij de mens en gecontamineerd varkensvlees is een belangrijke bron van *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*Salmonella* Typhimurium) besmetting. Vaccinatie kan een belangrijke rol spelen in de reductie van het aantal met *Salmonella* Typhimurium geïnfecteerde varkens. Aangezien het huidige monitoringsysteem gebaseerd is op serologie, met name detectie van antistoffen gericht tegen lipopolysacchariden (LPS), en er geen merkervaccins ter beschikking zijn, is het momenteel echter niet mogelijk om een onderscheid te maken tussen gevaccineerde en geïnfecteerde varkens. Bovendien kunnen levende vaccinstammen door gevaccineerde dieren worden uitgescheiden en persisteren in de stalomgeving. Het eerste doel van deze doctoraatsthesis was enerzijds het ontwikkelen van een *Salmonella* Typhimurium merkerstam die serologisch onderscheid toelaat tussen geïnfecteeerde en gevaccineerde varkens en die anderzijds minder lang overleeft in de stalomgeving. Vervolgens werd nagegaan of het uitschakelen van bepaalde genen van *Salmonella* Typhimurium, waarvan reeds werd aangetoond dat ze een rol spelen in cortisol-afhankelijke verhoogde intracellulaire groei van *Salmonella*, stress gerelateerde verhoogde vermeerdering van *Salmonella* Typhimurium kan inhiberen. Tot slot, evalueerden we de capaciteit van een levend *Salmonella* Typhimurium vaccin om te beschermen tegen stress gerelateerde *Salmonella* herexcretie in varkens.

In dit doctoraatsonderzoek was het in eerste instantie onze bedoeling om *Salmonella* Typhimurium mutantstammen te ontwikkelen die toelaten om een onderscheid te maken tussen geïnfecteerde en gevaccineerde varkens. Hiervoor werden een aantal LPS deletiemutanten (Δ*rfbA*, Δ*rfaL*, Δ*rfaJ*, Δ*rfaI*, Δ*rfaG* en Δ*rfaF*) aangemaakt en *in vitro* gekarakteriseerd aan de hand van SDS-polyacrylamide gel electroforese. Uit een *in vivo* experiment bleek dat de Δ*rfbA*, Δ*rfaL* en de Δ*rfaJ* stammen en niet de Δ*rfaI*, Δ*rfaG* en de Δ*rfaF* stammen, in staat waren BALB/c muizen te beschermen tegen een *Salmonella* Typhimurium infectie. Tenslotte toonden we aan dat varkens, gevaccineerd met Δ*rfaL* of Δ*rfaJ*, serologisch te onderscheiden waren van dieren die werden geïnfecteerd met een *Salmonella* Typhimurium veldstam, wanneer gebruikt gemaakt werd van de IDEXX ELISA om *Salmonella* antistoffen op te sporen. Dit was niet mogelijk voor varkens die gevaccineerd werden met Δ*rfbA*. Dit betekent dat deletie van *rfaJ* of *rfaL* in een bestaande vaccinstam

ervoor zal zorgen dat gevaccineerde varkens kunnen onderscheiden worden van geïnfecteerde varkens.

Een goede vaccinstam moet niet alleen serologisch onderscheid toelaten tussen gevaccineerde dieren en geïnfecteerde dieren, maar mag ook niet persisteren in de stalomgeving. Daarvoor werd een dubbel deletiemutant, Δ*lon*Δ*rfaJ*, geëvalueerd op zijn capaciteit om te overleven in de omgeving met behulp van een aantal *in vitro* experimenten. De Δ*lon*Δ*rfaJ* stam bleek gevoeliger te zijn aan het antibacteriële effect van ultraviolet licht en verschillende disinfectantia. Bovendien bleek dat deze dubbel deletiemutant, Δ*lon*Δ*rfaJ*, in een zelfde mate bescherming kan bieden tegen een *Salmonella* Typhimurium infectie in BALB/c muizen als de wild type stam en de Δ*rfaJ* stam. De Δ*lon*Δ*rfaJ* dubbelmutant kan bovendien als DIVA-merkerstam in varkens gebruikt worden. Bijgevolg kunnen we besluiten dat deletie van *lon* bijdraagt tot de optimalisatie van de Δ*rfaJ* DIVA stam.

