Comparison of host cell invasion and host innate immune responses to *Salmonella enterica* serovars Choleraesuis and Typhimurium infections and the effect of the *Salmonella* virulence effector protein AvrA in human and swine cells

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Zusammenfassung

Salmonella ist eins der wichtigsten zoonotischen Pathogene weltweit, das Wirbeltier-Wirte mit Folgen von subklinischen Infektionen bis hin zu lebensgefährlichen systemischen Erkrankungen infizieren kann. *Salmonella* Serovare variieren sowohl in ihrem Wirtsspektrum als auch in der von ihnen verursachten Pathogenese. Der Serovar *Salmonella enterica* Typhimurium kann ein breites Wirtsspektrum infizieren, darunter auch Menschen sowie Lebensmittel liefernde Tiere. Außerdem neigt er dazu akute, aber selbst-limitierte Enteritis zu verursachen. Der Serovar *Salmonella enterica* Choleraesuis ist an das Schwein adaptiert, kann aber auch systemische Infektionen beim Menschen verursachen.

Es wird davon ausgegangen, dass die genetischen Unterschiede zwischen den Serovaren, insbesondere innerhalb der *Salmonella* Pathogenitätsinseln eine Rolle bei der Begrenzung des Wirtsspektrums spielen. Es wurde berichtet, dass das innerhalb von SPI-1 kodierte *Salmonella* Effektor Protein AvrA in die Regulierung der Wirts-Immunantwort involviert ist. Das *avrA* Gen ist nicht innerhalb des *S.* Choleraesuis Genoms enthalten, im Genom von *S.* Typhimurium hingegen schon. Frühere Studien haben nahegelegt, dass das Fehlen des *avrA* Gens mit systemischen Formen einer *Salmonella* Infektionen assoziiert ist.

Um die möglichen Effekte der An- bzw. Abwesenheit des *avrA* Gens in *S.* Typhimurium und *S.* Choleraesuis zu analysieren, wurden ein *avrA+* *S.* Choleraesuis Stamm und ein Δ*avrA* *S.* Typhimurium Mutantenstamm konstruiert, mit denen anschließend epitheliale und Makrophagen-ähnliche Zelllinien humanen und porzinen Ursprungs infiziert wurden. Dabei wurden die Effekte des *avrA* Gens auf die Invasion, die intrazelluläre Persistenz und die Immunantwort analisiert.

In dieser Studie war der *S.* Typhimurium SL1344 Stamm invasiver, zeigte aber verglichen mit dem *S.* Choleraesuis SARB4 Stamm eine langsamere intrazelluläre Persistenz. Ferner war die Immunantwort auf die *S.* Choleraesuis Infektion bei porzinen wie auch bei humanen Zellen in der frühen Infektionsphase niedriger als bei einer *S.* Typhimurium Infektion. Dies änderte sich allerdings im Infektionsverlauf, insbes. bei den humanen Makrophagen-ähnlichen Zellen. Die spätere Wirts-Immunantwort gegen die Infektion mit *S.* Choleraesuis könnte mit seiner Wirtsadaptation und/oder seinem Infektionsergebnis zusammen hängen.

Trotz der komplexen Regulierung des *avrA* Gens konnte das Gen in dieser Studie erfolgreich in *S.* Choleraesuis geklont und exprimiert werden. Die Anwesenheit des *avrA* Gens hatte keinen Effekt auf die Invasion und die intrazelluläre Persistenz. Die möglichen Effekte einer Anwesenheit des *avrA* Gens auf die Regulierung der Wirts-Immunantwort bei einer *Salmonella* Infektion mit beiden Serovaren zeigte sich nur in humanen Zellen.

Abschließend lässt sich sagen, dass die Anwesenheit des *avrA* Gens in *Salmonella* einen möglichen Effekt auf die Immunantwort in humanen Zelllinien hat. Zusammenfassend lässt sich folgern, dass die beobachteten Unterschiede bezüglich der Invasion, intrazellulärer Persistenz und Wirts-Immunantwort zwischen *S.* Choleraesuis und *S*. Typhimurium durch die Abwesenheit des *avrA* Gens in *S*. Choleraesuis nich komplett erklärt werden können. Dies weist darauf hin, dass die Adaptation dieses Serovars an das Schwein sowie das häufige Vorkommen systemischer Infektionen beim Menschen von anderen Faktoren abhängt.

Abstract

Salmonella is one of the most important zoonotic pathogens worldwide, can infect and colonize vertebrate hosts with outcomes ranging from sub-clinical infections to life-threating systemic disease. *Salmonella* serovars differ in their host specificity and in the pathogenesis they cause. *Salmonella enterica* serovar Typhimurium can infect a wide range of hosts, including humans and food producing animals and it tends to cause acute but self-limited enteritis. *Salmonella enterica* serovar Choleraesuis is adapted to swine but can also cause systemic infection in humans.

Genetic differences between the serovars, especially within *Salmonella* pathogenicity islands have been proposed to play a role in conferring host specificity. The *Salmonella* effector protein AvrA, encoded within SPI-1 has been reported to modulate the host immune response. The *avrA* gene is not present within the genome of *S.* Choleraesuis but is present in *S*. Typhimurium. Prior studies have suggested that the absence of the *avrA* gene could be associated with systemic forms of *Salmonella* infections. In order to analyze the possible effects of the presence or absence of the *avrA* gene in *S.* Typhimurium and *S.* Choleraesuis, respectively, an *avrA*+ *S.* Choleraesuis strain and an Δ*avrA S.* Typhimurium mutant strain were constructed and used to infect epithelial and macrophage cell lines from human and porcine origin. These strains were used to analyze the effects of *avrA* gene on invasion, intracellular persistence and host immune response.

In this study *S.* Choleraesuis SARB4 strain was less invasive but showed a higher intracellular persistence compared to *S*. Typhimurium SL1344 strain. Furthermore, the immune response to *S.* Choleraesuis infection in porcine and human cells was lower in the early phase of infection (2 - 4 h. p.i.) compared with the response to *S*. Typhimurium, but this changed during the course of infection, particularly in human macrophages where the NFκB activation 24 hours p.i. were higher by *S*. Choleraesuis infection. This later response of the host immune system against infection with *S*. Choleraesuis could be related with the host adaptation in this *Salmonella* serovar and the higher rate of systemic infection.

Despite the complex regulation of the *avrA* gene, in the present study, this gene was successfully cloned into *S*. Choleraesuis and was successfully transcribed, allowing direct comparison of the influence of the presence or absence of the *avrA* gene in both serovars.

The presence of the *avrA* gene in both *Salmonella* serovars did not affect the invasion rate and the intracellular persistence in the eukaryotic cells. The possible effects of the presence or absence of the *avrA* gene in *Salmonella* serovars Choleraesuis and Typhimurium in modulation of the host immune response against *Salmonella* infection were only observed in human cell lines. The presence of the *avrA* gene in both *Salmonella* serovars had no effect on the immune response in the porcine cell lines tested.

Finally, the presence of the *avrA* gene in both *Salmonella* serovars appeared to have an effect in the immune response to infection in human cells. Despite this, it can be concluded that the differences observed in invasion, intracellular persistence and host immune response between *S.* Choleraesuis and *S*. Typhimurium are at least not totally explained by the absence of the *avrA* gene in *S*. Choleraesuis, indicating that the host-adaptation of this serovar for swine, and the high rates of systemic infections in humans is dependent upon other factors.

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ABBREVIATIONS

AP-1	Activator protein-1
ATCC	American Type Culture Collection
AvrA	*Salmonella* effector protein (avirulence factor)
BfR	Bundesinstitut für Risikobewertung
bp	Base pairs
CDC	Center for Disease Control and Prevention
cDNA	Complementary DNA
cfu	Colony forming units
CT	Threshold cycle
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EM	Electron microscopy
ERK	Extracellular signal regulating kinase
FBS	Fetal bovine serum
HIV-1	Human immunodeficiency virus type 1
IFNγ	Interferon γ
IL	Interleukin
IL-1β	Interleukin 1β
IL-8 or CXCL-8	Interleukin 8
IQR	Interquartile Range

IRF3	Interferon regulatory 3
IκB	Inhibitor of NFκB
JNK or SAPK	c-jun-NH2-terminal kinase or stress activated protein kinase
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
MAPK	Mitogen-activated protein kinase
M-cells	Specialized cells associated with the Peyer's patches
MKK	MAP kinase kinase
MOI	Multiplicity of infection
mRNA	Messenger Ribonucleic acid
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear transcription factor kappa B
NLRs	NOD-like receptors
OD	Optical density
P	Statistical significance level
p.i.	Post infection
p38	p38 MAPK
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate Buffered Saline
PMN	Polymorphonuclear leukocytes
PRR	Pattern recognition receptor
qRT-PCR	Quantitative reverse transcription PCR
RKI	Robert-Koch Institut
RNA	Ribonucleic Acid

S. Choleraesuis	*Salmonella enterica* serovar Choleraesuis
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
SCV	*Salmonella*-containing vacuole
Sif	*Salmonella*-induced filament
SPIs	*Salmonella* pathogenicity islands
SPSS	Statistical Package for Social Science
SseL	*Salmonella* secreted effector L
T3SS	Type III secretion system
T3SS1	Type III secretion system-1 encoded within SPI-1
T3SS2	Type III secretion system-2 encoded within SPI-2
TIR	Toll/interleukin-1 receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
VSV	Vesicular stomatitis virus
WHO	World Health Organization

1 INTRODUCTION

1.1 *Salmonella*

Salmonella is a Gram-negative, bacterium that can infect and colonize vertebrate hosts with outcomes ranging from sub-clinical infections to life-threating systemic disease (Lahiri *et al*., 2010; Jones *et al*., 2008a). *Salmonella* infection or salmonellosis constitutes a major public health burden and represents a significant cost to society in many countries. In the United States of America an estimated 1 million non-typhoidal *Salmonella* foodborne illness acquired in the same country resulting in 19336 hospitalizations and 378 deaths annually (Scallan *et al*., 2011). The total cost associated with *Salmonella* is estimated at US$ 3 billion yearly in the United States (WHO Fact sheet N°139). Worldwide infections with non-typhoidal *Salmonella* strains cause approximately 93.8 million gastroenteritis cases and 155000 deaths each year (Majowicz *et al*., 2010). *Salmonella* infections are principally acquired via oral ingestion of contaminated water or food (Haraga *et al.,* 2008). The quantity of *Salmonella* required causing disease, ranges from 30 to 10^9 organisms, depending on host conditions and the source of contamination (Foley and Lynne, 2008).

The genus *Salmonella* is currently classified into two species, *Salmonella enterica* and *Salmonella bongori. S. enterica* is further divided into six subspecies: *enterica, salamae, arizonae, diarizonae, houtenae, and indica* (Grimont and Weill, 2007). The serovars with clinical relevance are predominantly classified into *Salmonella enterica* subsp. *enterica*. More than 2600 antigenically distinct serovars have been identified within this subspecies. The *Salmonella* serovars are classified based on reactivity of antisera to somatic lipopolysaccharide (O) and flagellar (H) antigens (Grimont and Weill, 2007; CDC, 2011-National *Salmonella* Surveillance Overview). *Salmonella* serovars differ in both their host specificity and in the pathogenesis they cause in different species. The *Salmonella enterica* serovars Enteritidis and Typhimurium can infect a wide range of hosts, including humans and food producing animals and they tend to cause acute but self-limited enteritis. The host-restricted serovars such as Typhi and Gallinarum only infect a single species, humans and poultry, respectively. The host-adapted serovars such as Choleraesuis and Dublin are principally associated with disease in one species but can also infect a limited number of other species (Uzzau *et al*., 2000; Jones *et al*., 2008a; Jacobsen *et al*., 2011).

Another interesting characteristic of *Salmonella* serovars is that the same serovar can cause a different pathology depending on the host type and the host conditions such as age, genetic background and immune status (Coburn *et al*., 2007; de Jong *et al*., 2012). For example, *S. enterica* serovar Typhimurium typically causes enteritis however, in mouse it leads to systemic disease; *S. enterica* serovar Choleraesuis frequently causes enteritis in

adult pigs, but in young pigs and humans causes higher rate of systemic disease (Chiu *et al*., 2004; Jones *et al*., 2008a). The outcome of the *Salmonella* infection varies not only between the serovars and the host species, there are also differences among circulating strains of the same serovar (Coburn *et al*., 2007; Andrews-Polymenis *et al*., 2010).

Salmonella also has the ability to colonize the intestine in the absence of clinical signs in humans and animals and to develop a carrier state. In this carrier state, the hosts can excrete the bacteria in feces without clinical signs, and *Salmonella* can persist in its host for years or a life time, at this time, the bacteria can also be intermittent excreted in response to stress (Stevens *et al*., 2009). The carrier state allows *Salmonella* to remain in the environment and easily to spread between hosts (Stevens *et al*., 2009; Monack, 2012).

Salmonella is one of the most important zoonotic pathogens worldwide; in the European Union, it is the second most frequently reported zoonotic disease in humans, following *Campylobacter* spp. infections (EFSA and ECDC, 2013-Summary Report on Zoonoses in 2011). In the European Union and in the United States, human salmonellosis is frequently caused by the *Salmonella* serovars Enteritidis and Typhimurium (CDC, 2011-National *Salmonella* Surveillance Annual Data Summary 2009; EFSA and ECDC, 2013-Summary Report on Zoonoses in 2011). The human cases of infection with *Salmonella* serovar Enteritidis are principally related to consumption of contaminated eggs and poultry meat whereas the cases of infection with the serovar Typhimurium are mostly associated with consumption of contaminated pig, poultry and bovine meat products. *Salmonella enterica* serovar Typhimurium is a highly prevalent serovar in pig and pig meat in the European Union, making pork products an important vehicle for foodborne disease by *Salmonella* serovar Typhimurium (EFSA and ECDC, 2013-Summary Report on Zoonoses in 2011).

In the European Union, *Salmonella* serovar Choleraesuis is one of the ten most frequent reported serovar in pigs (EFSA and ECDC, 2013-Summary Report on Zoonoses in 2011). *Salmonella* serovar Choleraesuis is a highly invasive serovar, causing enterocolitis, pneumonia and septicemia in swine infections (Chiu *et al*., 2005; Shinkai *et al*., 2011), whereas serovar Typhimurium usually causes self-limited enterocolitis (Majowicz *et al*., 2010). *Salmonella* serovar Choleraesuis can also cause systemic infection in humans with little or no intestinal involvement (Chiu *et al*., 2004; Jones *et al*., 2008a).

The number and type of hosts that the different *Salmonella* serovars infect and the corresponding pathogenicity and/or pathologies likely depend on both bacterial and host factors. Genetic differences between the serovars, especially within *Salmonella* pathogenicity islands, virulence plasmid, pseudogenes, fimbrial operons and lysogenic phages have all been proposed to play a role in conferring host specificity and host-range restriction (Edwards *et al*., 2002; Eswarappa *et al.,* 2008; Suez *et al*., 2013;). Likewise, the genetic background of the host, their immune status and the particular immune response to

each *Salmonella* serovar plays an important role in the host specificity of the *Salmonella* serovars (Coburn *et al*., 2007). Not only differences in the presence of virulence genes among *Salmonella* serovars have been associated with the difference in host range and outcome of infection (Eswarappa *et al*., 2008; Soyer *et al*., 2009; Jacobsen *et al*., 2011) even also the differential regulation and expression of these genes among and intra serovars (Streckel *et al*., 2004; Rollenhagen and Bumann, 2006; Encheva *et al*., 2007; Patterson *et al*., 2012). This differential gene expression is in some cases dependent of *Salmonella*-specific regulation (Streckel *et al*., 2004; Ben Barak *et al*., 2006; Kerrinnes *et al*., 2009).

1.1.1 *Salmonella* pathogenicity islands

Pathogenicity islands are clusters of virulence genes on the chromosome of bacteria that have been acquired *via* horizontal gene transfer (Hall, 2010; Jacobsen *et al*., 2011). In *Salmonella*, these virulence genes are directly involved in manipulating the host system and may be at least partially responsible for the host specificity of different *Salmonella enterica* serovars (Hensel, 2004; Eswarappa *et al.,* 2008). Currently, at least 12 *Salmonella* pathogenicity islands (SPIs) have been described in the genome sequences of *Salmonella* serovars, many of these islands are found in all genomes within *Salmonella* subspecies *enterica*, with the exceptions of SPI-6 and SPI-7, which have only been found in *S. enterica* serovar Typhi (Jacobsen *et al*., 2011). Many studies have been carried out in order to analyze the genetic variation within SPIs, between different *Salmonella* serovars and their possible implication in the difference observed in host range and pathogenicity between these serovars (Edwards *et al*., 2002; Rollenhagen and Bumann, 2006; Eswarappa *et al*., 2008; Soyer *et al*., 2009; Suez *et al*., 2013).

1.1.2 *Salmonella* Type III Secretion Systems

The Type III secretion system (T3SS) is a specialized apparatus whose principal function is the delivery of bacterial proteins known as effectors into the eukaryotic cells. This secretion system is present in both animal and plant bacterial pathogens (Galán, 2001; Galán and Wolf-Watz, 2006; Ramos-Morales, 2012). The T3SS is evolutionarily related to the flagellar export system and comprises more than 20 different proteins (Enninga and Rosenshine, 2009; Buettner, 2012). This apparatus is composed of two rings one in the inner bacterial membrane composed of PrgH and PrgK protein subunits and the other in the outer bacterial membrane composed of InvG and InvH (Galán, 2001; Foley and Lynne, 2008). The cytoplasmic export machinery is localized adjacent to the inner ring and is composed of the InvA, InvC, SpaP, SpaQ, SpaR, and SpaS proteins (Galán, 2001; Foley

and Lynne, 2008). The externally localized needle-like structure protrudes approximately 20-50 nanometers from the bacterial surface (Schraidt *et al*., 2010) and is composed of the PrgI protein (Foley and Lynne, 2008). The effector proteins that are introduced (injected) into the host cell via this secretion system allow the bacteria to invade and colonize host tissues and are also essential for bacterial pathogenesis. These proteins have the capacity to modulate or alter host cellular functions such as cytoskeletal architecture, vesicle traffic, signal transduction and immune response (Galán, 2001; Figueira and Holden, 2012; Ramos-Morales, 2012).

Salmonella serovars possess two T3SS, encoded within the *Salmonella* pathogenesis islands 1 and 2. The SPI-1 encoded Type III secretion system-1 (T3SS1) (Figure 1) transfers *Salmonella* effector proteins from the bacterial cytosol through the host-cell plasma membrane into the host cytosol and is important for the invasion of non-phagocytic cells (Stevens *et al*., 2009; Bulmer *et al*., 2012). The Type III secretion system-2 (T3SS2) encoded within SPI-2 translocates *Salmonella* effector proteins across the membrane of the *Salmonella*-containing vacuole (SCV) into the host-cell cytoplasm and is mainly required for the intracellular survival and replication of *Salmonella* in epithelial cells and macrophages (Haraga *et al.,* 2008; Figueira and Holden, 2012).

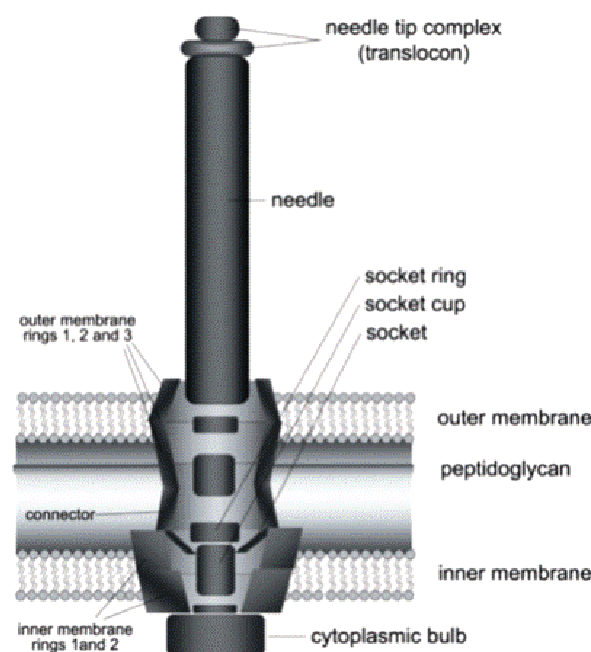

Figure 1: Imaging the type III secretion system (T3SS). Schematics of the T3SS based on data from single-particle analysis of purified needle complexes that were imaged by EM.

(Enninga and Rosenshine, 2009) License Number: 3150981294099.

1.1.3 Intestinal invasion by *Salmonella*

After oral ingestion, *Salmonella* traverses the intestinal mucous layer and contacts with the intestinal epithelium. *Salmonella* can be transport to the basolateral side of the intestinal epithelium by trancytosis mediated by the specialized M-cells associated with the Peyer's patches. After that, *Salmonella* is taken by the different phagocytes (macrophages, dendritic cells, and polymorphonuclear cells) within the lamina propia (Broz *et al*., 2012; Velge *et al*., 2012). This invasion pathway is independent of *Salmonella* invasion genes (Murray and Lee, 2000; Velge *et al*., 2012). *Salmonella* has also the ability to invade non-phagocytic cells such as intestinal enterocytes by bacterial-mediated endocytosis. After initial contact with the host cells, *Salmonella* is internalized through a mechanism mediated by the effector proteins translocated into the host cells via the type III secretion system-1 (Figure 2) (Srikanth *et al*., 2011). The *Salmonella* effector proteins, SopE, SopE2 and SopB promote the activation of the host-cell Rho GTPases: Cdc42, RhoG and Rac-1, which subsequently turn on signal transduction pathways that promote actin cytoskeletal rearrangements and membrane ruffling that leads to bacterial internalization by macropinocytosis (Haraga *et al*., 2008; Srikanth *et al*., 2011; Velge *et al*., 2012).

The internalization of *Salmonella* into the host cell is also promoted by the effector proteins SipA and Sip C. SipC is a component of the bacterial translocon and is anchored in the host membrane, modulating the actin dynamic by nucleating and bundling of actin. SipA mediates actin filament polymerization, decreasing the critical monomer concentration required for polymerization and increasing the stability of actin filaments (Haraga *et al.,* 2008; Srikanth *et al*., 2011).