Levende *Salmonella* Typhimurium vaccinstammen zouden in hogere mate kunnen vermeerderen in gestresseerde varkens, waardoor ze in de voedselketen terecht zouden kunnen komen. In varkens werd nagegaan of een verhoogde vermeerdering van een levende *Salmonella* Typhimurium vaccinstam opgewekt kan worden door injectie van dexamethasone. Uit onze resultaten bleek dat een levende *Salmonella* Typhimurium vaccinstam inderdaad in hogere mate vermeerdert onder invloed van stress. Vervolgens, werd de rol van *scsA, scsB, scsC*, *scsD* en het volledige *scs* locus in stress gerelateerde verhoogde vermeerdering van *Salmonella* Typhimurium nagegaan. Uit een voorgaande studie bleek namelijk dat stress-gerelateerde herexcretie van *Salmonella* Typhimurium in varkens gerelateerd is aan de aanwezigheid van het hormoon cortisol en dat dit hormoon een verhoogde intracellulaire proliferatie van *Salmonella* in varkensmacrofagen induceert. Uit dezelfde studie bleek dat *scsA* een belangrijke rol speelt in deze cortisol-afhankelijke verhoogde intracellulaire groei van *Salmonella*. In een DBA/2J muismodel, door ons op punt gesteld, werd het vermogen nagegaan van *Salmonella* Typhimurium en de verschillende deletiemutanten (*scsA, scsB, scsC*, *scsD* en het volledige *scs* locus) om onder invloed van stress (nagebootst door een subcutane injectie met dexamethasone) in hogere mate te vermeerderen. Daaruit bleek dat het uitschakelen van het *scsA* gen of de volledige *scs* locus en niet *scsB*, *scsC* of *scsD*, stress gerelateerde verhoogde excretie van *Salmonella* Typhimurium inhibeert. Deze resultaten suggereren dat het verwijderen van *scsA* of de volledige *scs* locus in *Salmonella*, verhoogde

vermeerdering van *Salmonella* vaccinstammen onder invloed van stress zou kunnen verhinderen.

Infectie van varkens met *Salmonella* Typhimurium resulteert vaak in het ontstaan van drager dieren die *Salmonella* tijdens perioden van stress, zoals bij transport naar het slachthuis, terug in grote aantallen gaan uitscheiden. Her-excretie van *Salmonella* is gelinkt aan een verhoogde cortisol concentratie in het varkensserum. Een intramusculaire injectie van dexamethasone veroorzaakt her-excretie van *Salmonella* Typhimurium in varkens. In deze studie werd nagegaan of een commercieel beschikbaar levend vaccin (*Salmoporc*®) deze stress gerelateerde *Salmonella* her-excretie kan tegengaan. Daarvoor werden tweeëntwintig biggen gebruikt, waarvan er elf gevaccineerd werden. Vervolgens werden alle dieren geïnfecteerd met *Salmonella* Typhimurium. Drieëntwintig dagen na infectie en 24 uur voor euthanasie werden alle dieren intramusculair geïnjecteerd met dexamethasone. Gevaccineerde dieren vertoonden significant lagere aantallen *Salmonella* Typhimurium kiemen in stalen van het ileum, ileum inhoud, caecum inhoud en het colon, in vergelijking met niet-gevaccineerde biggen. Bovendien werden er meer *Salmonella* positieve tonsillen en colonstalen aangetroffen bij de niet-gevaccineerde dieren. We kunnen dus besluiten dat vaccinatie met een commercieel beschikbaar levend vaccin (*Salmoporc*®) het aantal *Salmonella* Typhimurium kiemen, uitgescheiden door getresseerde biggen, reduceert maar niet elimineert. Bijgevolg is verdere optimalisatie van levende vaccinstammen aangewezen.

Als conclusie kan gesteld worden dat vaccinatie met een DIVA merkerstam kan bijdragen tot een reductie van het aantal met *Salmonella* Typhimurium geïnfecteerde varkens, en desgevallend ook tot een beperking van het aantal humane besmettingen. Niettegenstaande stemmen opgaan om de monitoring van *Salmonella* niet meer te baseren op serologie en om over te schakelen op een bacteriologische monitoring op populatieniveau van slachtvarkens, wordt serosurveillance in de meeste Europese landen nog steeds gebruikt. Bijgevolg, zijn DIVA merkerstammen, die serologisch onderscheid toelaten tussen geïnfecteerde en gevaccineerde dieren belangrijk. We benadrukken echter dat verder onderzoek noodzakelijk is om de efficientie en de werkzaamheid van merkerstammen in het veld te garanderen.

Curriculum vitae and bibliography

Curriculum vitae

Bregje Leyman werd geboren op 20 mei 1982 in Gent. Na het beëindigen van haar studies algemeen secundair onderwijs, richting Wetenschappen-Wiskunde aan het Sint-Franciscusinstituut te Melle, begon zij in 2000 aan haar studies Biologie aan de Universiteit Gent. In 2005 studeerde zij af als bioloog, optie dierkunde, met onderscheiding. In 2006 behaalde zij aan de Faculteit Psychologie en Pedagogische wetenschappen (UGent) het diploma van Academische Initiële Lerarenopleiding met onderscheiding. Van september 2006 tot september 2008 werkte zij als leerkracht fysica, chemie en wiskunde in de Stedelijke Academie voor Schone Kunsten te Brugge. In oktober 2008 startte zij haar doctoraatsonderzoek aan de Faculteit Diergeneeskunde (UGent) bij de vakgroep Pathologie, Bacteriologie en Pluimveeziekten, getiteld: "Optimizing vaccination of pigs against *Salmonella* Typhimurium" dat werd gefinancierd door de 'Federale Overheidsdienst Volksgezondheid, Veiligheid van de Voedselketen en Leefmilieu'. In november 2008 behaalde zij het certificaat van natuurgids aan het Centrum voor Natuur-en Milieueducatie (CVN). Bregje Leyman is auteur en medeauteur van meerdere wetenschappelijke publicaties in internationale tijdschriften. Zij nam deel aan internationale congressen en presenteerde resultaten van haar onderzoek in de vorm van posters en presentaties.

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