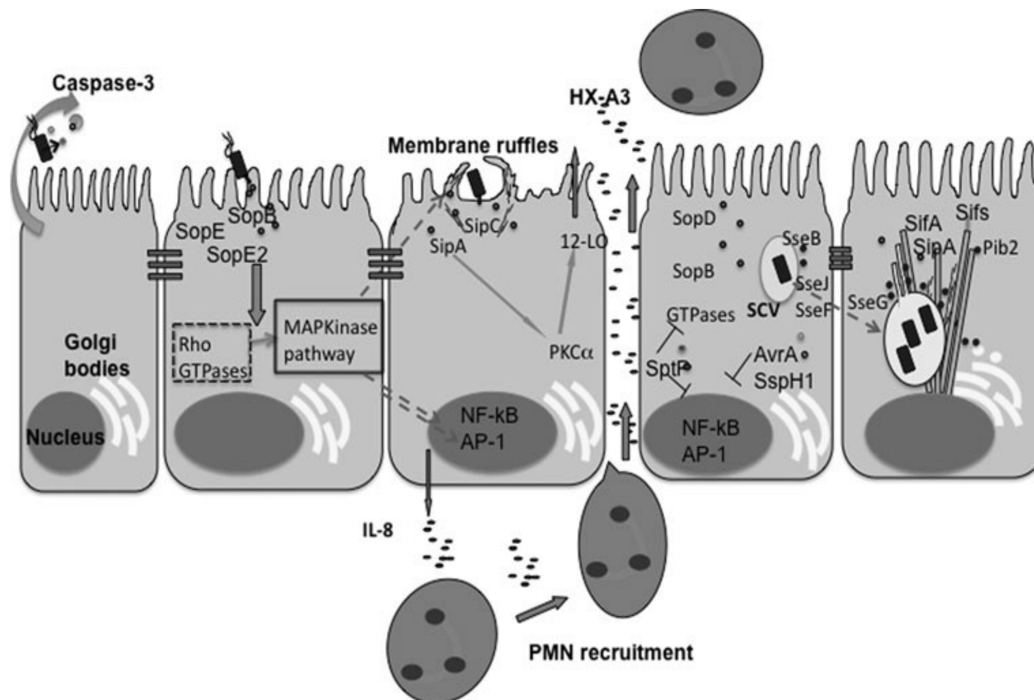

Figure 2: ***Salmonella* effector proteins during Infection.** For a description and function(s) of the indicated effector proteins see the text.

Springer and the original publisher (Cellular and Molecular Life Sciences, 68, 2011, 3690, *Salmonella* effector proteins and host-cell responses, C. V. Srikanth, Regino Mercado-Lubo, Kelly Hallstrom and Beth A. McCormick, Figure 1, original copyright notice). Reproduced with permission of Springer Science and Business Media. License Number: 313114112078.

1.1.4 Intracellular survival and replication of *Salmonella*

Shortly after *Salmonella* invasion, the T3SS1 effector protein SptP, reverses the cytoskeletal rearrangement, which had allowed the entry of *Salmonella*, returning cell architecture to normal (Velge *et al*., 2012). Once internalized into the host cell, *Salmonella* remains in a host membrane-bound compartment called the *Salmonella*-containing vacuole (SCV). This vacuole begins a maturation process that allows *Salmonella* to survive and to replicate within many cell types, including epithelial cells and macrophages (Haraga *et al.,* 2008; Srikanth *et al*., 2011). From within the SCV, *Salmonella* translocates additional effector proteins across this vacuolar membrane into the host cell cytoplasm through the functions of the second Type 3 Secretion System encoded within SPI-2 (T3SS2). These T3SS2-translocated effector proteins, in collaboration with T3SS1-effector proteins, allow the intracellular survival and replication of *Salmonella* within the SCV (Figure 2) (Agbor and McComick, 2011; Srikanth *et al*., 2011). The SP1-1 effector proteins SopB and SipA persist in the host cells after invasion and have a crucial role in the formation, perinuclear localization and maturation of the SCV (Agbor and McComick, 2011; Malik-Kale *et al*., 2011).

The T3SS2 translocated effectors most important for *Salmonella* intracellular survival, include SifA, SseJ, SseF, SseG, SopD2 and PipB2, as virulence defects are seen in strains with mutations in these genes (Srikanth *et al*., 2011). The maturation of the SCV necessary for *Salmonella* intracellular replication includes the formation of *Salmonella*-induced filaments (Sifs). Sifs are long filamentous membrane structures, extending from the SCV surface. These structures are essential for positioning the SCV close to the Golgi apparatus and the perinuclear region of the host cell (Srikanth *et al*., 2011). The formation of the *Salmonella*-induced filaments is induced by the effector protein SifA together with PipB2 and SopD. The effector proteins SseF and SseG are involved in the bundling of microtubules adjacent to the SCV, and direct Golgi-derived vesicle traffic toward the SCV (Haraga *et al.,* 2008). Together, these effector proteins permit to the SCV to localize close to the Golgi apparatus and acquire membrane constituents and nutrients necessary for the *Salmonella* survival and replication into the host cells (Haraga *et al.,* 2008; Agbor and McComick, 2011; Srikanth *et al*., 2011).

1.1.5 Intestinal inflammation mediated by *Salmonella* effector proteins

Salmonella effector proteins SopE, SopE2 and SopB induce the activation of the Rho GTPases, such as Cdc42 which initiates the activation of mitogen-activated protein kinase (MAPK) pathways, including ERK, JNK and p38 pathways, which subsequently activate the transcription factors AP-1 and NFκB (Bruno *et al*., 2009; Srikanth *et al*., 2011). These transcription factors initiate the production of pro-inflammatory cytokines such as IL-8, which stimulate the recruitment of polymorphonuclear leukocytes (PMN). Simultaneously, SipA induces in epithelial cells the apical release of hepoxilin A3, which promotes the migration of PMN across the epithelium into the intestinal lumen (McGhie *et al*., 2009), causing damage to the epithelial layer (Srikanth *et al*., 2011). The effector protein SipB contributes to the inflammation caused by *Salmonella* infection, by binding and activating the host protease Caspase-1, which leads to the production of the pro-inflammatory cytokines IL-1β and IL-18 (Haraga *et al.,* 2008). The effector proteins SopB, SopE, SopE2 and SipA are involved in the disruption of the tight junctions between the intestinal cells and alter the integrity of the epithelial monolayer causing the increase of fluid secretion into the intestinal tract (Layton and Galyov, 2007). SopB also promotes the cellular secretion of chloride and the fluid flux (Haraga *et al.,* 2008). In this manner *Salmonella*, through its effector proteins translocated by the T3SS1, produce damage to the epithelial monolayer, increase of fluid secretion and subsequent diarrhea. In addition to fluid loss, diarrhea also results in removal of *Salmonella* from the intestine, which is suggested to be a mechanism used by this pathogen in order to gain access to the environment and disseminate throughout the population (Layton and Galyov, 2007).

1.2 Host immune response to *Salmonella* infection

1.2.1 Recognition of *Salmonella* by the host immune system

Salmonella is recognized by the host innate immune system through pattern recognition receptors (PRRs) which bind to conserved molecules from microorganisms, called pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycans, lipoproteins and unmethylated bacterial DNA (Li and Verma, 2002; Broz *et al*., 2012). The best-known PRRs are the Toll-like receptors (TLRs), a group of transmembrane glycoproteins generally composed of domains containing variable numbers of leucine-rich-repeat (LRR) motifs, that recognize extracellular and vacuolar pathogen-associated molecular patterns, a single transmembrane domain and a cytoplasmic signaling region composed mainly of a Toll/interleukin-1 receptor (TIR) domain, which activates intracellular signaling pathways leading to induction of the host innate immune response in order to eliminate pathogens (Akira and Takeda, 2004; Shinkai *et al*., 2011; Song and Lee, 2012).

The Toll gene was first identified in Drosophila (Hashimoto *et al*., 1988) and associated with signaling pathways and immune response in Drosophila (Lemaitre *et al*., 1996). Shortly after that, the Toll homolog gene TLR4 was found in humans (Medzhitov *et al*., 1997; Uenishi and Shinkai, 2009). Currently, 13 TLR receptors that recognize different pathogen molecular patterns have been identified in mammalian cells (Kumar *et al*., 2011; Lee *et al*., 2012). The Toll-like receptors from TLR1 to TRL10 have been identified in humans and also in swine (Uenishi and Shinkai, 2009; Song and Lee, 2012). Twelve of the 13 TLRs have been identified in mouse TLR1 to TLR9 and TLR11 to TLR13 (Kang and Lee, 2011; Lee *et al*., 2012). The TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface whereas TLR3, TLR7, TLR8 and TLR9 are expressed on the membrane of intracellular vacuoles in order to recognize PAMPs that have been engulfed by the cell (Uenishi and Shinkai, 2009; Kumar *et al*., 2011; Song and Lee, 2012).

The Toll-like receptors 1, 2, 4-7 and 9 are primarily dedicated to the recognition of various bacterial components. TLR4 senses LPS which is a major cell wall component of gram-negative bacteria. TLR2 is responsible for the recognition of peptidoglycan a major component of Gram-positive bacteria; this receptor also senses diacylated lipopeptides from Gram-positive bacteria, in association with TLR6. TLR2 together with TLR1 senses triacylated lipopeptides from Gram-negative bacteria (Kawai and Akira, 2010). TLR5 senses the flagellin protein expressed by flagellated bacteria (Yoon *et al*., 2012) and the TLR9 senses bacterial genomic DNA rich in unmethylated CpG (Kumar *et al*., 2009a,b; Kumar *et al*., 2011). Bacterial RNA produced in the lysosomal compartment is likely to act as a PAMP for the receptor TLR7. The Toll-like receptors differ from each other not only in

the molecular patterns they recognize, but also in the host response that they induce. For example, the recognition of bacterial components by TLR1, TLR2, TLR4, TLR5 and TLR6 primarily induce the production of inflammatory cytokines, whereas the recognition by TLR7 and TLR9 mainly induce a type I interferon response (Kumar *et al*., 2009a,b; Kumar *et al*., 2011).

Among TLR, the Toll-like (TIR) domain, which is responsible for the intracellular signal cascade after the recognition of the pathogen components, is well conserved, also between different species. This TLR signaling is mainly mediated via the recruitment of the myeloid differentiation primary response gene 88 (MyD88), which is a TIR domain-containing adaptor protein. This association between the TIR domain and MyD88 leads to the activation of transcription factors such as NFκB, the interferon regulatory 3 (IRF3) and MAP kinases to induce the production of pro-inflammatory cytokines and type I interferons. TLR3 does not recruit MyD88, instead it recruits the TIR domain-containing adaptor molecule TRIF and initiates signaling to activate the transcription factor IRF3 and induce production of type I interferon (Kawai and Akira, 2010; Kumar *et al*., 2011).

Salmonella has been reported to be principally recognized by the host immune system through TLR4, TLR2, TLR5 and TLR9. TLR4, which recognizes lipopolysaccharide, has been suggested to be the dominant receptor in the detection of *Salmonella* by the host immune system (Weiss *et al*., 2004; Balaram *et al*., 2009). Flagellin is also a major pro-inflammatory determinant during intestinal *Salmonella* infections (Winter *et al*., 2009; Broz *et al*., 2012). *Salmonella*, but not commensal bacteria, is able to transport flagellin to the basolateral membrane and activate nuclear factor NFκB expression and interleukin IL-8 production via TLR5 (Gewirtz *et al*., 2001; Srinivasan and McSorley, 2006).

The intracellular NOD-like receptors (NLRs) are also important in the detection of *Salmonella* by the immune system. This receptor family comprises 23 members in humans and senses a wide range of ligands within the cytoplasm of cells. *Salmonella* is mainly recognized by the NOD-like receptors NOD2 and NLRC4 (IPAF, CLAN). After recognition of pathogen molecular patterns, the NOD-like receptors either activate NFκB or MAP kinases to induce the production of inflammatory cytokines, including IL-1β, IL-18, or initiates cell death (Kumar *et al*., 2011).

The activation of the host inflammatory and immune response by *Salmonella* infection is not only mediated by conserved innate immune receptors such as TLRs and NLRs, *Salmonella* also stimulates the activation of the host immune response through specific effector proteins such as SopE, SopE2 and SopB which lead to the activation of host transcription factors such as NFκB and MAP kinases. These transcription factors subsequently initiate the immune response leading to intestinal inflammation. This intestinal inflammation allows *Salmonella* to obtain essential nutrients that do not become

available at this site without the stimulation of the immune response (Stecher *et al*., 2007; Kaiser and Hardt, 2011). Thus, intestinal inflammation is crucial for *Salmonella*'s ability to growth in the intestinal tract. Furthermore, TLR-mediated *Salmonella* recognition by the host immune system have been described to be necessary for *Salmonella* growth into macrophages since the TLR-mediated signaling is required for acidification of the *Salmonella*-containing phagosome and the induction of SPI-2 genes (Arpaia *et al*., 2011; Keestra and Bäumler, 2011). The acidification of the *Salmonella*-containing phagosome is required for the assembly of the T3SS-2 system and the translocation of effector proteins into the host-cell cytosol (Beuzón *et al*., 1999). The TLR-mediated signaling seems to be necessary for *Salmonella* replication within host macrophages, which is crucial in *Salmonella* virulence (Arpaia *et al*., 2011; Keestra and Bäumler, 2011).

1.2.2 Activation of host transcription factor NFκB during *Salmonella* infection

Nuclear transcription factor kappa B (NFκB) is a major eukaryotic transcriptional regulator which is involved in both innate and adaptive immune responses and consists of five related transcription factors, RELA (p65), NFκB1 (p50), NFκB2 (p52), c-REL and RELB. Each member of the NFκB family, except for RELB, can form homodimers, as well as heterodimers with one another. The main activated form of NFκB is a heterodimer of the p65 subunit associated with either the p50 or the p52 subunit (Li and Verma, 2002; Vallabhapurapu and Karin, 2009).

After host recognition of *Salmonella* by the pattern recognition receptors such as TLRs and NOD proteins, a signaling cascade is initiated that primarily induces the phosphorylation of the inhibitors of NFκB (IκBs), which is followed by their deubiquitination and subsequent degradation by the proteasome (Ghosh and Hayden, 2008; Broz *et al*., 2012; Lee *et al*., 2012). IκB binds the NFκB dimers, p50 and p65 and sequesters them in the cytoplasm in the absence of stimuli such as bacterial infection. Activation of the PRRs during infection results in the release of IκB from NFκB dimers and allows NFκB to translocate to the cell nucleus where it binds κB sites in the promoter regions of target genes, leading to activation of the transcription of pro-inflammatory proteins in order to control infection (Ghosh and Hayden, 2008). As mentioned above, the activation of the transcription factor NFκB can also be mediated by *Salmonella* effector proteins.

1.2.3 Activation of JNK signaling pathway during *Salmonella* infection

The mitogen activated protein kinases (MAPKs) are important components of signalling pathways that transduce stimuli leading to intracellular responses. The MAP kinases phosphorylate various substrates, including transcription factors, which regulate the expression of specific genes depending on the type of stimulus (Minden and Karin, 1997). There are three major forms of MAPKs: extracellular signal regulating kinase (ERK), c-jun-NH2-terminal kinase (JNK) or stress activated protein kinase (SAPK), and the p38 MAPK (Das and Muniyappa, 2010).

The JNK/SAPK pathway is activated in response to cellular stress such as heat shock, UV irradiation, or inflammatory cytokines (Minden and Karin, 1997). The JNK signaling cascade works through the activation of an initiating kinase such as MAP kinase kinase kinase (MEKK1) that, in turn, phosphorylates the MAP kinase kinase (MKK4/SEK1 or MKK7), which finally activates JNK by phosphorylating serine and threonine residues (Figure 3). The JNKs phosphorylate specific sites on the amino terminal trans-activation domain of transcription factor c-Jun, which is an important part of the AP-1 transcription factor (Weston and Davis, 2007). The JNKs also phosphorylate and activate several other transcription factors such as ATF-2, Elk-1, p53, and c-Myc, as well as non-transcription factors such as Bcl-2, Bcl-xL, paxillin, and MAP2 (Ip and Davis, 1998; Nishina *et al*., 2004).

The activator protein-1 (AP-1) family of transcription factors is composed of homodimers and heterodimers of JUN (v-Jun, c-Jun, JunB, and JunD), Fos (v-Fos, c-Fos, FosB, Fra 1, and Fra2) or activating transcription factor (ATF2, ATF3/LRF1, B-ATF) family proteins (Angel and Karin, 1991; Karin *et al*., 1997). The activation of AP-1 by the JNK pathway induces the transcription of genes involved in inflammation, immune responses, DNA repair, cell survival, cell proliferation and apoptosis (Nishina *et al*., 2003). The phosphorylation of the c-Jun protein by JNK stimulates its transcription activity and the autoregulatory induction of c-jun and subsequently, other AP-1 dependent genes (Karin and Gallagher, 2005). The JNK signaling pathway is activated during *Salmonella* infection in response to inflammatory cytokines and activation of immune receptors such as TLRs and NLRs. The activation of the JNK signaling cascade is also affected by *Salmonella* effector proteins (Weston and Davis, 2007; Du and Galán, 2009).

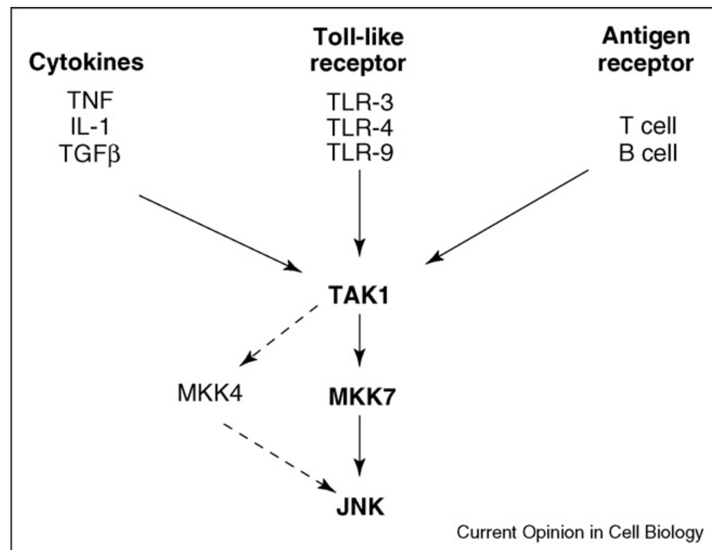

Figure 3: Activation of the JNK signaling Pathway.

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The activation of host transcription factors such as NFκB and AP-1 lead to the production of cytokines and chemokines which are small proteins produce by many types of cells and they have a central role in inflammation and immune response.

The most important cytokines secreted in response to *Salmonella* infection include Interleukin 1β (IL-1β), Interleukin 6 (IL-6), CXCL8 (IL-8), Interleukin 12 (IL-12) and tumor necrosis factor α (TNF-α). The immune response against *Salmonella* Typhimurium is characterized by the release of IL-1β and TNF- α by macrophages. IL-12 and interferon-γ have also been shown to be relevant in the control of *Salmonella* infection by the host immune system (Broz *et al*., 2012; de Jong *et al*., 2012). These cytokines are required for macrophage activation and efficient killing of *Salmonella* in systemic infections (Srikanth *et al*., 2011; de Jong *et al*., 2012). IL-1β activates vascular endothelium, lymphocytes and mediates local tissue destruction increasing access of effector cells to the infection site. IL-8 is also a chemotactic factor that recruits neutrophils, basophils and T cells to the site of infection and is also important in the host immune response to *Salmonella* infection (Vitiello *et al*., 2004; Murphy *et al*., 2008).

1.3 Modulation of the host innate immune response by *Salmonella*

As mentioned above, inflammation is important for *Salmonella*'s growth in the intestinal tract but the activation of the host immune system can also affect cell homeostasis and subsequently, the intracellular replication and surviving of *Salmonella*. Thus, *Salmonella* has acquired the ability to regulate the host immune response in order to counter its own *Salmonella*-induced inflammation, allowing the bacteria to avoid elimination by the host immune system and to maintain its intracellular niche (Sun, 2009; Kaiser and Hardt, 2011). In particular, the *Salmonella* effector proteins AvrA and SseL have been suggested to down-regulate the host inflammatory response through the NFκB pathway (Ye e*t al*., 2007; Le Negrate e*t al*., 2008; Sun, 2009).

1.3.1 *Salmonella* effector protein AvrA

AvrA is a *Salmonella* effector protein encoded within SPI-1 and translocated into the host cell *via* the T3SS1. The AvrA protein was originally identified and characterized by Hardt and Galán (1997). These authors found that AvrA shared sequence similarity with the protein YopJ from *Yersinia pseudotubercolosis* and the avirulence factor AvrRXV from *Xanthomonas campestris*. The AvrA protein was initially not associated with virulence, hence the nominal designation Avr from proteins that mediate avirulence functions (Hardt and Galán, 1997; Wu e*t al*., 2011). The *avrA* gene is present in approximately 80% of the *Salmonella enterica* serovars and encodes a 32 kDa protein, AvrA. Interestingly, this gene is not present in *Salmonella enterica* serovars Choleraesuis, Typhi and Paratyphi A, which are host-restricted or host-adapted serovars and are frequently associated with systemic disease (Hardt and Galán, 1997; Streckel *et al*., 2004).

Although the *avrA* gene is present in the majority of *Salmonella* serovars, the expression of the AvrA protein is highly variable among strains. Ben-Barak and coworkers grouped *Salmonella* strains into four classes according to their expression of the AvrA protein: the class 0 are the strains which do not contain the *avrA* gene, such as serovar Choleraesuis and Typhi; strains with constitutive synthesis of AvrA belong to class 1; class 2 is composed of strains with acid-inducible AvrA synthesis; and class 3 strains are those harbouring the gene but which show no AvrA production. The differences in expression between class 1, 2 and class 3 are not due to differences in their promoter or structural gene sequences, all 3 classes present similar transcription rates (Streckel *et al*., 2004; Ben-Barak *et al*., 2006). This has suggested a *Salmonella*-specific post-transcriptional control which regulates the production of the effector protein AvrA (Ben-Barak *et al*., 2006). In addition, SPI-1 regulators like HilA, InvF and PhoP/PhoQ, that normally regulate the expression of secreted effector proteins have no effect on AvrA (Eichelberg e*t al*., 1999; Ellermeier and

Slauch, 2007; Kerrinnes *et al*., 2009). Kerrinnes and co-workers identified a post-transcriptional activation factor for the synthesis of AvrA, the CsrA protein. *Salmonella* mutants harbouring a deletion in the *csrA* gene do not show translation of the AvrA protein. The CsrA protein binds to target mRNA molecules and alters their stability or blocks the Shine-Dalgarno sequence from recognition by ribosomes to prevent translation and production of the target protein (Wei e*t al*., 2001; Kerrinnes *et al*., 2009). Thus, it is suggested that the CsrA protein can directly regulate the *avrA* mRNA and that this regulation (activation or inhibition) depends on a critical or effective relative concentrations of CsrA to the *avrA*-mRNA concentrations. This active/effective concentration of CsrA can be adjusted by the untranslated RNA CsrB, the second element of the Csr control system, which sequesters or 'neutralizes' the action of CsrA (Liu *et al*., 1997). The inhibition of AvrA production in *Salmonella enterica* strains can therefore be altered or modulated either by the absence of CsrA or CsrB or by their overproduction. The CsrA/CsrB regulatory system therefore controls the translation of AvrA protein without affecting *avrA* transcription. The inhibition of the AvrA production by the CsrA/CsrB regulatory system has been shown to be reverted by increasing of the *avrA*-mRNA copy number in the *Salmonella* strains. Therefore, this suggests that the expression of AvrA by *Salmonella* involve a delicate control of the levels of CsrA, CsrB and *avrA*-mRNA. It has been suggested that this regulatory control of the AvrA production provides *Salmonella* a rapid and extremely sensitive means to alter virulence gene expression in response to environmental conditions (Kerrinnes *et al*., 2009).

The AvrA protein has been reported to have multiple functions involved in modulating the host immune response to *Salmonella* infection. The first function described for AvrA was a deubiquitinase activity. Ye and coworkers showed that AvrA blocks degradation of IκB and β-catenin in epithelial cells by deubiquitination. This lead to inhibition of the NFκB activation and promoted β-catenin activity, which subsequently inhibited apoptosis and augment *Salmonella* proliferation in epithelial cells (Ye e*t al*., 2007).

A second activity assigned to AvrA has been an acetyltransferase activity, using acetyl-coenzyme A (CoA) to modify the key signaling pathway mitogen-activated protein kinase (MAPK). AvrA was found to interact with the MAPK signaling pathway through the kinase MKK 4/7, blocking phosphorylation and activation of the JNK pathway (Jones *et al*., 2008b). AvrA expression resulted in a potent blockade of the JNK pathway, which prevented apoptosis and the activation of the immune response normally activated by JNK pathway during *Salmonella* infection (Jones *et al*., 2008b; Wu *et al*., 2011). Du and Galán showed that the AvrA protein in the context of *Salmonella* infection in intestinal epithelial cells is capable of inhibiting MAP kinase signaling leading to the inhibition of the *Salmonella*-induced activation of the JNK pathway. However, in these experiments, AvrA

did not interfere with the bacterial infection-induced NFκB activation (Du and Galán, 2009). This latter observation differs from other studies which have reported the inhibition of the NFκB pathway by AvrA (Collier-Hyams *et al*., 2002; Ye e*t al*., 2007). The difference between these results could be due to differences in the experimental model (cell line, mouse), the *Salmonella* strains used or the level of AvrA expression.

Liu and co-workers found that cell signaling pathways such as NFκB, platelet-derived growth factors, vascular endothelial growth factor, oxidative phosphorylation, and mitogen-activated protein kinase are specifically regulated by AvrA *in vivo* (Liu e*t al*., 2010). They analyzed the protein expression during the early (8 hours post-infection) and late stages (4 days post-infection) and found that AvrA function is differentially regulated in host cells depending on the infection time. NFκB activation was found to be inhibited during early stage of infection, but at the late stage of infection, the expression of genes involved in both inhibition and activation of NFκB were affected by AvrA. Furthermore, Liu and co-workers showed that AvrA may inhibit the activation of JNK pathway in the early stage of *Salmonella* infection, but found no evidence that AvrA affects the JNK pathway at late times (4 days) post-infection (Liu e*t al*., 2010). Hence, the function of AvrA at the late stage of infection was more complex and the full effects on NFκB activation are still not entirely clear.

It has also been reported that AvrA promotes the intracellular survival of *Salmonella* in macrophages by preventing macrophage apoptosis through inhibition of apoptosis mediated by the JNK signaling pathway. The suppression of apoptosis in macrophages has been proposed to allow *Salmonella* to establish a stable intracellular niche but also prevent systemic dissemination (Wu e*t al*., 2011). Finally, another function described for AvrA is the stabilization of the tight junctions in the intestinal epithelium, leading to decrease in cell permeability and inhibition of the inflammation response (Liao e*t al*., 2008).

In summary, the AvrA protein modulates (inhibits) the host immune response and the apoptosis of the infected host cells in order to assure a protected niche for intracellular growth and replication. The effect of AvrA in the host cell appears to favor the enteric manifestation of salmonellosis rather than systemic disease. This would be consistent with the absence of the *avrA* gene in *Salmonella* serovars commonly associated with systemic salmonellosis such as Choleraesuis, Typhi and Paratyphi A (Hardt and Galán, 1997; Prager *et al*., 2000; Streckel *et al*., 2004). However, the *avrA* gene is present in *Salmonella* enterica serovar Dublin which also cause systemic disease in humans. For example, in *Salmonella* surveillance data from FoodNet (USA) between 1996 and 2006, the 6% of the human infection caused by *Salmonella* serovar Typhimurium ended in invasive disease and the 57% and 64% of the infection caused by serovars Choleraesuis and Dublin respectively (Jones *et al*., 2008a). Also in the EuroSurveillance Report 2007, the serovar Dublin and

Choleraesuis presented higher rate of systemic disease (41% and 33% respectively) compared with the serovars Typhimurium and Enteritidis (0-4%) (Wollin, 2007). Nevertheless, the expression of AvrA showed a great variation among strains and *Salmonella* serovar Dublin have been reported to present a lower expression level of the *avrA* gene (Streckel *et al*., 2004). *Salmonella* AvrA effector protein shows a complex and sensitive regulatory control that could lead to variations in the AvrA effect depending on AvrA expression and environmental conditions (Streckel *et al*., 2004; Ben-Barak *et al*., 2006; Kerrinnes *et al*., 2008).

1.3.2 *Salmonella* effector protein SseL

The *Salmonella* secreted effector L (SseL) is an effector protein translocated into the host cytoplasm through the TTSS encoded within SPI2 (Figueira and Holden, 2012). SseL was described and named for the first time in *Salmonella enterica* serovar Typhimurium by Coombes and co-workers in 2007 (Coombes *et al*., 2007). Homologues of *sseL* were also identified in the serovars Typhi, Paratyphi A and Choleraesuis. The SseL protein possesses deubiquitinase activity (Rytkonen *et al*., 2007), which cause the deubiquitination of IκBα suppressing its degradation by the proteasome, preventing the subsequent NFκB activation induced by *Salmonella* infection (Le Negrate *et al.*, 2008). *In vivo*, SseL decreases the inflammatory response to *Salmonella* infection, since mice infected with SseL-deficient bacteria showed a more severe inflammatory response that mice infected with the wild type strain. The stronger immune response was shown by increased PMN infiltration into intestinal tissue and increased production of NFκB-dependent cytokines such as IL-1β (Le Negrate *et al.*, 2008; Sun, 2009).

1.4 Variation between *Salmonella* serovar Choleraesuis and *Salmonella* serovar Typhimurium within *Salmonella* pathogenicity islands

Amavisit and co-workers found no significant genetic variation between *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Choleraesuis within the full length of the *Salmonella* pathogenicity islands 2, 3, 4 and 5 (Amavisit *et al*., 2003). However, in another study, variations within SPI-1, SPI-3 and SPI-5 between *S. enterica* serovar Choleraesuis and serovar Typhimurium were found (Chiu *et al*., 2005). SPI-3 harbours 10 genes, including the operon *mgtCB* which is important for survival in macrophages and growth under low Mg 2+ conditions (Blanc-Potard *et al*., 1999). In SPI-3, the genes *sugR* and *rhuM* present in *S. enterica* serovar Typhimurium were not found in *Salmonella* serovar Choleraesuis (Chiu *et al*., 2005). In SPI-5, two genes (STM1092 and STM1093) between *sopB* and *pipD*, were absent in the genome of *S. enterica* serovar

Choleraesuis compared with the genome of *S. enterica* serovar Typhimurium (Chiu *et al*., 2005). However, the major genetic difference within the *Salmonella* pathogenicity islands between these two serovars was found in SPI-1 (Amavisit, *et al*., 2003; Chiu *et al*., 2005).

As mentioned in sections 1.1.1 and 1.1.2, *Salmonella* pathogenicity island 1 (SPI-1) harbours genes encoding a type III secretion system and virulence effector proteins involved in host cell invasion (Srikanth *et al*., 2011; Que *et al*., 2013). *Salmonella* serovar Choleraesuis lacks approximately 900 base pairs (bp) of sequence compared to *S*. Typhimurium, corresponding to the *avrA* gene. In *S*. Choleraesuis, the sequence that would encode the AvrA protein was replaced by a 200 bp sequence which was not significantly similar to any protein sequence in the GenBank database (Amavisit, *et al*., 2003). Additionally, two genes which encode for a putative permease and a LysR-type transcriptional regulator in *Salmonella* serovar Typhimurium were found as pseudogenes in *Salmonella* serovar Choleraesuis (Chiu *et al*., 2005). These genetic differences within the *Salmonella* pathogenicity islands could be responsible for the difference in the host range and in the pathogenicity observed between *Salmonella* serovar Typhimurium and *Salmonella* serovar Choleraesuis. As noted above (section 1.3.1), the absence of the *avrA* gene has been proposed to be related to systemic infection and host adaptation (Streckel *et al*., 2004; Ben Barak *et al*., 2006). This gene is present in broad host range serovars such as *Salmonella* serovar Typhimurium and Enteritidis which are generally associated with gastroenteritis and enteric forms of salmonellosis, but is absent in host-restricted or host-adapted *Salmonella* serovars such as Typhi and Choleraesuis, which cause systemic diseases (Hardt and Galán, 1997; Streckel *et al*., 2004). The *avrA* gene is also present in *Salmonella* serovar Dublin (Hardt and Galán, 1997; Amavisit *et al*., 2003, Suez *et al*., 2013) even though this serovar is adapted to or persists in cattle populations however, this serovar have been reported to present a very low production (< 2 μg/ml) of the AvrA protein (Streckel *et al*., 2004).

In the following sections of this study *Salmonella enterica* serovar Choleraesuis will be referred as *S*. Choleraesuis and *Salmonella enterica* serovar Typhimurium as *S*. Typhimurium. This format will also apply for others *Salmonella* serovars in the following text.

1.5 Goals of this study

Salmonella enterica is one of the most important agents of foodborne disease worldwide, in part due to the ability of *Salmonella* serovars to infect different host species, including food-producing animals, enabling the spread of infection to humans through the food chain. In addition to differences in the host-specificities of different *Salmonella* serovars to invade and proliferate within different hosts, different outcomes of infection in different host species, *i.e.* enteric or systemic forms of disease, has also indicated that the host immune response to infection varies dependent upon the infecting *Salmonella* serovar. An understanding of this aspect of *Salmonella* infections is particularly important in host-pathogen interactions and could reveal new aspects in the management of zoonotic diseases.

The ability to invade and grow within host cells is vital for *Salmonella* infection. One aim of the present study was to compare the invasiveness and intracellular persistence of the *Salmonella* host-adapted serovar Choleraesuis and the broad host-range *Salmonella* serovar Typhimurium in human and porcine intestinal epithelial and macrophages cell lines. Porcine and human cell lines were chosen as infection models since *S*. Choleraesuis is a host-adapted serovar of *Salmonella enterica*, which persists in swine populations, but can cause severe systemic infections in humans. In contrast, *S*. Typhimurium is a broad host-range serovar, infecting many different species, including swine and humans. Meat products from swine are therefore an important source of infection for humans by both *S*. Typhimurium and *S*. Choleraesuis. However, the pathogenesis of infections in humans by these two serovars differs. *S*. Typhimurium infections are generally limited to self-resolving gastroenteritis, whereas *S*. Choleraesuis shows high rates of systemic infections, approaching that of human-restricted serovars such as *S*. Typhi and *S*. Paratyphi (Wollin, 2007). In the present study, we compared the invasion rates and intracellular persistence of these two serovars in both human and porcine intestinal epithelial and macrophage cell lines in an effort to clarify whether these aspects of virulence are associated with the differences in host-range and outcome of infections caused by *Salmonella* serovars Choleraesuis and Typhimurium.

Another aim of this study was to determine whether the differential presence of the *avrA* gen in *S*. Typhimurium and *S*. Choleraesuis is at least in part responsible for the differences in host specificity and pathogenesis between these two *Salmonella* serovars. The *Salmonella* effector protein AvrA has been shown in other studies to affect the activity of the host cell transcriptional activator, NFκB, a key regulator of genes involved in the host innate immune response to infections. Interestingly, the *avrA* gene is not present within the genome of *S*. Choleraesuis but is present in *S*. Typhimurium within the SPI-1. Prior studies have suggested that the absence of the *avrA* gene could be associated with

systemic forms of *Salmonella* infections. To achieve this goal, the *avrA* gene from *S.* Typhimurium strain LT2 was cloned and introduced into the *S.* Choleraesuis strain SARB4. In parallel, the *avrA* gene from the strain *S.* Typhimurium SL1344 was deleted and complemented with the *avrA* gene from *Salmonella* serovar Typhimurium strain LT2. These strains were then used to analyze the effects of the absence or presence and transcription of the *avrA* gene in both *Salmonella* serovars on invasion, intracellular persistence, activation of NFκB and transcription of the genes JUN, IL-1β and IL-8 which are known to be important in the host immune response against *Salmonella* infections.

Finally, an additional goal of the present study was to evaluate possible overlapping functions of the *Salmonella* virulence effector SseL and AvrA, as SseL is also suggested to modulate the host immune response during *Salmonella* infections, and like the AvrA protein, possesses a deubiquitinase activity affecting the activation of NFκB, and is present in both *Salmonella* serovars Typhimurium and Choleraesuis. By comparing the roles of host cell invasion, intracellular persistence and modulation of the activities of the host innate immune regulator NFκB by these two serovars, we hoped to clarify the roles of these aspects in host-adaptation and pathogenesis, and open new areas of investigation.

2 MATERIALS

2.1 Cell lines

Table 1: List of eukaryotic cell lines used in this study

Cell line	Cell type	origin	Source or reference*
THP-1 Human acute monocytic leukemia cell line	monocyte	Human blood	DSMZ Cat. Nr. ACC-16 (Tsuchiya *et al.*, 1982)
THP-1/NFκB	THP-1 Transfected with NFκB Cignal Lenti reporter (luc) SABiosciences		S. Maurischat
THP-1-AP-1	THP-1 Transfected with AP1-1 Cignal Lenti reporter (luc) (SABiosciences)		This study
LoVo	Intestinal epithelial	Human colon	ATCC Cat. Nr. CCL-229 (Drewinko *et al.*, 1976)
LoVo/ NFκB	LoVo Transfected with NFκB Cignal Lenti reporter (luc) (SABiosciences)		S. Maurischat
LoVo/ AP-1	LoVo Transfected with AP1-1 Cignal Lenti reporter (luc) (SABiosciences)		This study
PLN/C2	monocyte/macrophage	Porcine blood	K. Tedin
PLN/C2-G6	PLN/C2 Transfected with NFκB Cignal Lenti reporter (luc) (SABiosciences)		B. Siepert
PLN/C2/ AP-1	PLN/C2 Transfected with AP1-1 Cignal Lenti reporter (luc) (SABiosciences)		This study
IPEC-J2 Intestinal Porcine Epithelial Cell - Jejunum 2	Intestinal Epithelial	Porcine Jejunum	A. Blikslager, (Berschneider, 1989)
IPEC-J2/K3	IPEC-J2 Transfected cells with NFκB Cignal Lenti reporter (luc) (SABiosciences)		S. Maurischat
IPEC-J2/ AP-1	IPEC-J2 Transfected with AP1-1 Cignal Lenti reporter (luc) (SABiosciences)		This study

*Abbreviations: ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen

2.2 Bacterial strains and plasmids

Table 2: List of bacterial strains used in this study

Strain	Genotype/Relevant features	Source or reference*
LT2	*S*. Typhimurium virulent prototroph	*Salmonella* Genetic Stock Centre (SGSC)
JR501	*S*. typhimurium LT2 *fla*-66 *galE719 hsdSA29 hsdSB121 hsdL6* (r-m+) *ilv*-452 *metA22 metE551 nml rpsL120 trpC2 xyl*-404	*Salmonella* Genetic Stock Centre (SGSC)
4/74	*S*. Typhimurium virulent prototroph, progenitor strain of SL1344. aka S2337/65	A. Thompson/S. Bowden, IFR, Norwich UK
SL1344	*S*. Typhimurium *hisG46 rpsL*	(Hoiseth and Stocker, 1981)
14028	*S*. Typhimurium virulent prototroph	ATCC
A50	*S*. Choleraesuis A50	(Chadfield *et al*., 2003)
SARB4	*S*. Choleraesuis var. kunzendorf SARB4	M. Achtman
2950	*S*. Choleraesuis SARB4 rough mutant	K. Tedin
06-00244	*S*. Choleraesuis 6,7:c:1,5	BfR - B. Malornyi
07-01292	*S*. Choleraesuis 6,7:c:1,5	RKI - E. Tietze
4200	*S*. typhimurium LT2(pKD46)	Laboratory strain
6210	SL1344 Δ*avrA*::*kan*	This study
6214	SL1344 Δ*avrA*	This study
6604	*S*. Typhimurium JR501 (pWSK29)	This study
6605	*S*. Typhimurium JR501 (pWSK-IB)	This study
6606	*S*. Typhimurium JR501 (pWSK-IIA)	This study
6607	*S*. Typhimurium JR501 (pWSK-IIB)	This study
6611	*S*. Typhimurium JR501 (pWSAM-C1)	This study
6650	SL1344 (pWSK-IIA)	This study
6750	SL1344 Δ*avrA* (pWSK29)	This study
6752	SL1344 Δ*avrA* (pWSK-IB)	This study
6754	SL1344 Δ*avrA* (pWSK-IIA)	This study
6756	SL1344 Δ*avrA* (pWSK-C1)	This study
6922	SL1344 Δ*avrA* Δ*sseL*::*kan*	This study
6978	*S*. Choleraesuis var. kunzendorf SARB4 (pWSK29)	This study

Continuation Table 2: List of bacterial strains used in this study

Strain	Genotype/Relevant features	Source or reference*
6980	*S*. Choleraesuis var. kunzendorf SARB4 (pWSK-IIA)	This study
6982	*S*. Choleraesuis 06-00244 (pWSK29)	This study
6984	*S*. Choleraesuis 06-00244 (pWSK-IIA)	This study
6986	*S*. Choleraesuis 07-01292 (pWSK29)	This study
6988	*S*. Choleraesuis 07-01292 (pWSK-IIA)	This study
7154	SL1344 Δ*avrA* Δ*sseL*::*kan* (pWSK-IIA)	This study
7212	SL1344 Δ*avrA* Δ*sseL*::*kan* (pWSK29)	This study
DH5α	*E. coli deoR endA1 gyrA96 hsdR17*(rK-mK*+) Δ(lacZYA-argF)*U169 *phoA recA1 relA1 spoT1 supE44 thi*-1 ϕ*80dlacZ*ΔM15	Laboratory strain
JM109λpir	*E. coli endA1 gyrA96 hsdR17*(rK-mK+) Δ(*lac-proAB*) *recA1 relA1 supE44 thi*. λ*pir*+	J. Slauch
357	DH5α (pWSK-IB)	This study
358	DH5α (pWSK-IIA)	This study
363	DH5α (pWSAM-C1)	This study

*Abbreviations: ATCC, American Type Culture Collection; BfR, Bundesinstitut für Risikobewertung; RKI, Robert-Koch-Institut

Table 3: List of plasmids used in this study

Plasmids	Description	Source or Reference
pKD4	*bla kan* oriRγ (pir-dependent replication)	(Datsenko and Wanner, 2000)
pKD46	*bla araC* pBAD-γ-β-*exo repA101*(ts) *oriR101*	(Datsenko and Wanner, 2000)
pCP20	*bla cat* pSC101(ts) *c*I857(ts) pL-FLP+	(Cherepanov and Wackernagel, 1995)
pWSK29	*bla lacZ*α T7/T3 f1ori pSC101ori	(Wang and Kushner, 1991)
pWSK-IB	*bla* PavrA-*avrA*$^{+}$(4/74) pSC101ori	This study
pWSK-IIA	*bla* PavrA-*avrA*$^{+}$(LT2) pSC101ori	This study
pWSAM-C1	*bla* PavrA-*avrA*$^{+}$(14028) pSC101ori	This study

2.3 Oligonucleotide primers

The oligonucleotides (primers) used in this study were synthesized by Eurofins MWG Operon, Ebersberg and Sigma-Aldrich, Steinheim.

Table 4: List of oligonucleotides used in this study

Oligonucleotide	Target region	Primer Sequence (5'-3')	Tm (ºC)	Reference
AVRAHF	*Salmonella avrA* gene	TATCGTAAGAAGCT TATGTATCGTTTGG	55,0	K. Tedin
AVRABR	Salmonella *avrA* gene	GGCGCTGGAAGGAT CCTCTGGCAGGCAA CC	55,0	K. Tedin
DNAAFor	*Salmonella dnaA* gene	GGCGAAGTAGCGTT CTTTAT	55,0	K. Tedin
DNAARev	*Salmonella dnaA* gene	ACGCAGTTGCTCAAT TTTAC	55,0	K. Tedin
huGAPDH_FW	Human GADPH gene	GAGTCAACGGATTT GGTCGT	63,9	(Shervington and Patel, 2008)
huGAPDH_RV	Human GADPH gen	TTGATTTTGGAGGGA TCTCG	63,8	(Shervington and Patel, 2008)
hIL1bFW	Human IL-1β gene	TTGTTGCTCCATATC CTGTCC	63,8	K. Tedin
hIL1bRV	Human IL-1β gene	CACCTTCTTTCCCTT CATCTTTG	64,7	K. Tedin
huIL8FW	Human IL-8 (CXCL8) gene	CAAAAACTTCTCCA CACAACCCTC	58,4	(Salinthone *et al.*, 2004)
hIL8RV	Human IL-8 (CXCL8) gene	TCCAAACCTTTCCAC CCCAAA	57,9	(Salinthone *et al.*, 2004)
poGADPH-F4	Porcine GADPH gene	CAGCAATGCCTCCTG TACCA	59,4	
poGADPH-R4	Porcine GADPH gene	ACGATGCCGAAGTT GTCATG	57,3	
poIL8F-4	Porcine IL-8 (CXCL8) gene	AACTGAGAGTGATT GAGAGTGGA	58,9	(Hyland *et al.*, 2006)

Continuation Table 4: List of oligonucleotides used in this study

Oligonucleotide	Target region	Primer Sequence (5'-3')	Tm (ºC)	Reference
poIL8R-4	Porcine IL-8 (CXCL8) gene	GCTGTTGTTGTTGCT TCTCAGTT	58,9	(Hyland *et al*., 2006)
mwg 256	*Salmonella avrA* gene	CTAAACACCGAAGC ATTGACC	57,9	(Kerrinnes *et al*., 2009)
mwg 257	*Salmonella avrA* gene	GGAAACAAGCTCAT GGACTGAC	60,3	(Kerrinnes *et al*., 2009)
AVRAF	*Salmonella avrA* gene	CCTGGCTCAATCATT GAGGC	60,2	K. Tedin
AVRAR2	*Salmonella avrA* gene	AGGCAGTTGTTCAC GTTCAAGAG	60,6	K. Tedin
hJUNF	Human Jun gene (c-jun)	TGCCTCCAAGTGCC GAAAAA	57,3	Du and Galán, 2009
hJUNR	Human Jun gene (c-jun)	TGACTTTCTGTTTAA GCTGTGCC	58,9	Du and Galán, 2009

2.4 Commercial kits

Cignal Lenti AP1 Reporter (luc) Kit: CLS-011L for develop stable cell line reporters to monitoring AP-1 activity in mammalian cells	SABiosciences/QIAGEN, Hilden
Bright-Glo™ Luciferase Assay System, for quantification of luciferase expression in mammalian cells	Promega, Mannheim
QIAprep Spin Midi and Mini prep kit, for DNA plasmid purification	Qiagen, Hilden
RNeasy mini kit, for purification of total RNA from animal cells	Qiagen, Hilden

2.5 Enzymes

Pfu DNA Polymerase (3 U/ µl)	Promega Madison, WI
AccuPrimer^{TM} Pfx DNA Polymerase (2,5 U/ µl)	Invitrogen
Taq Polymerase (5 U/ µl	Rapidozym
RNase free DNase	Qiagen
Hind III (10 U/ µl)	Promega
Bam HI (10 U/ µl)	Promega
T4 DNA ligase	Promega
Power SYBR® Green PCR Master Mix	Applied Biosystems
M-MLV-reverse transcriptase (5 U/µl)	Promega

2.6 Antibiotics

Antibiotic	**Final concentration**	**Stock solution**
Carbenicilin	100 µg/ml	10 mg/ml
Kanamycin	50 µg/ml	10 mg/ml
Gentamicin	50 µg/ml and 10 µg/ml	10 mg/ml
Puromycin	5 µg/ml	1 mg/ml

2.7 Culture media

2.7.1 Cell culture media

DMEM/Ham's F12-FKS:	Dulbecco's modified Eagle's Medium (DMEM)/Ham's F-12 salts (1:1) (Biochrom, Berlin) DMEM/Ham´s, 5 - 10% fetal calf serum (heat inactivated at 56°C for 30 min.)
Iscove´s (IMDM)-FKS:	Iscove's modified Dulbecco's modified Eagle's medium (IMDM, Biochrom) supplemented with 10% fetal calf serum (Biochrom, Berlin)

2.7.2 Bacterial culture media

The bacterial culture media were prepared with double distilled water and sterilized by autoclaving at 121°C for 20 minutes.

Lennox Broth (Lennox, 1955)	10 g/L Tryptone 5 g/L yeast extract 5 g/L NaCl pH 7
Lennox Agar (Lennox, 1955)	10 g/L Tryptone 5 g/L yeast extract 5 g/L NaCl 15 g/L Agar
X-Gal Agar	Autoclaved Lennox agar supplemented with 40 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside)

2.8 Solutions

2.8.1 Solutions for *E. coli* plasmid preparation

Solution I:	Tris-HCl 25,0 mM (pH 8,0) Glucose 50,0 mM EDTA 2,0 mM pH 7,0 - 7,5
Solution II (lysis buffer):	0,2 M NaOH 1,0% SDS ddH2O to 200,0 ml
Solution III:	Potassium acetate (KOAc) 58,9 g Acetic acid (HOAc) 50,0 ml ddH_2O to 200,0 ml pH 5,0

2.8.2 Solutions for *Salmonella* plasmid preparation (Kado)

10X Kado TE:	Tris base 42,5 g EDTA 7,5 g ddH2O to 1,0 liter pH to 7,9 and autoclaved
Kado Lysis Buffer:	Kado Lysis Buffer: Tris base 0,6 g SDS 3,0 g ddH2O to 100 ml Filter sterilized (0,45 µm filter) Before use: 80 µl of 10N NaOH / 10 ml of lysis buffer added
Kado KOAc (Solution III):	5 M KOAc 6,0 ml glacial HOAc 1,15 ml ddH2O 2,85 ml

2.8.3 Solutions for RNA preparation

Stop solution:	95% absolute ethanol, 5% phenol
Resuspension buffer:	25 mM Tris (pH 7,0 - 7,2), 1 mM EDTA in DEPC-treated water
Lysis solution:	0,6 M NaOAc (pH ≈ 5,0) 4,0 mM EDTA 1,0% (w/v) SDS for *E. coli* or 3,0% SDS for *S*. Typhimurium or 0,1% SDS for eukaryotic cells
10X DNAse buffer:	1,0 M NaOAc (pH 5,0 - 5,5) 0,05 M $MgSO_4$ (or $MgCl_2$)
DEPC-treated water:	1:1000 DEPC (Diethylpyrocarbonate) in double-distilled or MilliQ water, mixed and incubated overnight at room temperature and autoclaved for 20 min. at 121°C

2.8.4 Solutions for Agarose gel electrophoresis

1% - 2% Agarose Gel	Agarose 1-2 g 1X Tris Borate EDTA (TBE) buffer 100 ml Ethidium Bromide 5 µl
1% Tris Borate EDTA (TBE) Buffer	Tris base 10,8 g Boric acid 5.5 g 0.5M EDTA (pH 8.0) 4 ml to 1L with ddH$_2$O
100 bp DNA-ladder, extended	Carl Roth GmbH, Karlsruhe
Sample buffer (Tris/EDTA/glycerol)	Carl Roth GmbH, Karlsruhe

2.8.5 Chemicals and stock solutions

Table 5: List of Chemicals used in this study

Chemicals and stock solutions	Manufacturer
Triton X-100	Roth, Karlsruhe
10X PBS	Biochrom, Berlin
Sodium dodecyl sulfate (SDS)	Roth, Karlsruhe
Glycerol	Roth, Karlsruhe
Sodium chloride (NaCl)	Roth, Karlsruhe
Trypsin/EDTA (10X)	Biochrom, Berlin
Sodium acetate (NaOAc)	Roth, Karlsruhe
Chloroform	Merck, Darmstadt
Phenol, water equilibrated	Roth, Karlsruhe
Ethanol absolute	Merck, Darmstadt
EDTA: ethylenediamino-N,N,N',N'-tetraacetic acid	Roth, Karlsruhe
EGTA: ethylene-glycol-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid	Roth, Karlsruhe
10% dimethylsulfoxide (DMSO)	Roth, Karlsruhe
Tris base	Sigma-Aldrich, München

2.9 Technical equipment and instruments

Table 6: List of the laboratory equipment used

Equipment and instruments	Manufacturer
Agarose gel electrophoresis tank	B1A and GE-B2, AGS, Heidelberg
Aspirator	Vacuboy, neolab, Heidelberg
Autoclave	GTA16, Gössner, Hamburg
Centrifuges	Rotina 46R, Hettich Zentrifugen, Tuttlingen 3K30, Sigma Laborzentrifugen, Osterode 5415D, Eppendorf Zentrifugen, Engelsdorf
Nanodrop	PeqLab, Erlangen
Electronic balance	BL3100, Sartorius, Göttingen
Electronic precision weighing instrument	BP210S, Sartorius, Göttingen
Electroporator	EasyjecT Prima, PeqLab, Erlangen
Electrophoresis power supply	PS 250, Hybaid, Lexington, USA
Heating block	Thermomixer compact, Eppendorf, Hamburg
Image-Documentation instrument	UV-Transilluminator E.A.S.Y. 429 K, Documentationssystem Herolab, Wiesloch
Incubators	CB150, Binder, Tuttlingen VT 5042 EK/N2, Heraeus, Hanau
Labmate Precision pipettors	Abimed GmbH, Langenfeld
Magnetic stirrer	IKAMAG RET, Th. Karow, Berlin
pH-Meter	Mikroprozessor pH-Meter 740, Knick, Berlin
Shaking incubator	GFL3031, GFL, Burgwedel
Spectrophotometer	Ultrospec 3000 pro, Amersham, Freiburg
Sterile work bench	LaminAir HB 2448 and LB-48-C, Heraeus,Hanau
Thermocycler	T3 Thermocycler, Biometra, Göttingen
Vaccum pump	N735 AN18, KNF Neuberger, Freiburg

Continuation Table 6: List of the laboratory equipment used

Equipment and instruments	**Manufacturer**
Vortex	MS2 Minishaker, IKA, Staufen Vortex-Gene 2, Scientific Induestries, New York USA
Waterbath	Gr E-5, Julabo, Seelbach ant OLS 200, CLF Laborgeräte, Burgwedel
StepOne Plus Real-Time PCR System	Applied Biosystems, USA
Plattenphotometer – BioTek	Synergy HT, BioTek, Bad Friedrichshall
Scanner	DUOSCAN T1200, AGFA, Köln
Pipetting aid	Pipetus-akku, Hirschmann, Eberstadt

2.10 Consumables

96-Well Assay Microplate, White, Clear Bottom with Lid, sterile	Corning, Cat. Nr. 3610
24-Well, Multiwell Plate, Corning® CellBIND® Surface, sterile	Corning, Cat. Nr. 3337
Cell culture flasks, 25 cm^2	Corning, Cat. Nr. 430639
Cell scraper	Corning, Cat. Nr. 3010
Disposable cuvettes	MBT, Giessen
Disposable pipettes	Costar, Bodenheim
Electroporation cuvettes	2 mm, PeqLab, Erlangen
Borosilicate glass Pasteur pipettes	Roth, Karlsruhe

3 METHODS

3.1 Cell culture methods

3.1.1 Maintenance and cultivation of eukaryotic cell lines

Epithelial cell lines were maintained in DMEM/Ham's F-12 salts (1:1) cell culture medium with heat- inactivated fetal bovine serum (FBS). For cultivation of IPEC-J2 porcine epithelial cells the medium was supplemented with 5% FBS. For cultivation of LoVo human epithelial cells, the cell culture medium was supplemented with 10% FBS. The porcine (PLN/C2) and human (THP-1) macrophage cell lines were maintained in Iscove´s Modified Eagle Medium (Iscove´s/IMDM) supplemented with 10% FBS. All cell lines were incubated at 37°C with 5% CO_2 in a cell culture incubator and the cell culture medium was replaced each two to three days. Confluent cell culture monolayers were passaged by detachment with Trypsin/EDTA treatment. On the day of passage, the cell culture medium was removed and the cells were washed once with Phosphate Buffered Saline (PBS). The PBS was removed and replaced with one culture volume of Trypsin/EDTA (0,5%/0,2% w/v in PBS) and incubated 5 to 10 minutes at 37°C. The detached cells were transferred to a sterile, 15 ml centrifuge tube and the cell suspensions diluted 1:1 with cell culture medium containing FBS. The cells were collected by centrifugation in a swinging bucket rotor (Sigma 3K30) at 155 x g for 5 min. at 25°C. The supernatants were removed by aspiration, and the resulting cell pellets were resuspended in fresh medium at the original cell culture medium volume. Flasks or well plates were seeded at empirically determined dilutions depending on the cell line and the desired cell concentrations for maintenance (passage) or experimental cultures.

3.1.2 Cryopreservation of eukaryotic cell lines

Stocks of each cell line were preserved in liquid nitrogen in the appropriate cell culture medium supplemented with 10% dimethylsulfoxide (DMSO). At passage, from the cell pellet resuspensions in cell culture medium, 1 ml was added to 0,1 ml of DMSO, mixed gently, and placed immediately to ice. The cryogenic vials with the cells were first stored one to three days at -80°C and then transferred to liquid nitrogen for long-term storage.

3.1.3 Gentamicin protection assays

The gentamicin protection assay or invasion assay is frequently used to study eukaryotic cell-pathogen interaction as an infection model (Shaw and Falkow, 1988; Elsinghorst,

1994; Edwards and Massey, 2011). Briefly, the respective cell line was seeded into 24-well or 96-well plates with 1 ml or 100 µl of the appropriate cell culture medium, respectively. The cells were incubated at 37°C in 5% CO_2 until a confluent cell culture monolayer was achieved, approximately 2 x 10^5 cells per well in the 24-well plate format and 3 x 10^4 cells/well in the 96-well format. One hour prior to infection the cell culture medium was replaced with fresh medium.

One day before the invasion assay, the bacterial strains were streaked from the frozen -80°C stocks to a Lennox agar plate and incubated overnight at 37°C. After incubation, 2 to 5 colonies from the agar plate were inoculated in Lennox broth and were incubated at 37°C with agitation until the bacterial culture had reached a late log growth phase, which corresponds to an optical density (OD) at 600 nm of approximately 2-3. One ml of this bacterial culture was centrifuged and the cell pellet was suspended in 1 ml of the cell culture medium; a dilution 1:20 of this bacterial culture was made and the OD at 600 nm was measured and the expected bacterial counts per ml of suspension were calculated according to the assumption that an OD at 600 nm of 1 corresponds to approximately 1 x 10^9 *Salmonella* cfu/ml. The bacterial cultures were diluted in cell culture medium until a concentration of approximately 10^6 cfu/ ml, 50 µl of this suspension were used to inoculate each well of a 24 well plate contained approximately 2 x 10^5 eukaryotic cells obtaining a ratio bacteria: eukaryotic cell of approximately 1. The 96-well plates contained approximately 3 x 10^4 eukaryotic cells per well, used to analyze NFκB activation were inoculated with 100 µl of the 10^6 cfu/ ml bacterial culture obtaining a MOI of approximately 5. This bacterial culture was plated onto Lenox agar and incubated overnight at 37°C in order to calculate the number of colony forming units used to infect the eukaryotic cells.

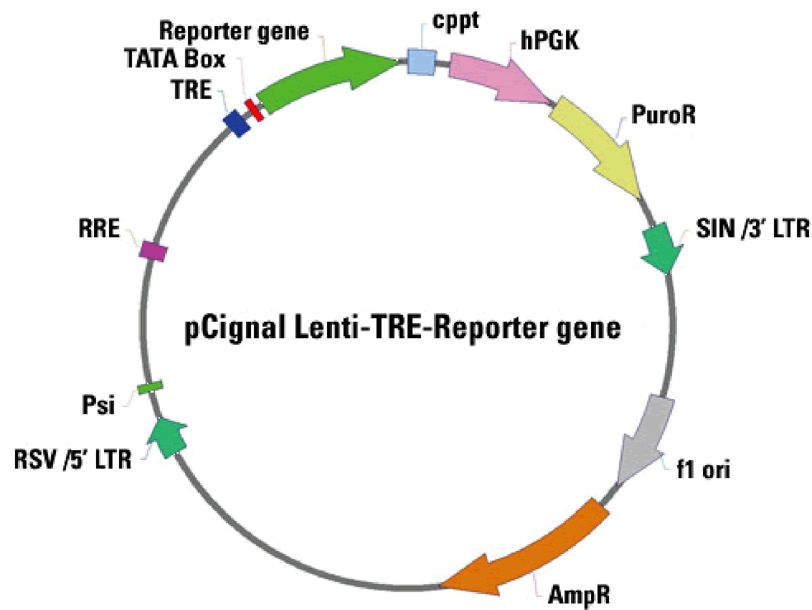
After infection, the plates were centrifuged at 250 x g for 10 minutes in order to bring cells and bacteria in contact and initiate infection (synchronization of infection). The plates harbouring the infected cells were incubated for one hour at 37°C in 5% CO_2 to allow the bacteria to penetrate the cells. After infection, 50 µg / ml gentamicin was added to the plates for 60 minutes to kill all remaining extracellular bacteria that had not penetrated the host cells. After one hour of co-incubation in the presence of gentamicin (2 hours post-infection), the medium was replaced by fresh medium containing 10 µg/ml gentamicin. At 2, 4, and 24 hours post-infection, cells were washed with 1X PBS and lysed by addition of 0,5 ml of 0.1% Triton X-100 in deionized, distilled water, a concentration which causes lysis of host cells but, which does not affect the bacterial cell wall. Various dilutions were plated directly onto Lennox agar plates and colony forming units (cfu) were counted after overnight incubation at 37°C. The percentage of bacteria that were able to invade the eukaryotic cells was calculated as the ratio of cfu at 2 hours post infection and the cfu used to infect the cells. The intracellular persistence 24 hours p.i. was determined

using the ratio of cfu at 24 hours post infection and cfu at 2 hours post infection. The assays were repeated at least three times for each strain and each cell line.

3.1.4 Transfection of eukaryotic cells

In order to compare the effect of the *Salmonella* serovars Typhimurium and Choleraesuis and furthermore the effect of the effector proteins AvrA and SseL in modulation of eukaryotic transcription factors such as NFκB and AP-1 eukaryotic cells were transfected using Cignal Lenti reporter assays from SABiosciences/Qiagen. The Cignal Lenti reporter assays are a preparation of self-inactivating, replication-incompetent lentiviral particles, based in the human immunodeficiency virus type 1 (HIV-1) and pseudotyped with glycoproteins from the vesicular stomatitis virus (VSV) (see Figure 4). The NFκB and AP-1 responsive luciferase vector encodes the firefly luciferase gene under the control of a minimal cytomegalovirus (CMV) promoter and tandem repeats of the NFκB transcriptional response element or TPA transcriptional response element, respectively. This allows monitoring of the activity of a transcription factor through a luciferase assay (Qiagen, 2012). The Cignal Lenti reporter vector also harbours a puromycin resistance gene which allows puromycin selection for transduced cells and clones.

The cell lines IPEC-J2, LoVo, PLN/C2 and THP-1 were seeded in a 96-well plate at 5×10^3 cells per well and incubated overnight at 37°C in 5% CO_2. After incubation, the medium was removed from the wells and the cells were infected with the lentiviral particles at an MOI of 10-50 in a final volume of 50 µl and incubated 18 to 20 hours. Subsequently, the medium with the lentiviral particles was removed from wells and replaced by fresh cell culture medium. The cells were incubated for 2 days at 37°C in 5% CO_2. The cell culture medium was replaced by fresh medium with 5 µg / ml puromycin and incubated 24 to 48 hours. The cells were detached with Trypsin/EDTA and seeded at a concentration of 5×10^4 cells per well in a new 96-well plate and incubated until a confluent monolayer was formed and tested for luciferase activity.



Feature	Function
RSV-5' LTR; Hybrid Rous sarcoma Virus (RSV) enhancer/promoter-U5 long terminal repeat	Permits viral packaging and reverse transcription of viral mRNA
Psi; Packaging signal	Allow viral packaging
RRE; Rev response element	Involved in packaging of viral transcript
cppt; Central polypurine tract	Involved in nuclear translocation and integration of transduced viral genome
Reporter gene (firefly luciferase or GFP)	Allow quantification of transcription
hPGK; human phosphoglycerate kinase eukaryotic promoter	Permits high-level expression of the mammalian selection marker (puromycin)
PuroR; puromycin resistance gene	Can be used for mammalian selection
SIN/3'LTR; 3' self-inactivating long terminal repeat	Modified 3'LTR that allows viral packaging but self-inactivates the 5'LTR for biosafety purpose. The element also contains a polyadenylation signal for efficient transcription termination
f1 ori; f1 origin of replication	Origin of DNA replication for bacteriophage f1
AmpR; ampicillin resistance gene	Allows selection of the plasmid in E.coli
TRE; Transcription response element	Permits regulation of reporter gene expression by a specific transcription factor
TATA box	Act as an minimal promoter

Figure 4: Schematic of Cignal Lenti lentiviral vector used to generate NFκB reporter cell lines (Qiagen, 2012).

3.1.5 NFκB activation assays

In order to determine the effect of the presence or lack of the *avrA* gen in *S*. Choleraesuis and *S*. Typhimurium on host NFκB activity during infection, porcine (IPEC-J2) and human (LoVo) intestinal epithelial and porcine (PLN/C2) and human (THP-1) macrophage cell lines containing chromosomally-integrated, NFκB dependent reporter luciferase fusions were infected at a multiplicity of infection of approximately 5 with the strains: *S*. Choleraesuis *avrA*+ (SARB4/pWSK29-$avrA_{LT2}$), *S*. Choleraesuis wild type (SARB4/pWSK29), *S*. Typhimurium *avrA*+ (SL1344 Δ*avrA*/pWSK29-$avrA_{LT2}$) and *S*. Typhimurium Δ*avrA* mutant (SL 1344 Δ*avrA*/pWSK29) in 96-well plates for 60 minutes, using the gentamycin protection assay protocol described in the section 3.1.3. NFκB-dependent luciferase expression was monitored by determination of luminescence with a Bright-Glo™ Luciferase Assay System from Promega and a Synergy™ HT Microplate Reader from BIO-TEK, at 2, 4 and 24 hours post-infection. In parallel, invasion and intracellular persistence of these serovars in each cell line were determined.

The cell lines containing chromosomally-integrated, NFκB dependent reporter luciferase fusions were maintained in DMEM/Ham's F-12 medium for epithelial cells and Iscove's medium for macrophages, both with 10% FBS and 5 µg / ml puromycin and incubated at 37 °C in 5% CO_2. One hour prior infection the cell culture medium was removed and the cells were washed with PBS and 50 µl of the respective cell culture medium without puromycin was added to each well. The cells were infected with the respective *Salmonella* strain at MOI of 5 as described in the section 3.1.3 for the 96 plate format. At 2, 4 and 24 hours post infection the Bright-Glo™ luciferase assay from Promega was carried on following the manufacturer's instructions: 75 µl of cell culture medium were removed from each well and 25 µl of Bright-Glo™ luciferase reagent were added to obtain a concentration 1:1 cell culture medium and luciferase reagent, the plate was mixed and 5 minutes after the luminescence was measured with the Synergy™ HT Microplate Reader from BIO-TEK). The luminescence of the infected cells was normalized with the luminescence obtained in the uninfected cells in the same well plate; the uninfected cells and the infected cells were identically treated.

3.2 Microbiological methods

3.2.1 Preparation of bacterial stocks

The bacterial strains listed in the Table 2 were conserved at -80 °C in order to avoid mutations. A single colony from Lennox agar was incubated overnight at 32 or 37 °C, depending on the strain, and suspended in Lennox broth an incubated until an OD at 600 nm of 2-3, 1 ml of this bacterial culture was transferred to a cryo-tube containing 300 µl of

a sterile 80% Glycerol solution, gently mixed and preserved at -80 °C.

3.2.2 Blue White screening for clones

"Blue-White" screening was used to select for putative positive clones after transformation of bacteria by electroporation. The vector pWSK29 harbours multiple cloning sites within the coding sequence for the β-galactosidase N-terminal α fragment. Successful insertions into the cloning sites inactivates the α-fragment coding region, and recombinant colonies (white - non-complementing) can be identified among non-recombinant ones (blue - complementing) after transformation of bacteria expressing the β-galactosidase C-terminal Ω-fragment and plating onto selective plates containing the appropriate antibiotics and the chromogenic substrate for β-galactosidase, X-Gal (Snyder and Champness, 2007). White colonies were selected and the plasmids were purified, screened and sequenced.

3.3 Molecular methods

3.3.1 Isolation of bacterial DNA for PCR reactions

Total genomic DNA from *Salmonella* strains was obtained by boiling. The bacteria were streaked onto Lennox broth agar plates and incubated at 37°C overnight. The following day, a single colony was used to inoculate a Lennox broth liquid culture tube which was incubated at 37°C in agitation to an OD 600 of 2 - 4. A 1 ml aliquot of the bacterial culture was centrifuged at 16100 x g for 5 minutes; the pellet was resuspended in double distilled water and incubated at 99°C for 10 minutes, centrifuged and the supernatant was stored at -20°C.

3.3.2 Polymerase Chain Reaction (PCR)

PCR was used to amplify specific DNA sequences for cloning or analytical purposes. The composition of the PCR reaction mixture, the standard protocol and the Thermocycler program used are described in the tables 7 and 8. The AccuPrime^{TM} *Pfx* DNA polymerase from Invitrogen and the Pfu DNA polymerase from Promega, were used in PCR reactions that require high fidelity such as cloning PCR applications. The Taq DNA polymerase from Rapidozym was used for testing primers and other simple analytical purposes. The boiled DNA solution described in section 3.3.1 was used as DNA template if no other specification is mentioned. The PCR reactions were performed in a T3 Thermocycler from Biometra. The steps 2 to 4 of the PCR protocol were repeated 35 to 40 times depending on the analytical purpose. The PCR products were analysed by agarose gel electrophoresis.

Table 7: PCR reaction mixture and protocol using AccuPrime^{TM} *Pfx* DNA polymerase

PCR reaction mixture			**PCR protocol**		
Components	**Volume**		**PCR step**	**Temperature**	**Time**
10X AccuPrime ^{TM} *Pfx* reaction mix	5 µl		Initial Denaturation	95°C	180 s
Forward Primer (10 pmol)	1,5 µl		Denaturation	95°C	60 s
Reverse Primer (10 pmol)	1,5 µl		Primer Annealing	55°C	60 s
AccuPrime ^{TM} *Pfx* DNA Polymerase (2,5 U/ µl)	0,5 µl		Extension	68°C	60 s
DNA template	variable		Final Extension	68°C	60 s
Sterile double distilled water	to 50 µl				

Table 8: PCR reaction mixture and protocol using *Pfu* DNA polymerase and Bio Therm Taq DNA polymerase

PCR reaction mixture			**PCR protocol**		
Components	**Volume**		**PCR step**	**Temperature**	**Time**
dNTP mix (10 mM)	1 µl		Initial Denaturation	95°C	180 s
10X PCR buffer with MgSO4 (20 mM)	5 µl		Denaturation	95°C	60 s
Forward Primer (10 pmol)	1,5 µl		Primer Annealing	55°C	60 s
Reverse Primer (10 pmol)	1,5 µl		Extension	72°C	60 s
Pfu DNA Polymerase (3 U/µl) / Taq Polymerase (5 U/µl)	0,5 µl / 0,2 µl		Final Extension	72°C	60 s
DNA template	variable				
Sterile double distilled water	to 50 µl				

3.3.3 Gel electrophoresis

DNA and RNA samples were visualized and analyzed using agarose gel electrophoresis. Agarose gel was made diluting 1,0 - 1,5% agarose in 100 ml of 1X TBE by boiling, after cooling to ca. 65 ℃, 5µl of ethidium bromide (1%) was added and mixed. The samples were mixed with the loading buffer and loaded into the pockets of the solidified agarose gel and separated under an electric field of 110-130 V for 30 – 60 minutes. The nucleic acid fragments were visualized under ultraviolet light and the DNA-fragment sizes were estimated by comparison to a standard 100 bp DNA-ladder from Carl Roth.

3.3.4 Isolation and purification of DNA

3.3.4.1 Isolation of chromosomal DNA (Phenol/Chloroform extraction)

Phenol/chloroform extractions were used to purify DNA after PCR amplification and enzyme restriction. 100 µl of the DNA-probe was brought to 300 µl with sterile, double distilled water and 500 µl of phenol was added and mixed by vortexing. The DNA mixture was centrifuged at 16100 x g for 3 minutes. The organic phase (phenol, lower phase) was discarded and 600 µl of chloroform was added to re-extract and remove remaining phenol from the nucleic acids. The DNA-solution was centrifuged at 16100 x g for 3 minutes and the organic lower phase (chloroform) was discarded. The chloroform extraction step was repeated two additional times. After removal of the chloroform, 33 µl of a 3 M NaOAC (pH 4,0 - 5,0) solution were added to the DNA-mixture and mixed. 900 µl of ice-cold absolute ethanol was added to the mixture and incubated at -20°C overnight. The precipitated DNA was collected by centrifugation at 31500 x g at 4°C for 1 hour. The resulting DNA pellet was washed with 70% ethanol and dried for ca. 10 minutes at 37°C. The DNA-pellet was suspended in 20-100 µl of sterile double distilled water and stored at -20°C.

3.3.4.2 Isolation of DNA plasmids

Plasmids were isolated from bacteria using different methods, depending on the bacterial strain and the subsequent analyses. QIAprep Spin Midi and Mini Kits (Qiagen) were used to purify plasmid DNA from bacterial cultures for use as templates for cloning or sequencing. For the Midi prep kit, one bacterial colony from a Lennox agar plate was inoculated in 5 ml Lennox broth and incubated at 37°C with agitation until an OD_{600} of 2 was reached, 200 µl of this initial culture were transferred to a flask containing 200 ml Lennox broth with the appropriated selective antibiotic and incubated overnight at 37°C

with agitation. The following day, the bacterial culture was split into four centrifuge tubes and the Qiagen protocol was followed. For preparing the bacterial culture for the Spin Miniprep purification one colony from a Lennox agar plate was inoculated in 5 ml L- broth and incubated overnight at 37°C with agitation, the culture was centrifuged at 16100 x g for 3 minutes at room temperature and the manufacturer's instructions were followed.

In order to isolate DNA plasmids from *Escherichia coli* for further bacteria transformation 5 ml bacterial culture was incubated overnight at 37°C under agitation. Following incubation 1,25 ml of the bacterial culture were centrifuged for 5 min at 16100 x g, the pellet was resuspended in 1,25 ml of the same culture and the centrifugation was repeated. The bacterial pellet was dissolved in 100 µl of solution I and 200 µl of freshly prepared lysis buffer were added. The mixture was briefly mixed using a vortex and incubated 20 - 30 minutes at room temperature. After lysis 150 µl of solution III were added, the mixture was briefly mixed using a vortex and incubated for 5 - 10 minutes on ice. The mixture was mixed again and centrifuged 3 minutes at 16100 x g; the supernatant was removed to a new microfuge tube and the plasmid was isolated using Phenol/Chloroform extraction as described in section 3.3.4.1. DNA, including plasmids, was isolated from *Salmonella* strains using the method described by Kado and Liu (Kado and Liu, 1981), the method is similar to the mentioned above for *Escherichia coli* but using the Kado TE buffer, Kado lysis buffer and Kado KOAc instead of solution I, lysis buffer and solution III, respectively and the lysis incubation was carried out for 30 minutes at 65°C. The formulations of the solutions are described in Chapter 2.

3.3.5 Enzyme restriction

In order to open DNA plasmids, prepare PCR products for ligation and cloning or testing cloned-plasmids, the DNA was cleavaged at specific places using the endonucleases *Bam*HI (10 U/ µl) and *Hind*III (10 U/ µl) from Promega. If not otherwise mentioned, 35 µl of sterile double distilled water, 5 µl DNA, 5 µl of the corresponding buffer and 5 µl of the respective enzyme were incubated at 37°C for 6 hours and controlled by gel electrophoresis.

3.3.6 Ligation

After enzyme restriction, the DNA fragments were joined with the selected vector, for further bacterial transformation using the enzyme T4 Ligase from Promega and two different protocols.

A. After the endonuclease restriction, the DNA fragments were added to a concentration ca. 1:1 insert: vector, mixed with 2 -3 µl ligase buffer (10% of the final volume) and double distilled water to a final volume of 20-30 µl and incubated at 37°C for 10 minutes, after incubation 2 µl of ligase were added, mixed and incubated at 37°C for 20 minutes and overnight at room temperature or 4°C.

B. DNA fragment and vector were mixed and dried with vacuum. 16 µl of double distilled water, 2 µl ligase buffer and 2 µl ligase were added and the mixture was incubated overnight at room temperature.

3.3.7 Bacterial transformation

The ligation reaction products or plasmid vector were introduced into *Escherichia coli* DH5α or *Salmonella* strains using electroporation. The successfully transformed bacteria were further selected by Blue White selection. The plasmids were purified, screened and sequenced commercially by LGC AGOWA Genomics and the data were analyzed using DNA and protein databases (www.ncbi.nlm.nih.gov).

3.3.7.1 Preparation of electrocompetent cells

The bacterial cells were prepared for introduction of plasmids or ligation products by electroporation; the recipient bacteria were washed extensively in glycerol/double distilled water solution to remove trace of salt that could cause heating of the cells resulting in cell death when the electric pulse field is applied to them.

5 ml of LB broth containing the appropriated antibiotic were inoculated with one bacterial colony from Lennox agar plate, this culture was incubated at 37°C and 200 rpm until OD_{600} approx. 2. The bacterial culture was distributed in 4 microfuge tubes and centrifuged at 8000 x g for 5 min at room temperature. The bacterial pellet was resuspended in 2 ml of ice-cold 10% glycerol and equally distributed in 2 microfuge tubes on ice and centrifuged at 8000 x g for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 1 ml of ice-cold 10% Glycerol and centrifuged at 6000 x g for 5 min at 4°C, the supernatant was discarded. The suspension in ice-cold 10% glycerol on ice and the centrifugation at 4°C for 5 min, were repeated two more times at 3000 x g and at 1500 x g, the pellets were resuspended in 100 µl of 10% ice-cold glycerol each, pooled and stored at -80°C. For preparation of major amounts of electrocompetent cells and for further introduction of ligation products, quantity of bacterial culture and 1X 10% ice-cold glycerol were scaled up and the centrifugation time was raised to 10 min.

3.3.7.2 Electroporation

In the electroporation process, the bacteria are mixed with DNA and briefly exposed to a strong electric field. The electric fields across the cellular membranes could create artificial hydrophilic pores that permit the entry of DNA; the transformed bacteria recover and continue to grow (Snyder and Champness, 2007). 5-10 µl of the ligation products or 1 µl of plasmids, were added to approximately 100 µl of electrocompetent cells (thawed on ice) and transferred to a precooled electroporation cuvette and briefly exposed to an electric field pulse (25 µF, 400 Ω, 2500 V) in an electroporator. The electroporation mixture was taken with 900 µl of L- broth and incubated at room temperature for 60 minutes. After incubation, the mixture was platted on X-Gal agar or Lennox agar with the respective selective antibiotic for further screening of the successfully transformed bacteria.

3.3.8 Mutagenesis

The *S. enterica* serovar Typhimurium strain SL1344 harbouring the complete non-polar deletion of the *avrA* gene was constructed using the method described by Datsenko and Wanner (Datsenko and Wanner, 2000). Owing to the high sequence homology between *S.* Typhimurium strains, the Δ*avrA* deletion was first constructed in *S.* Typhimurium strain LT2 and transferred by *Salmonella*-specific bacteriophage P22 transduction into strain SL1344. Briefly, mutagenic primers containing 3′ sequence homology to the regions flanking a kanamycin resistance cassette present on plasmid pKD4 (Datsenko and Wanner, 2000) and 5′ flanking sequence homology to the *avrA* gene were used to amplify the kanamycin cassette and FRT recombination sites present immediately upstream (5′) and downstream (3′) of the kanamycin resistance cassette by PCR. After determination of the quality of the PCR product (single band on agarose gel electrophoresis of the correct size of 1,1 kilobases), the PCR product was used to transform a derivative of strain LT2 harbouring the plasmid pKD46, which contains the genes encoding the bacteriophage λ Red recombination system.

The λ red recombination genes present on plasmid pKD46 under control of the arabinose-inducible P_{BAD} promoter. In the absence of arabinose, the P_{BAD} promoter is repressed by the AraC protein, also encoded on plasmid pKD46. Prior to transformation with the PCR product, electrocompetent cells were first prepared from arabinose-induced cultures of strain LT2 harbouring pKD46 to induce the expression of the λ red recombination genes. PCR products generated using pKD4 as the template contain 5′ and 3′ regions with sequence homology to flanking regions outside of the gene being targeted, and immediately downstream of these regions, a copy of the FRT recombination targeting site for the λ Red recombinase. In strains expressing the Red recombinase, the recombinase

recognizes the FRT sites and mediates a homologous recombination between the flanking homologous sequences of the PCR product and sequences present on the chromosome, effectively exchanging/deleting the coding sequence of the targeted gene for the kanamycin cassette present on the PCR product. Plasmid pKD46 also harbours a temperature-sensitive *repA* gene, and is therefore is only capable of replication at temperatures at or below 30°C.

After electroporation with the mutagenic PCR products, the transformed cells were diluted 1:10 in Lennox broth, and plated to Lennox agar plates containing 25 µg/ml kanamycin at 37°C for selection of recombinants and loss of pKD46. Kanamycin-resistant colonies were picked and inoculated for preparation of lysates for PCR screening verification of the recombination using combinations of primers specific for chromosomal regions flanking the insertion site and internal primers complementary to regions within the kanamycin cassette. Both the 5′ and 3′ regions of the targeted gene are verified in this manner to assure both the successful deletion of the targeted gene as well as the correct orientation in the chromosome. Positive PCR reactions are generated only when the kanamycin cassette has successfully replaced the targeted gene. Of four kanamycin-resistant colonies obtained from the mutagenesis of the *avrA* gene, all four were found to show the correct PCR amplification products. From one of the isolates, bacteriophage P22 lysates were prepared for transduction of kanamycin-resistance into strain SL1344 by infection of strain SL1344 with P22 lysates prepared on the LT2 deletion strain and plating to Lennox agar plates containing kanamycin. Putative transductants were purified in a second step on Green plates to assure the absence of infected cells and/or lysogens, and bacteriophage-free colonies were inoculated into Lennox broth containing kanamycin for preparation of bacterial stocks and lysates for a second PCR screening for the presence of the gene replacement/deletion.

After PCR verification of the gene deletion/replacement with the kanamycin cassette, the kanamycin cassette was removed by introduction of the plasmid pCP20 (Cherepanov and Wackernagel, 1995), which harbours the FLP-recombinase, the bacteriophage λ *c*I857 gene, and a temperature-sensitive origin of replication. The FLP-recombinase is under transcriptional control of the bacteriophage λ P_L promoter, which is in turn repressed by the cI protein. The cI857 allele is also temperature-sensitive, being inactivated at or above 37°C. After electroporation of the kanamycin-resistant deletion strain with pCP20, if the transformants are plated in the absence of kanamycin at 37°C, the cI857 protein is inactivated, and the λ P_L promoter can express the FLP-recombinase. The FLP-recombinase recognizes the FRT sites flanking the kanamycin cassette in the chromosome, and recombines out all DNA sequences between the FRT sites, effectively removing the kanamycin cassette, and leaving only one FRT sites of approximately 30 nucleobases. Plasmid pCP20 is also lost during incubation owing the temperature-sensitive origin of replication. In this manner, both the deletion strains harbouring the kanamycin

cassette (designated Δ*avrA*::*kan*) and deletion strains in which the kanamycin cassette had been removed (designated Δ*avrA*) were generated.

3.3.9 RNA extraction

3.3.9.1 Bacterial RNA extraction

In order to determinate the transcription of the *avrA* gen in different *Salmonella* strains, total bacterial RNA was extracted for reverse transcription-PCR analyses. 5 ml of LB broth containing the appropriate antibiotic were inoculated with one bacterial colony and incubated at 37°C under agitation until OD_{600} of approximately 2, this bacterial culture was added to a glass 15 ml Corex tube containing 1 ml stop solution (5% phenol in ethanol) and placed on ice for approx. 30 min. The glass Corex tubes were previously heat sterilized in an oven at 150°C for 4 hours to assure the absence of RNAse. The bacteria were collected by centrifugation at 3220 x g and 4 °C for 15 min. For bacterial lysis, the pellet was resuspended in 25 mM Tris-HCl (pH 7,0 – 7,5) by vortexing and an equal volume of lysis solution preheated at 100°C were added, the mixture was boiled for 30 s - 1 min to obtain a clear solution and maintained at room temperature. The cell lysate was diluted with 1 ml DEPC-water and distributed in 4 microfuge tubes with 500 µl each. The Phenol/Chloroform extraction was used as described in section 3.3.4.1 using 500 µl of phenol and 500 µl of chloroform and repeating both precipitation steps twice, after each centrifugation the upper phase containing the nucleic acids was recovered in a new microfuge tube. The nucleic acids were precipitated by addition of 2 - 2,5 volumes of ice cold absolute ethanol and 10% NaOAc 3M and incubated overnight, the nucleic acids were collected by centrifugation at 16100 x g and 4°C for 1 hour, the pellet was washed with 1 ml 70% ethanol-DPEC and dried. The nucleic acid pellet was resuspended in 50 µl DPEC water and 25 µl of this solution were used to DNase digestion adding 5 µl of RNAse-free DNAse, 5 µl DNAse Buffer (10 X) and 15 µl RNAse free DPEC water and incubated at 37°C for 2 - 4 hours, 200 µl DPEC water were added and the Phenol/Chloroform extraction and the precipitation overnight in absolute ethanol were repeated as described above. The total RNA pellet was resuspended in 20 - 30 µl DPEC water and stored at - 20°C. The samples were verified for intact ribosomal RNA (rRNA) by electrophoresis in 2% agarose gels containing ethidium bromide.

3.3.9.2 RNA isolation from eukaryotic cells

In order to analyse the effect of *Salmonella* infection in the mRNA expression levels of different eukaryotic genes relevant in the host immune response against infection, porcine

and human eukaryotic cell lines seeded in 24 well plates, were infected with *Salmonella* strains at MOI of approximately 10, inoculating 100 µl of a 10^6 cfu/ ml bacterial dilution in each cell-well as described in the Gentamicin protection assay. The total RNA from porcine macrophage (PLN/C2), porcine intestinal epithelial (IPEC-J2), human macrophage (THP-1) and human intestinal epithelial (LoVo) cell lines were extracted 4 and 24 hours after infection with the respective *Salmonella* strain.

The eukaryotic cells were direct lysed in the 24 well-plate using the RNeasy mini kit from Qiagen according to the instructions given by the manufacturer. The cells were washed with 1X PBS and lysed adding 350 µl of RLT Buffer to each well, the RNA was isolated in the RNeasy spin column, eluted with RNase-free water and stored at -80°C for further measure of the RNA concentration and qRT-PCR analysis.

3.3.10 Determination of nucleic acids concentration

The concentration of DNA and RNA were measured using Nanodrop spectrophotometer ND-1000 from peqlab, according to the manufacturer's instructions.

3.3.11 Real-time quantitative reverse transcription PCR (qRT-PCR)

Quantitative reverse transcription PCR (qRT-PCR) was used to quantify the relative amounts of IL-8, IL-1β, JUN mRNA in the human LoVo and THP-1 cell lines and IL-8 in porcine cells IPEC-J2 and PLN/C2 after *Salmonella* infection. The transcription of *avrA* gene in *Salmonella* strains were confirmed using reverse transcription PCR. The RNA was isolated from eukaryotic and bacterial cells as described above and the RNA concentration was measured using a Nanodrop device. The mRNA was reverse transcribed into complementary DNA (cDNA) using M-MLV-reverse transcriptase (Promega). The RNA solution containing 2 µg of RNA were mixed with 2 µl Random primer (500 µg / ml) and denatured at 70°C for 5 minutes, then 13,4 µl of a reaction mix containing 5 µl reverse transcriptase buffer, 6,8 µl dNTPs (2 mM), 0,6 µl RNase inhibitor (40 U/ µl) and 1 µl M-MLV (200 U/ µl) reverse transcriptase were added to the RNA mixture and incubated at 37°C for 1 hour. The resulting cDNA solution was diluted 1:10 in HPLC water and used to perform Real Time PCR in a StepOne Plus Real-Time PCR System (Applied Biosystems) and using the Power SYBR® Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions.

In eukaryotic cells, Glyceraldehyde-3-phosphate dehydrogenase gene (GADPH) was used as a reference gene for the qRT-PCR. GADPH catalyses the oxidative phosphorylation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate during glycolysis and the reverse reaction during gluconeogenesis and is also implicated in DNA

replication, repair and apoptosis; therefore, GADPH is essential for the cells and is consecutively and universally expressed (Corbin *et al*., 2002; Zainuddin *et al*., 2010). The *dna*A gene involved in bacterial DNA replication was used as reference gene or internal control for the reverse transcription PCR using bacterial RNA. The quantification of the expression of the eukaryotic target genes was made applying the comparative threshold cycle (CT) method and using for the eukaryotic qRT-PCR the uninfected cells as reference sample or calibrator (Livak and Schmittgen, 2001). First, the difference in CT for the target gene and the reference gene (ΔCT) was calculated in order to normalize for differences in nucleic concentration and other experimental variations. The ΔCT of each sample was subtracted from the ΔCT of the reference sample (uninfected cells) obtaining the ΔΔCT value. Finally, the level of expression of the normalized target gene in the infected cells relative to its expression in the uninfected cells was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The results were repeated in three independent experiments. In the real time PCR, a denaturation and extension temperature of 95°C and 72°C were used, respectively, and the annealing temperature was adjusted for each cell line and gene as following:

Parameter	**Annealing temperature**
Human cells: THP-1 and LoVo Genes: IL-8 and JUN	55°C
Human cells: THP-1 and LoVo Gene: IL-1β	60°C
Porcine cells: PLN/C2 and IPEC-J2 Gene: IL-8	55°C
Bacterial cells: *Salmonella* Gene: *avrA*	55°C

3.4 Statistical analysis

The statistical analysis in this study was performed using the software Statistical Package for Social Science SPSS 19.0 and applying linear models with Tukey's post-hoc test. In order to adjust for possible plate effects we included well-plate as a factor in all models. We did not perform further adjustments for multiple testing. The level of significance was $\alpha = 0{,}05$. When it was necessary data was transformed to achieve normality and equal variance within the groups. In particular we applied following transformations:

Data	**Transformation**
Rel. Invasion (MOI = 1)	log(x)
Intracellular Persistence 24h post infection (MOI = 1)	log(x)
IL-8 4 h post infection	log10(x+1)
IL-8 24 h post infection	log10(x+1)
IL-1β 4 h post infection	log10(x+1)
IL-1β 24 h post infection	log10(x)
JUN 4 h post infection	log10(x+1)
JUN 24 h post infection	log10(x+1)
NFκB 2 h post infection	None
NFκB 4 h post infection	None
NFκB 24 h post infection	None
Relative Invasion (MOI = 5)	Sqrt
Intracellular Persistence 24 h post infection (MOI = 5)	log10

4 RESULTS

4.1 Comparison of invasiveness and intracellular persistence of *S.* Typhimurium and *S.* Choleraesuis in porcine and human intestinal epithelial and macrophage cell lines

The ability to invade host intestinal epithelial cells and macrophages and the capacity to replicate within these cells is crucial for *Salmonella* infection and virulence. In this study, the invasion and intracellular persistence of *S.* Typhimurium and *S.* Choleraesuis were compared to determine whether differences in these two important aspects of infection are at least in part responsible for the variation in host specificity and pathogenesis between these two *Salmonella* serovars. Moreover, a *Salmonella* serovar Typhimurium Δ*avrA* mutant and a *Salmonella* serovar Choleraesuis spontaneous rough mutant strain were used to explore the influence of these variations in invasion and intracellular persistence 24 hours p.i.

Gentamicin resistance invasion assays (Shaw and Falkow, 1988) were performed to compare the invasiveness and intracellular persistence of *S.* Typhimurium and *S.* Choleraesuis. Gentamicin is only poorly capable of entering eukaryotic cells, and therefore preferentially targets extracellular bacteria, thus only the bacteria that are able to invade and survive within the eukaryotic cells will be protected against its microbicidal activity.
S. Typhimurium and *S.* Choleraesuis are able to infect both humans and swine causing infection with different outcomes; therefore, in this study the intestinal porcine epithelial cell–jejunum 2 (IPEC-J2), the porcine macrophage cell (PLN/C2) and the human colorectal epithelial cell line (LoVo) were selected. These cell lines provide species- and cell type-relevant *in vitro* model systems for porcine and human pathogen-host interactions (Drewinko *et al.*, 1976; Tsuchiya *et al.*, 1982; Schierack *et al.*, 2006; Brosnahan and Brown, 2012). The PLN/C2 cell line is a monocyte/macrophage cell line derived from porcine peripheral blood mononuclear cell fractions (PBMCs). The cell line was found to be positive for characteristic macrophage markers such as CD14 (LPS-receptor), CD16 (Fc-receptor), MHC-I and -II, the porcine monomyeloid marker SWC3a, and SWC9, the latter a marker for fully differentiated macrophages (unpublished results, C. Tölke/K. Tedin).

The strains *S.* Typhimurium SL1344 wild type and its Δ*avrA* isogenic mutant were used to compare invasion and persistence 24 hours p.i. with *S.* Choleraesuis strains and also to investigate the possible role of the presence or absence of the *avrA* gene in host specificity in these serovars. Moreover, The *S.* Choleraesuis strain A50 wild type and the *S.* Choleraesuis strain SARB4 spontaneous rough mutant with a substantially reduced or absent LPS *O*-chain was used in these assays to explore also the impact of this spontaneous mutation in invasion and intracellular persistence.

Cell monolayers were infected with the *Salmonella* strains at a 1:1 ratio of bacteria to eukaryotic cell (multiplicity of infection, MOI = 1). At 2, 4 and 24 hours post-infection, the eukaryotic cells were lysed and the intracellular bacteria were calculated. The assays were repeated at least three times for each cell line and strain tested.

4.1.1 Invasiveness *S.* Typhimurium versus *S.* Choleraesuis in human and porcine intestinal epithelial and porcine macrophage cell lines

The invasion of eukaryotic cells is a dynamic process depending not only on the invasion ability of each bacterial strain so furthermore the specific susceptibility of the host cell to each strain, taking this into consideration, the invasiveness of the above described strains *S.* Typhimurium and *S.* Choleraesuis were compared in porcine (IPEC-J2, PLN/C2) and in human cell lines (LoVo). Invasion or internalization of *Salmonella* in the cells is calculated as the ratio of the number of recoverable colony forming units 2 hours post infection and the number of bacteria (cfu) used to infect the cells and is expressed as a percentage of this initial bacterial inoculum.

According to the invasion assay results, the host-adapted *S.* Choleraesuis (A50 wild type) showed a significantly lower ability to invade human (LoVo) and porcine (IPEC-J2) intestinal epithelial cells as well as porcine macrophage (PLN/C2) cells compared to the broad host range *S.* Typhimurium (Figure 5; $P \leq 0{,}001$). This result suggests that the higher virulence associated with *S.* Choleraesuis is not dependent on enhanced invasiveness compared to *S.* Typhimurium as previously reported in *in vivo* experiments in swine (Paulin *et al.*, 2007). As it is not involved in the invasion process, as expected, the deletion of the *avrA* gene in *S.* Typhimurium did not impair host cell invasion in the cell lines tested.

In contrast, the *S.* Choleraesuis strain SARB4 spontaneous rough mutant showed a significantly higher capacity to invade human intestinal epithelial cells (LoVo) and porcine macrophage (PLN/C2) cells than the *S.* Choleraesuis A50 wild type strain (Figure 5; $P \leq 0{,}001$). Furthermore, the *S.* Choleraesuis rough mutant and *S.* Typhimurium strains were equally invasive in both LoVo and PLN/C2 cells, in contrast with the results obtained in invasion assays performed to analyze the role of the presence or lack of the *avrA* gene (section 4.2.2) where the *S.* Choleraesuis SARB4 wild type strain was less invasive than *S.* Typhimurium in all cell lines tested. In porcine intestinal epithelial cells IPEC-J2 no significant difference was observed between *S.* Choleraesuis strain A50 and the strain SARB4 rough mutant. These results suggest that the spontaneous mutation of LPS O-antigen may facilitate the invasion of *S.* Choleraesuis in human intestinal epithelia (LoVo) and porcine macrophage (PLN/C2) cell lines.

When comparing the invasiveness of the *Salmonella* strains in the porcine and human intestinal epithelial cells, the human intestinal epithelial cells were significantly less susceptible to invasion by the strain *S.* Typhimurium compared to the porcine intestinal epithelial cells (Figure 5; $P < 0,05$). Here, human intestinal epithelial cell line was significantly more susceptible to the invasion by the strain *S.* Choleraesuis SARB4 rough mutant than the porcine intestinal epithelial cell line tested (Figure 5; $P \leq 0,001$).

These results highlight that the invasiveness of these *Salmonella* serovars depends not only on the serovar or strain which causes the infection, but also on the host and the specific cell type infected, consistent with the complexity of host pathogen interactions in the context of *Salmonella* infection. Additionally, these results indicate that the virulence and higher rate of systemic disease of *S.* Choleraesuis in swine and humans is not directly related to enhanced invasion of human and porcine intestinal epithelial and porcine macrophage cells.

The invasiveness of both *Salmonella* serovars in porcine epithelial and macrophage cell lines was also compared. Both *S.* Choleraesuis strains showed a significant higher ability to invade the porcine macrophage cell line than the porcine intestinal epithelial cell line (Figure 5; $P < 0,05$). In contrast, *S.* Typhimurium showed a significantly lower ability to invade porcine macrophage than porcine intestinal epithelial cells (Figure 5; $P \leq 0,001$). The observed higher ability of *S.* Choleraesuis to invade porcine macrophage rather than epithelial cells together with the observed affinity of *S.* Typhimurium to invade porcine epithelial cells rather than macrophages, could be associated with the systemic and gastrointestinal manifestation of infection frequently caused by these serovars, respectively.

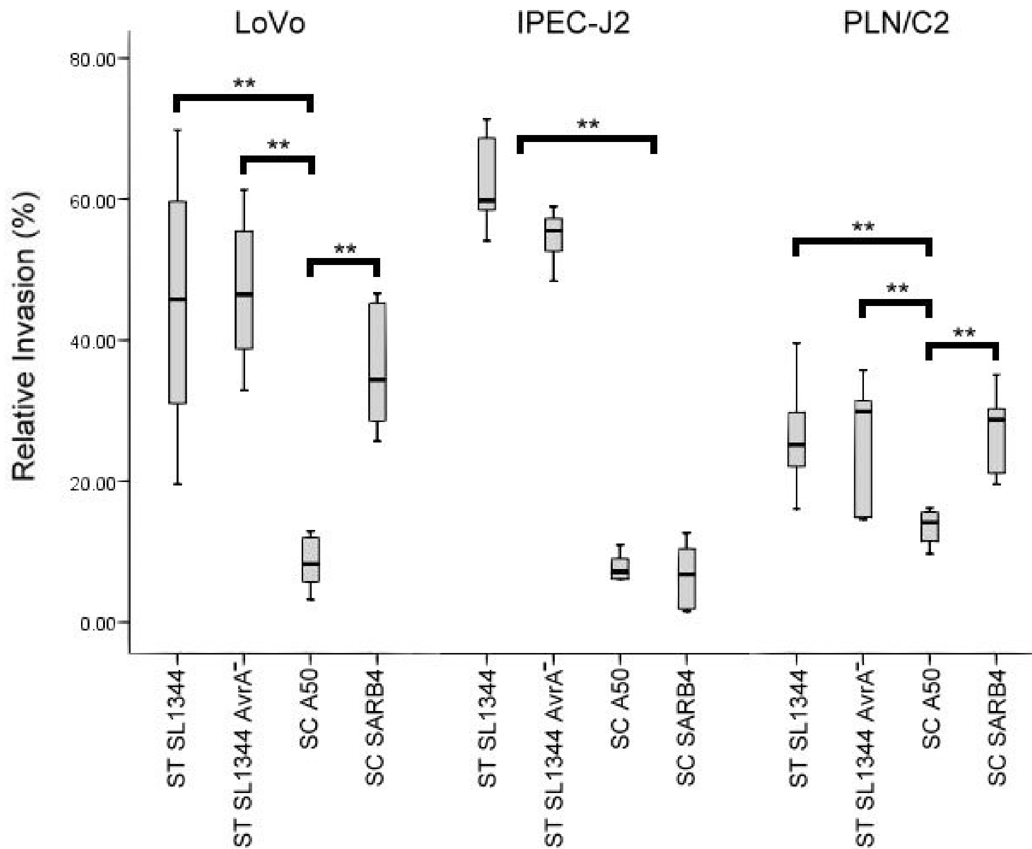


Figure 5: Comparison of invasiveness *S.* Typhimurium versus *S.* Choleraesuis in human and porcine intestinal epithelial and porcine macrophage cell lines. Human intestinal epithelial (LoVo), porcine intestinal epithelial (IPEC-J2) and porcine macrophage (PLN/C2) cell lines were infected with *S.* Typhimurium (ST) SL1344 wild type and an Δ*avrA* mutant strain (AvrA-) and *S.* Choleraesuis (SC) strains SARB4 and A50 at a MOI of 1. The eukaryotic cells were lysed 2 hours post-infection and the intracellular bacteria were calculated. Values represent the ratio of cfu 2 hours post infection to the initial bacterial inoculum and are expressed as a percentage of this initial inoculum. The diagram represent the distribution of data of at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentiles, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line inside the box represents the median, and the ends of the whiskers show minimum and maximum values. Transformation: log (x). Statistic test: Bonferroni. ** = P ≤ 0,001; * = P < 0,05.

4.1.2 Intracellular persistence *S.* Typhimurium versus *S.* Choleraesuis in human and porcine intestinal epithelial and porcine macrophage cell lines

The gentamicin protection (invasion) assays described were also used to compare the intracellular persistence of *S.* Typhimurium and *S*. Choleraesuis in human and porcine intestinal epithelial and porcine macrophage cell lines 24 hours post-infection. The intracellular persistence of each strain was determined as the number of bacteria cfu 24 hours post-infection compared to the bacteria cfu 2 hours post infection, for each cell line.

In the porcine intestinal epithelial cell line, both strains of *S*. Choleraesuis (A50 and SARB4 rough mutant) presented significantly less intracellular persistence than the strain *S*. Typhimurium (Figure 6; $P \leq 0{,}001$). However, in the human intestinal epithelial cell line, the strain *S*. Choleraesuis A50 showed significantly more intracellular persistence than the strains *S*. Typhimurium (Figure 6; $P \leq 0{,}001$) and the strain *S*. Choleraesuis SARB4 rough mutant (Figure 6; $P \leq 0{,}001$). Also, *S*. Choleraesuis SARB4 rough mutant showed lower persistence in the LoVo cell lines than the strains *S*. Typhimurium (Figure 6; $P \leq 0{,}001$). In contrast, the *S.* Choleraesuis rough mutant strain showed higher intracellular persistence in porcine macrophage compared to the *S.* Choleraesuis strain A50 wild type (Figure 6; $P < 0{,}05$) and the *S*. Typhimurium strains (Figure 6; $P \leq 0{,}001$).

The absence of the *avrA* gene did not affect the intracellular persistence of *S.* Typhimurium compared with the wild type in the porcine and human cell lines tested. *S.* Choleraesuis A50 strain showed a lower ability to invade all the cell lines tested, but a better capacity to persist within human epithelial cells than serovar Typhimurium. The intracellular persistence in the human and porcine epithelial and porcine macrophage cell lines tested seems to be host-, serovar- and strain-specific. Thus, in order to further investigate and compare the difference in invasion, intracellular persistence and activation of the host immune response between *Salmonella* serovars Typhimurium and Choleraesuis the strain *S.* Choleraesuis SARB4 wild type strain was used rather than the rough mutant derivative of SARB4.

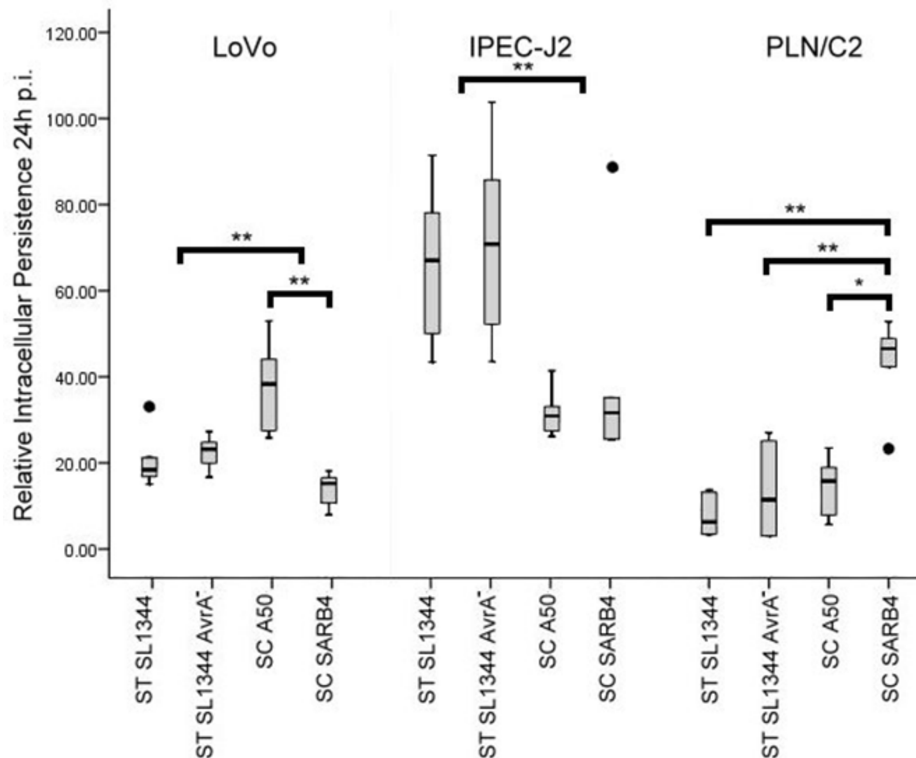


Figure 6: Comparison of intracellular persistence *S.* Typhimurium versus *S.* Choleraesuis in human and porcine intestinal epithelial and porcine macrophage cell lines. Porcine intestinal epithelial (IPEC-J2), human intestinal epithelial (LoVo) and porcine macrophage (PLN/C2) cell lines were infected with *S.* Typhimurium (ST) SL1344 wild type and an Δ*avrA* mutant strain (AvrA-) and the *S.* Choleraesuis (SC) strains SARB4 and A50 at a MOI of 1. At 2 and 24 hours post-infection, the eukaryotic cells were lysed and the intracellular bacteria were calculated. Values represent the ratio of cfu 24 hours post infection to the cfu 2 hours post infection. The diagram represent the distribution of data of at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentiles, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line inside the box represents the median, and the ends of the whiskers show minimum and maximum values, the circles represent the Outliers, values greater than 1.5 times the IQR. Transformation: log (x). Statistic test: Bonferroni. ** = $P \leq 0,001$; * = $P < 0,05$.

4.2 Effects of the differential presence of the *avrA* gene in *S.* Choleraesuis and *S.* Typhimurium on host invasion and persistence and host innate immune response

Salmonella effector proteins such as AvrA have been shown to be involved in manipulating host processes and play important roles in the outcomes of infection (Wu e*t al*., 2011; Liu e*t al*., 2010). Furthermore, the absence of the *avrA* gene in *Salmonella* serovars has been suggested to be related with higher rate of systemic infection and host range restriction, since it is present in *Salmonella* serovars with a broad host range such as serovar Typhimurium but is missing in the host-restricted *Salmonella* serovar Typhi and the host-adapted serovar Choleraesuis, both of which are associated with high rates of systemic infections (Hardt and Galán, 1997; Streckel *et al*., 2004; Wollin, 2007). The *avrA* gene is also present in *S.* Dublin which is also a host-restricted serovar which causes high rates of systemic disease (Wollin, 2007; Suez *et al*., 2013), but have been reported to produce low levels of AvrA protein (Streckel *et al*., 2004). The role of the presence or lack of the *avrA* gene in host range, outcome of infection and lower rate of systemic disease is therefore not entirely clear. Therefore, to analyze the possible effects of the presence or absence of the *avrA* gene in *S.* Typhimurium and *S.* Choleraesuis, respectively, an *avrA*+ *S.* Choleraesuis strain and an Δ*avrA S.* Typhimurium mutant strain were constructed and used to infect epithelial and macrophage cell lines from human and porcine origin. These strains were used to analyze the effects of *avrA* gene on invasion, intracellular persistence and immune response such as activation of the transcription factor NFκB and mRNA expression levels of inflammatory cytokines IL-8 and IL-1β and Jun.

4.2.1 Construction of an *avrA*+ *S.* Choleraesuis strain and a Δ*avrA* and an *avrA*+ *S.* Typhimurium mutant strains

In order to analyze the effects of the differential presence of the *avrA* gen in *S*. Typhimurium and *S*. Choleraesuis and whether this factor could be involved in the difference in host-specificity and pathogenesis between these serovars in humans and swine, the *avrA* gene from *S*. Typhimurium was cloned and introduced into *S.* Choleraesuis, which does not have this gene. The *avrA* gene including its own promoter from three different *S*. Typhimurium strains LT2, 4/74 and ATCC 14028 were amplified by PCR for cloning. These three different *S*. Typhimurium strains were chosen as the source for the *avrA* gene as strain-specific differences in the sequences and/or activities of AvrA had previously been reported (Jarvik *et al*., 2010; Du and Galán, 2009). The PCR products were purified and the resulting PCR fragments were restricted with the enzymes *Bam*HI and *Hin*dIII for cloning into the low-copy vector pWSK29, also restricted with these enzymes. The ligation reaction products were introduced into the strain *Escherichia coli*

DH5α using electroporation. Since the vector pWSK29 harbours multiple cloning sites within the coding sequence for the β-galactosidase α fragment, recombinant colonies (white) were identified among non-recombinant ones (blue) after plating of the transformation mixtures onto selective plates containing the chromogenic substrate 5-bromo-4chloro-3indolyl-β-D-galactopyranoside (X-Gal) (Snyder and Champness, 2007). White colonies were selected and the plasmids were purified. Plasmids containing the cloned genes were screened, sequenced and compared with the published AvrA amino acid sequence of *S*. Typhimurium strain LT2 (Figure 7).

A. Predicted amino acid sequence of AvrA from *S.* Typhimurium strain LT2 (GenBank)

MIFSVQELSCGGKSMLSPTTRNMGASLSPQPDVSGELNTEALTCIVERLESEIIDGSWIHISYEETDLEMMPF
LVAQANKKYPELNLKFVMSVHELVSSIKETRMEGVESARFLVNMGSSGIHISVVDFRVMDGKTSVILFEPAAC
SAFGPALLALRTKAALEREQLPDCYFAMVELDIQRSSSECGIFSLALAKKLQLEFMNLVKIHEDNICERLCGE
EPFLPSDKADRYLPVSFYKHTQGAQRLNEYVEANPAAGSSIVNKKNETLYERFDNNAVMLNDKKLSISAHKKR
IAEYKSLLKP-

B. Predicted amino acid sequence of AvrA cloned from *S.* Typhimurium strain 4/74

MIFSVQELSCGGKSMLSPTTRNMGASLSPQPDVSGELNTEALTCIVERLESEIIDGSWIHISYEETDLEMMPF
LVAQANKKYPELNLKFVMSVHELVSSIKETRMEGVESARFLVNMGSSGIHISVVDFRVMDGKTSVILFEPAAC
SAFGPAL—ALRTKAALEREQLPDCYFAMVELDIQRSSSECGIFSLALAKKLQLEFMNLVKIHEDNICERLCGE
EPFLPSDKADRYLPVSFYKHTQGAQRLNEYVEANPAAGSSIVNKKNETLYERFDNNAVMLNDKKLSISAHKKR
IAEYKSLLKP-

C. Predicted amino acid sequence of AvrA cloned from *S.* Typhimurium strain LT2

MIFSVQELSCGGKSMLSPTTRNMGASLSPQPDVSGELNTEALTCIVERLESEIIDGSWIHISYEETDLEMMPF
LVAQANKKYPELNLKFVMSVHELVSSIKETRMEGVESARFLVNMGSSGIHISVVDFRVMDGKTSVILFEPAAC
SAFGPALLALRTKAALEREQLPDCYFAMVELDIQRSSSECGIFSLALAKKLQLEFMNLVKIHEDNICERLCGE
EPFLPSDKADRYLPVSFYKHTQGAQRLNEYVEANPAAGSSIVNKKNETLYERFDNNAVMLNDKKLSISAHKKR
IAEYKSLLKP-

D. Predicted amino acid sequence of AvrA cloned from *S.* Typhimurium strain ATCC 14028

MIFSVQELSCGGKSMLSPTTRNMGASLSPQPDVSGELNTEALTCIVERLESEIIDGSWIHISYEETDLEMMPF
LVAQANKKYPELNLKFVMSVHELVSSIKETRMEGVESARFLVNMGSSGIHISVVDFRVMDGKTSVILFEPAAC
SAFGPALLALRTKAALEREQLPDCYFAMVELDIQRSSSECGIFSLALAKKLQLEFMNLVKIHEDNICERLCGE
EPFLPSDKADRYLPVSFYKHTQGAQRLNEYVEANPAAGSSIVNKKNERFMSDSITMPLC

Figure 7: Predicted amino acid sequence of cloned *avrA* gene used to construct *avrA* + *S.* Choleraesuis and *S.* Typhimurium strains. The *avrA* gene from *S.* Typhimurium strains LT2, 4/74 and ATCC 14028 was amplified by PCR and cloned into the vector pWSK29. Plasmids containing the cloned genes were screened and sequenced. The Figure shows the predicted amino acid sequences of the AvrA gene product encoded on these plasmids. The gray boxes indicate the Leu139 residue. **A.** Amino acid sequence AvrA from LT2 taken from the published sequence in GenBank and verified in this work. **B.** Amino acid sequence of AvrA from strain 4/74 indicating the missing Leu139 residue. **C.** Amino acid sequence AvrA from strain LT2. **D.** Amino acid sequence AvrA from strain ATCC 14028, indicating the truncated, altered C-terminal region of AvrA in this strain.

The sequence of the *avrA* gene from *S.* Typhimurium strain 4/74 showed a three nucleotide deletion resulting in the absence of a leucine residue at position 139 (Leu139) (Figure 7B), as previously described by Du and Galán for the strain *S.* Typhimurium SL1344 which is derived from the strain 4/74 (Du and Galán, 2009). This leucine deletion is located in a conserved region of the AvrA protein family, raising the possibility that this mutation may be functionally significant. Du and Galán found that the AvrA protein from the *S.* Typhimurium strain LT2 is capable of inhibiting MAP kinase signaling in yeast and that the *S.* Typhimurium strain SL1344 harbours an allele incapable of exerting this activity (Du and Galán, 2009). In the sequence analysis it was found that the *avrA* gene from *S.* Typhimurium strain ATCC 14028 was truncated compared to the *avrA* gene from the strain LT2, resulting in a 36 amino acid deletion and 12 amino acid, non-homologous replacement of the C-terminal region of the AvrA protein in this strain (Figure 7D). This truncation has also been reported by Jarvik and co-workers but its effect in the activity of the AvrA protein is unclear (Jarvik *et al*., 2010). In this study, the plasmid harbouring the *avrA* gene from *S.* Typhimurium LT2 was selected to transform the *S.* Choleraesuis strain SARB4 and the *S*. Typhimurium SL1344 *avrA* gene deletion strain. The *S.* Typhimurium strain SL1344 harbouring the complete non-polar deletion of the *avrA* gene was constructed by using the method described by Datsenko and Wanner (Datsenko and Wanner, 2000). The strain *S.* Typhimurium SL1344, was selected for these experiments rather than the strain LT2 due to the attenuation of virulence in LT2 (Swords *et al*., 1997; Jarvik *et al*., 2010). In contrast, strain SL1344 is a standard, virulent strain used as a model for bacterial pathogenesis, and causes systemic infections in mice. The deletion of the *avrA* gene of strain *S.* Typhimurium SL1344 and the subsequent complementation with the cloned gene from LT2 on plasmid pWSK29-$avrA_{\text{LT2}}$ was performed due to the previously described missing activity reported for AvrA in SL1344 (Du and Galán, 2009). Both the wild type *S*. Choleraesuis strain SARB4 and *S*. Typhimurium SL1344 Δ*avrA* mutant strain were transformed with the plasmid harbouring the cloned wild type *avrA* gene (pWSK29-$avrA_{\text{LT2}}$) or the vector plasmid (pWSK29), in order to compare the effects of the presence or absence of the *avrA* gene in these two *Salmonella* serovars. The transformed strains were used to analyze the virulence properties of the presence of the *avrA* gene in both *Salmonella* serovars, as well as to determinate the effects on NFκB and JNK activity and expression of pro inflammatory cytokines during infection in epithelial and macrophage cell lines from porcine and human origin. Prior to use in invasion assays and determination of the effects on NFκB and/or AP-1 activation, the mRNA expression of the *avrA* gene in the *avrA*+ strains as well as the no *avrA* mRNA expression in the *S*. Choleraesuis wild type strain and in the *S*. Typhimurium Δ*avrA* mutant were confirmed using reverse transcription PCR (RT-PCR) (Figure 8).

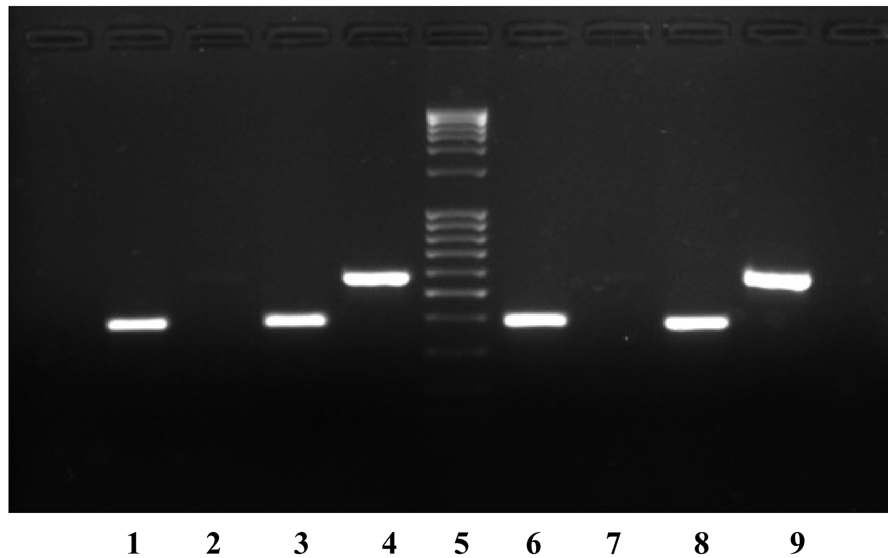


Figure 8: Expression of the *avrA* gene in the constructed strains *S.* Typhimurium and *S.* Choleraesuis. Total RNA of the strains *S.* Typhimurium *avrA*+ (SL1344 Δ*avrA/*pWSK29-*avrA*$_{\text{LT2}}$), *S.* Typhimurium *avrA*- (SL1344Δ*avrA/*pWSK29), *S.* Choleraesuis *avrA*+ (SARB4/pWSK29-*avrA*$_{\text{LT2}}$) and *S.* Choleraesuis *avrA*- (SARB4/pWSK29) were extracted and the *avrA* mRNA expression was confirmed by reverse transcription PCR using the primers AvrAF and AvrAR2 and using the gene *dna*A as reference gene**.** The Figure shows the Photo of Gel-Electrophorese of the respective PCR products. **1**: ST *avrA*- (*dnaA*); **2**: ST *avrA*- (*avrA*); **3:** ST *avrA*+ (*dnaA*); **4:** ST *avrA*+ (*avrA*); **5**:100 bp Marker; **6**: SC *avrA*- (*dnaA*); **7**: SC *avrA*- (*avrA*); **8:** SC *avrA*+ (*dnaA*); **9:** ST *avrA*+ (*avrA*).

4.2.2 Role of the presence or absence of the *avrA* gene in *S.* Typhimurium and *S.* Choleraesuis in invasion and intracellular persistence in human and porcine cell lines

In order to determine the effects of the presence or lack of the *avrA* gene in the *Salmonella* serovars Typhimurium and Choleraesuis on host invasion and intracellular persistence during infection, porcine (IPEC-J2) and human (LoVo) intestinal epithelia and porcine (PLN/C2) and human (THP-1) macrophage cell lines were infected with the strains; *S.* Typhimurium SL1344 Δ*avrA*, *S.* Typhimurium SL1344 *avrA*+ (Δ*avrA/*pWSK29-*avrA*$_{\text{LT2}}$), *S.* Choleraesuis wild type SARB4 (pWSK29) and *S.* Choleraesuis SARB4 *avrA*+ (pWSK29-*avrA*$_{\text{LT2}}$) at an MOI of 5:1 bacteria:host cells in 96-well-plates using standard invasion / gentamycin protection assay protocols.

No significant differences were observed either in the rate of host cell invasion or in the intracellular persistence between *S.* Typhimurium *avrA*+ and the strain harbouring a mutation in the *avrA* gene. Also no significant differences were observed between *S.*

Choleraesuis wild type and the *avrA*+ derivative (Figures 9 and 10). These results indicated that the presence of the *avrA* gene in both *Salmonella* serovars does not affect either the invasion process or the intracellular persistence during infection of the human and porcine cell lines tested. These results were consistent with other studies where AvrA was shown to play a role in post-invasion host cell immune responses (Collier-Hyams *et al*., 2002; Ye *et al*., 2007; Jones *et al*., 2008b; Du and Galán, 2009).

The *Salmonella* host adapted serovar Choleraesuis presented a significant lower relative invasion than the broad host-range serovar Typhimurium in each cell line tested (Figure 9; $P \leq 0,001$). These results are consistent with the results obtaining in this study with the strain serovar Choleraesuis A50 (section 4.1.1). In addition, *S.* Choleraesuis showed a significant higher intracellular persistence than the serovar Typhimurium in each cell line tested (Figure 10; $P \leq 0,001$).

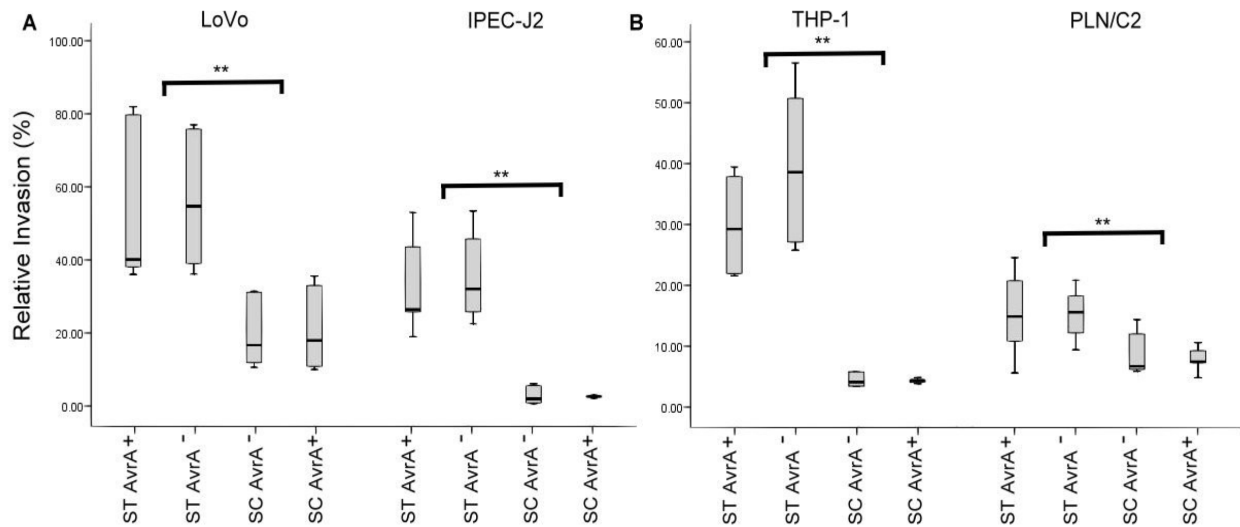


Figure 9: Role of *Salmonella avrA* gene in invasion of host cells. A. Human intestinal epithelial (LoVo), porcine intestinal epithelial (IPEC-J2), **B.** human macrophage (THP-1) and porcine macrophage (PLN/C2) cell lines were infected with the strains: ST AVRA+: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-*avr*ALT2), ST AVRA-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29), SC AvrA-: *S.* Choleraesuis (pWSK-29) and SC AVRA+: *S.* Choleraesuis SARB4 (pWSK29-*avrA*LT2) at a MOI of approximately 5. At 2 hours post-infection, the eukaryotic cells were lysed and the intracellular bacteria were calculated. Values represent the ratio of CFU 2 hours post infection to the initial bacterial inoculum and are expressed as a percentage of this initial inoculum. The diagram represent the average of at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentiles, resp., the box is the Interquartile Range (IQR) between percentile 75th and 25th, the heavy black line represents the median, and the ends of the whiskers show minimum and maximum values. Transformation: Sqrt. Statistic test: Bonferroni.
** = $P \leq 0{,}001$; * = $P < 0{,}05$.

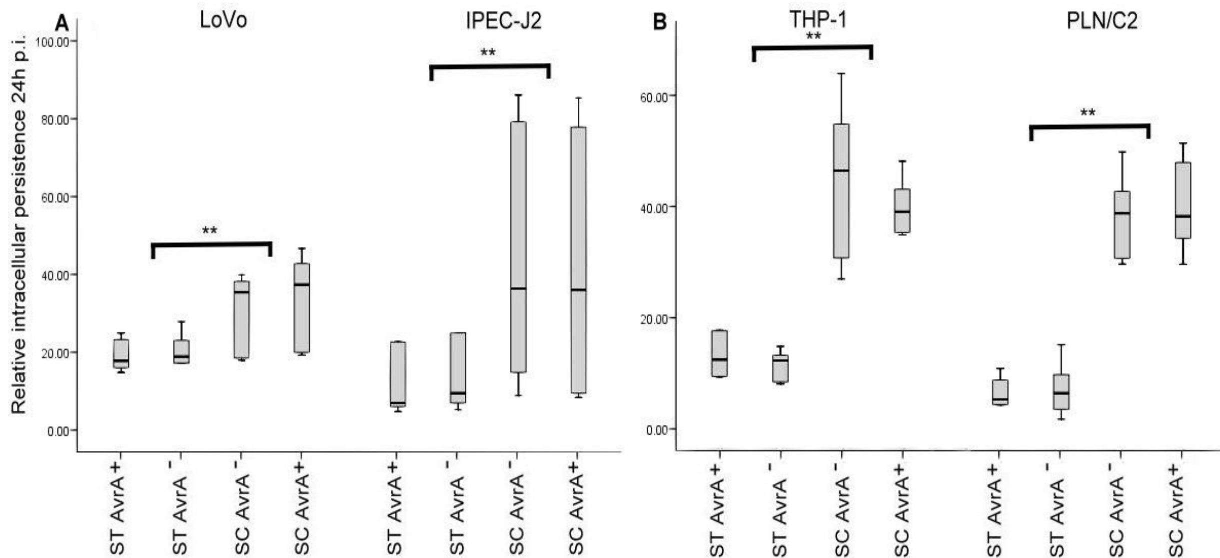


Figure 10: Effect of *avrA* on intracellular persistence of *Salmonella enterica* serovars Typhimurium and Choleraesuis. A. Human intestinal epithelial (LoVo), porcine intestinal epithelial (IPEC-J2), **B.** human macrophage (THP-1) and porcine macrophage (PLN/C2) cell lines were infected with the strains: ST AVRA+: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2), ST AVRA-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29), SC AvrA-: *S.* Choleraesuis (pWSK-29) and SC AVRA+: *S.* Choleraesuis SARB4 (pWSK29-*avrA*LT2) at a MOI of approximately 5. At 2 and 24 hours post-infection, the eukaryotic cells were lysed and the intracellular bacteria were calculated. Values represent the ratio of cfu 24 hours post infection to the cfu 2 hours post infection. The diagram represent the distribution of data of at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentiles, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line inside the box represents the median, and the ends of the whiskers show minimum and maximum values. Transformation: log 10. Statistic test: Bonferroni. ** = $P \leq 0{,}001$; * = $P < 0{,}05$.

4.2.3 Human and porcine innate immune response to *Salmonella* serovars Typhimurium and Choleraesuis / Influence of presence or absence of the *avrA* gene

4.2.3.1 Human and porcine NFκB activation during *Salmonella* infection

The eukaryotic transcription factor NFκB is crucial for the host immune responses to pathogens and its activation is known to be modulated by *Salmonella* (Sun, 2009; Agbor and McComick, 2011; Pilar *et al*., 2012). In order to analyze the possible effects of the absence or presence (and transcription) of the *avrA* gene in *Salmonella* serovars Typhimurium and Choleraesuis on NFκB activation in response to infection in human and porcine cells and on the serovar-specific host immune response, porcine (IPEC-J2) and human (LoVo) intestinal epithelia and porcine (PLN/C2) and human (THP-1) macrophage cell lines containing chromosomally-integrated, NFκB-dependent luciferase reporter fusions were infected with *S*. Choleraesuis wild type and an isogenic *avrA*+ mutant and *S*. Typhimurium *avrA*+ and Δ*avrA* mutant strains using the standard invasion / gentamycin protection assays. NFκB-dependent luciferase activity was determined at 2, 4 and 24 hours post-infection with a Bright-Glo™ Luciferase Assay System (Promega) using a Synergy™ HT Microplate Reader (BIO-TEK). In parallel, the relative invasion and intracellular persistence of these strains were also determined (Section 4.2.2).

Salmonella serovar Choleraesuis infection resulted in a statistically significantly lower NFκB activation 2 and 4 hours post-infection in human and porcine intestinal epithelial and human and porcine macrophage cells, compared to *Salmonella* serovar Typhimurium (Figures 11A-B, 11C-D; $P \leq 0{,}001$). Additionally, 24 hours post-infection, *Salmonella* serovar Choleraesuis also showed a statistically significant lower NFκB activation in porcine intestinal epithelial ($P < 0{,}05$) and porcine macrophage cells ($P \leq 0{,}001$). In contrast, 24 hours post-infection in both human intestinal epithelial cells, both *Salmonella* serovars generated similar NFκB activation. Furthermore, 24 hours post-infection *Salmonella* serovar Choleraesuis showed a statistically significantly higher NFκB activation in human macrophages (Figure 11F; $P \leq 0{,}001$) despite a lower intracellular CFU (two-fold). This suggests that the difference observed in the NFκB activation between the serovars Typhimurium and Choleraesuis in human macrophages is not only due to the difference in the invasiveness and intracellular persistence between the serovars. This higher NFκB activation in human macrophages by *Salmonella* serovar Choleraesuis could be involved in the higher rate of systemic disease in humans caused by this strain. Also the differences observed between both *Salmonella* serovars in each cell line tested were independent of the presence or absence of the *avrA* gene, indicating that the differential presence of the *avrA* gene in *S*. Typhimurium and *S*. Choleraesuis could not along explain the serovar-specific NFκB activation observed in this study.

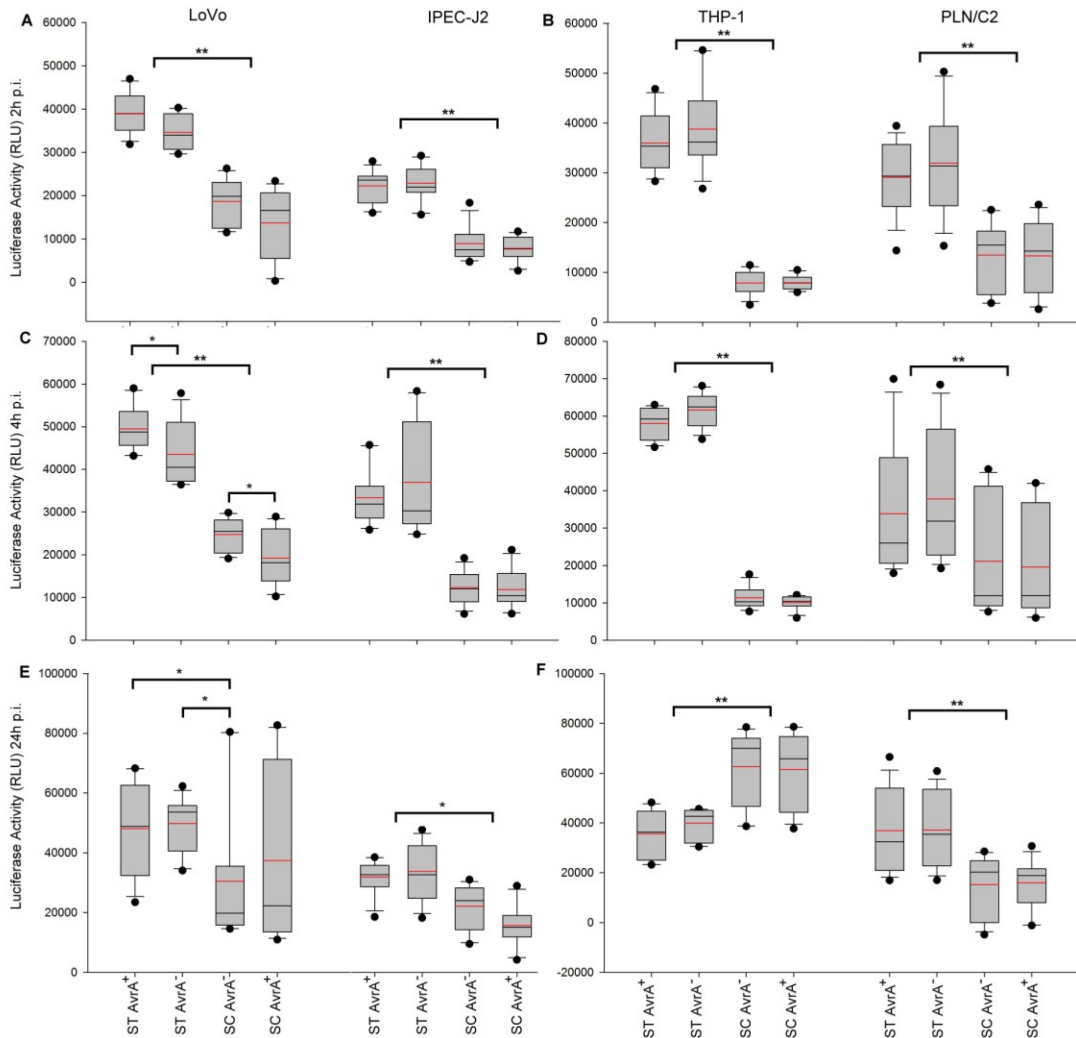


Figure 11: Host and serovar specific NFκB activation during *Salmonella* infection. Human intestinal epithelial (LoVo), porcine intestinal epithelial (IPEC-J2), human macrophage (THP-1) and porcine macrophage (PLN/C2) cell lines were infected with the strains: ST AVRA+: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2), ST AVRA-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29), SC AvrA-: *S.* Choleraesuis (pWSK-29) and SC AVRA+: *S.* Choleraesuis SARB4 (pWSK29-*avrA*LT2) at a MOI of approximately 5:1. Shown are the NFκB activities at 2 h (**A-B**), 4 h (**C-D**) and 24 h (**F-G**) post-infection the expression of the NFκB-dependent luciferase enzyme was monitored by measured of luminescence intensity with a Bright-Glo™ Luciferase Assay System (Promega) using a Synergy™ HT Microplate Reader (BIO-TEK). The diagram represents data of at least three independent determinations. The bottom and the top of the box represent the 25th and 75th percentiles, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line and the red line inside the box represent the median and the mean, and the ends of the whiskers show minimum and maximum values. Statistic test: Bonferroni.

** = P ≤ 0,001; * = P <0,05.

No significant differences were observed in the NFκB activation between *S.* Choleraesuis (wild type *avrA*-minus) and the *avrA*+ derivative in macrophage cell lines (THP-1 and PLN/C2), nor were there significant differences between *S.* Typhimurium wild type *avrA*+ and the Δ*avrA* mutant in these cells (Figures 11B, 11D and 11F). These results suggest that the presence of the *avrA* gene in both *Salmonella* serovars does not directly affect the activation of the transcription factor NFκB in human and porcine macrophages, and further suggests that the higher NFκB activation observed in human macrophage cell line during *S.* Choleraesuis infection is not directly related to the lack of *avrA* gene in this serovar.

On the other hand, the *S.* Choleraesuis *avrA*+ strain produced a statistically significantly lower NFκB activation compared to the wild type 4 hours post-infection in human intestinal epithelial cell line but this difference was absent 24 hours following infection (Figures 11C; $P < 0,05$ and 11E). In contrast, in the human epithelial cell line, *S.* Typhimurium *avrA*+ showed a significantly higher NFκB activation than the Δ*avrA* mutant, 4 hours post infection, but which was also absent 24 hours post-infection (Figures 11C; $P < 0,05$ and 11E). The presence or absence of the *avrA* gene in both *Salmonella* serovars showed no effect on the activation of NFκB during infection in porcine intestinal epithelial cells.

These results could indicate that the AvrA effector protein has the ability to modify the activation of the important immune transcription factor NFκB in human intestinal epithelial cells rather than in porcine intestinal epithelial cells or macrophages and that this effect varies depending on the infection conditions such as the specific strain of *Salmonella* causing the infection or a particular time point post-infection. This conclusion would be consistent with the study of Liu and co-workers in which AvrA specifically regulated NFκB activity in both an inhibitory and stimulatory manner depending on the time point after infection and involving a complex regulatory network (Liu e*t al.*, 2010).

4.2.3.2 Human JNK/AP-1 activation during *Salmonella* infection

The modulation of the host JNK signaling pathway and the subsequent regulation of apoptosis in host cells is another important strategy for intracellular pathogens such as *Salmonella* to avoid elimination (Wu *et al.*, 2011; Ashida *et al.*, 2011). To analyze the effects of the presence or lack of the *avrA* gene in *S*. Typhimurium and *S*. Choleraesuis on the activation of the host JNK pathway, human intestinal epithelial (LoVo) and macrophage cell lines (THP-1) were infected with the strains *S.* Typhimurium *avrA*+, *S.* Typhimurium Δ*avrA* mutant, *S*. Choleraesuis wild type and *S*. Choleraesuis *avrA*+ mutant. Following 4 and 24 hours post infection, the total RNA of the eukaryotic cells was extracted and the complementary DNA was obtained by reverse transcription PCR and the level of mRNA expression of the gene *jun* which is important in the JNK signaling pathway and part of the

AP-1 transcription factor family, was measured by Quantitative Real-Time PCR.

The infection of human intestinal epithelial cell line by the strain *S.* Typhimurium Δ*avrA* mutant generated significantly higher levels of JUN mRNA at 4 and 24 hours post-infection compared to the wild type strain (Figures 12A; $P < 0,05$ and 12B; $P \leq 0,001$). These results were consistent with those reported by Du and Galán where the activation of the JNK pathway was counteracted in human epithelial cells infected with *S*. Typhimurium *avrA*+ strain compared with the JNK activation caused by the *S*. Typhimurium isogenic Δ*avrA* mutant strain (Du and Galán, 2009). Therefore the presence and transcription of the *avrA* gene in *S*. Typhimurium appears to have an effect on modulation of the JNK pathway in human intestinal epithelial cells.

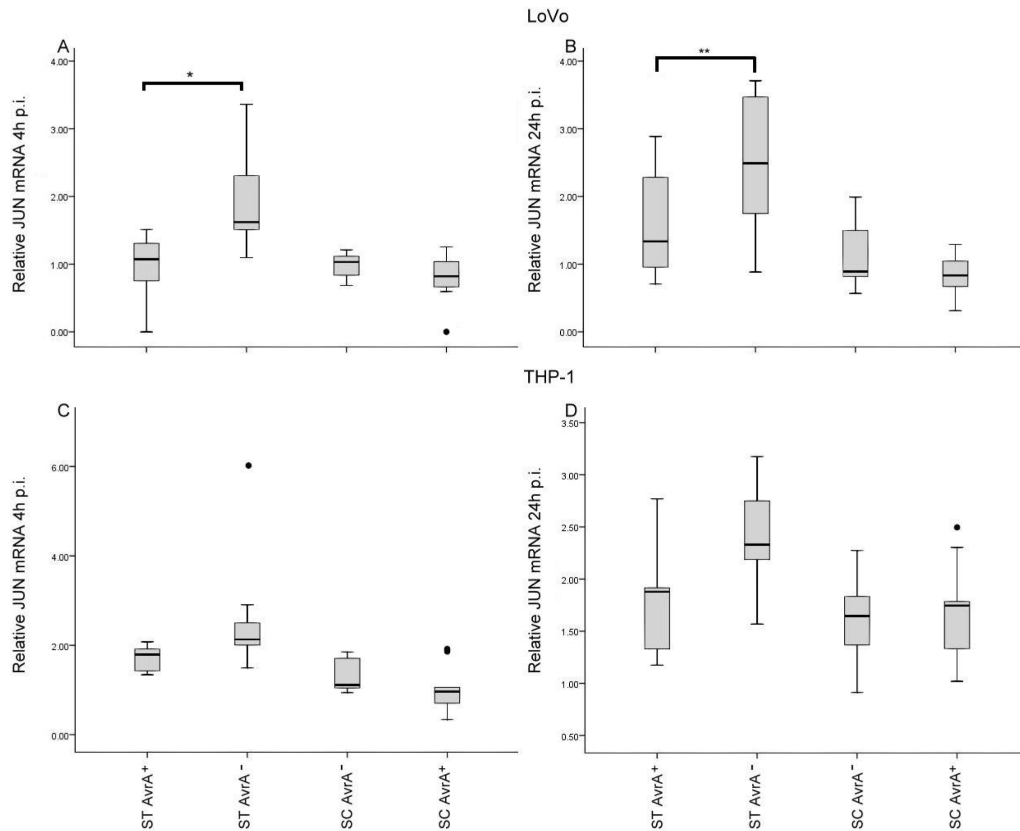


Figure 12: Relative JUN mRNA levels in human intestinal epithelial and macrophages in response to *S.* Typhimurium and *S.* Choleraesuis infections. A-B Human intestinal epithelial (LoVo) and **C-D** macrophage (THP-1) cell lines were infected at MOI of approximately 10 with strains of *S.* Typhimurium and *S.* Choleraesuis with and without the *avrA* gen. The total RNA of these cells was extracted at 4 hours (**A-C**) and 24 hours (**B-D**) p.i., reverse transcribed and quantified by qRT-PCR. The threshold cycles of the *jun* gen were normalized again the reference gene GADPH. The data represent JUN mRNA fold-induction in infected cells relative to that of uninfected cells. The diagram represent the data of at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentiles, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line inside the box represents the median, and the ends of the whiskers show minimum and maximum values, the circles represent the Outliers, values greater than 1.5 times the IQR. Transformation: log(10x+1). Statistic test: Tukey-HSD. ** = $P \leq 0{,}001$; * = $P < 0{,}05$.

4.2.3.3. Cytokines expression during *Salmonella* infection

The expression of the cytokines IL-8 and IL-1β are known to be regulated by both the NFκB and the JNK pathways (Hoffmann *et al*., 2002; Roman *et al*., 2000). In order to further analyze the effects of the differential presence of the *avrA* gene in *S*. Typhimurium

and *S.* Choleraesuis on the host innate immune response to *Salmonella* infection the expression of IL-8 was determined by qRT-PCR in the porcine and human epithelial and macrophage cell lines (LoVo, IPEC-J2, PLN/C2 and THP-1) at 4 and 24 hours post infection. The presence or absence of the *avrA* gene in both *Salmonella* serovars Choleraesuis and Typhimurium did not affect the mRNA expression of IL-8 in all the porcine and human cell lines tested (Figure 13).

Moreover, the relative IL-8 mRNA expression levels were significantly lower in response to *S.* Choleraesuis infection than by *S.* Typhimurium in all the cell lines tested, 4 hours post-infection and independent of the presence of the *avrA* gene (Figures 13A, 13C, 13E, 13G; $P \leq 0,001$). In addition, the relative IL-8 mRNA expression levels were significantly lower in response to *S.* Choleraesuis infection than by *S.* Typhimurium in the porcine macrophage cell line PLN/C2, at 24 hours post-infection also independent of the presence of the *avrA* gene (Figure 13H; $P \leq 0,001$). However, 24 hours post-infection, the difference between both *Salmonella* serovars showed variations depending on the presence of the *avrA* gene and the cell line infected. Thus, *S.* Choleraesuis with and without *avrA* gene showed a significantly lower IL-8 expression than the *S.* Typhimurium strain *avrA*+ ($P \leq 0,001$); but no difference compared to the *S.* Typhimurium Δ*avrA* strain in porcine intestinal epithelial cells (Figure 13D). The levels of IL-8 expression showed significant differences in human intestinal epithelial cells only between the strains *S.* Choleraesuis *avrA*+ and *S.* Typhimurium Δ*avrA*, where the latter showed a higher IL-8 expression (Figure 13B; $P < 0,05$). This apparently *avrA*-dependent difference between *S.* Choleraesuis and *S.* Typhimurium was also observed in human macrophages (THP-1), where *S.* Choleraesuis, which does not have the *avrA* gene, showed a significantly lower IL-8 expression than the strain *S.* Typhimurium without the *avrA* gene (Figure 13F; $P \leq 0,001$).

Due to the effects of the presence or absence of the *avrA* gene in *S.* Choleraesuis and *S.* Typhimurium on NFκB activation, previously observed in this study in human intestinal epithelial cells (Section 4.2.3.1) and the involvement of NFκB in activation of IL-1β transcription, the level of expression of IL-1β mRNA was determined in human intestinal epithelial and macrophage cell lines (LoVo and THP-1) at 4 and 24 hours post-infection. Significant differences associated with harbouring the *avrA* gene were only observed at 24 hours post infection in the human macrophage cell line THP-1, where the infection with *S.* Typhimurium Δ*avrA* mutant generated significantly higher levels of IL-1β mRNA than the wild type strain (Figure 14D; $P < 0,05$). This observation would be consistent with the hypothesis that AvrA plays a role in counteracting the host immune response during *Salmonella* infection.

In addition to lower IL-8 levels compared to *S*. Typhimurium, *S.* Choleraesuis also showed significantly lower IL-1β expression levels than *S*. Typhimurium in human macrophages 4 and 24 hours following infection (Figures 14C and 14D; $P \leq 0,001$), but independent of the presence of the *avrA* gene. On the other hand, the strain *S.* Choleraesuis wild type (without *avrA* gene) induced in human epithelial cells 24 hours post-infection higher IL-1β mRNA levels than *S.* Typhimurium *avrA*+ (Figure 14B; $P < 0,05$).

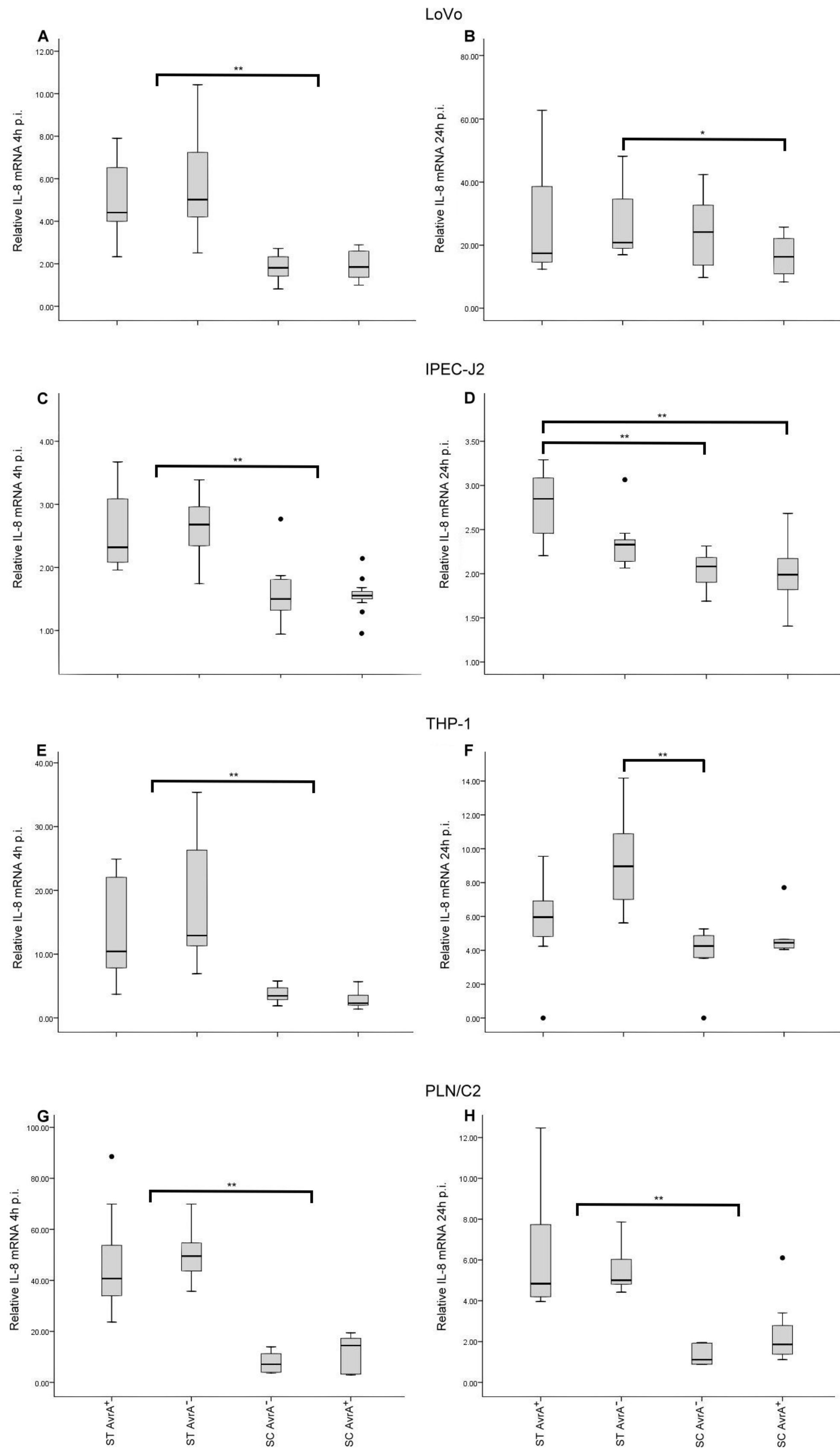

LoVo
A
B
Relative IL-8 mRNA 4h p.i.
Relative IL-8 mRNA 24h p.i.
IPEC-J2
C
D
THP-1
E
F
PLN/C2
G
H
ST AvrA+
ST AvrA−
SC AvrA−
SC AvrA+

Figure 13: Relative IL-8 mRNA levels in human and porcine intestinal epithelial and macrophages in response to *S.* Typhimurium and *S.* Choleraesuis infections. Human and porcine intestinal epithelial (LoVo, IPEC-J2) and macrophages (THP-1, PLN/C2) cell lines were infected at MOI of approximately 10 with strains of *S.* Typhimurium and *S.* Choleraesuis with and without the *avrA* gen. The total RNA of these cells was extracted **A, C, E, G** at 4 hours and **B, D, F, H** at 24 p.i., reverse transcribed and quantify by qRT-PCR. The threshold cycles of the porcine and human IL-8 gen were normalized again the reference gene GADPH (human and porcine). The data represent IL-8 mRNA fold induction in infected cells relative to that in uninfected cells. The diagram represent the data for at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentiles, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line inside the box represents the median, and the ends of the whiskers show minimum and maximum values, the circles represent the Outliers, values greater than 1.5 times the IQR. Transformation: log(10x+1). Statistic test: Tukey-HSD. ** = $P \leq 0{,}001$; * = $P < 0{,}05$.

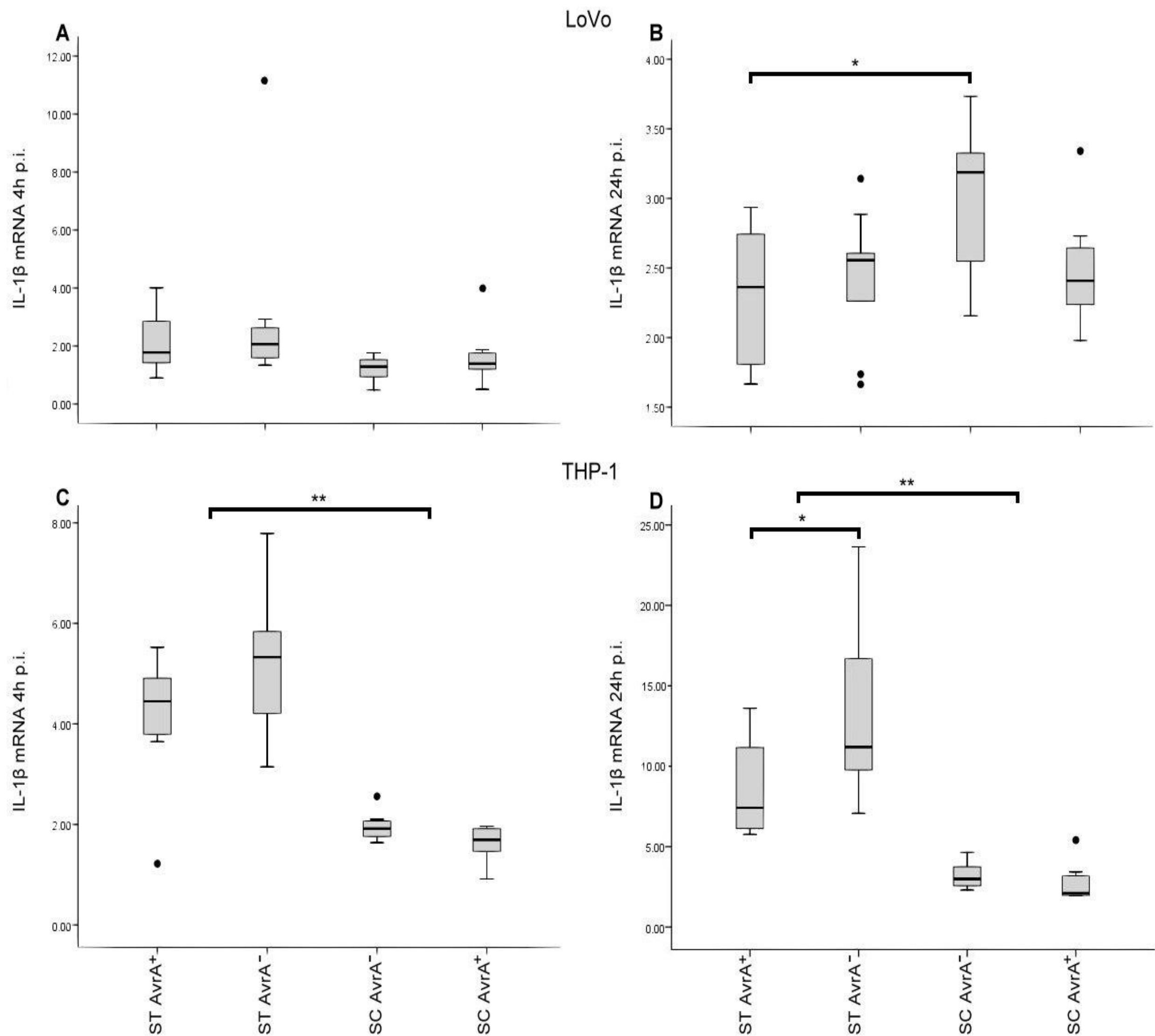


Figure 14: Relative IL-1β mRNA levels in human intestinal epithelial and macrophages in response to *S.* Typhimurium and *S.* Choleraesuis infections. Human intestinal epithelial (LoVo) and macrophages (THP-1) cell lines were infected at MOI of approximately 10 with strain of *S.* Typhimurium and *S*. Choleraesuis with and without the *avrA* gen. The total RNA of these cells was extracted **A-C** at 4 hours and **B-D** at 24 p.i., reverse transcribed and quantify by qRT-PCR. The threshold cycles of the porcine and human *IL-1β* gen were normalized again the reference gene GADPH. The data represent IL-1β mRNA fold induction in infected cells relative to that in uninfected cells. The diagram represent the distribution of Data for at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentiles, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line inside the box represents the median, and the ends of the whiskers show minimum and maximum values, the circles represent the Outliers, values greater than 1.5 times the IQR. Transformation: 4 hours p.i. = log(10x+0,1); 24 hours p.i.= log10(x). Statistic test: Tukey-HSD.
** = P ≤ 0,001; * = P < 0,05.

4.2.3.4 Effect of SseL on host immune response by *S.* Typhimurium infection in human and porcine macrophage - overlapping functions with AvrA

Like AvrA, the *Salmonella* effector protein SseL has been reported to cause the deubiquitination of IκBα, thereby inhibiting NFκB activation in macrophages (Le Negrate *et al*., 2008). The possibility of overlapping functions of the effector proteins AvrA and SseL during *S.* Typhimurium infection were analyzed in porcine and human macrophage cell lines harbouring the chromosomally-integrated NFκB-dependent reporter fusion. Cells were infected with *S.* Typhimurium strain SL1344 *avrA*+ and *S.* Typhimurium SL1344 harbouring deletion mutations of Δ*avrA*, Δ*sseL* or deletions of both genes (Δ*avrA* Δ*sseL*), at a multiplicity of infection (MOI) of approximately 5. The complete non-polar deletion of the *sseL* gene was obtained using the method described by Datsenko and Wanner (Datsenko and Wanner, 2000).

No significant differences on NFκB activation were observed 4 and 24 hours after infection between *S.* Typhimurium *avrA*+ and the Δ*avrA* mutant strain in the presence or absence of the *sseL* gene in porcine and human macrophages. However, 2 hours post-infection the strain *S.* Typhimurium Δ*avrA* Δ*sseL* double mutant showed significantly higher NFκB activation compared to the *avrA*+ Δ*sseL* mutant strain in porcine macrophage cells (Figure 15A; $P < 0,05$). Thus, the lack of effect of the presence or lack of the *avrA* gene in the *Salmonella* strains tested on the NFκB activation in macrophages, (observed in our previous results) could be associated to overlapping functions between *Salmonella* effector proteins genes *avrA* and *sseL*.

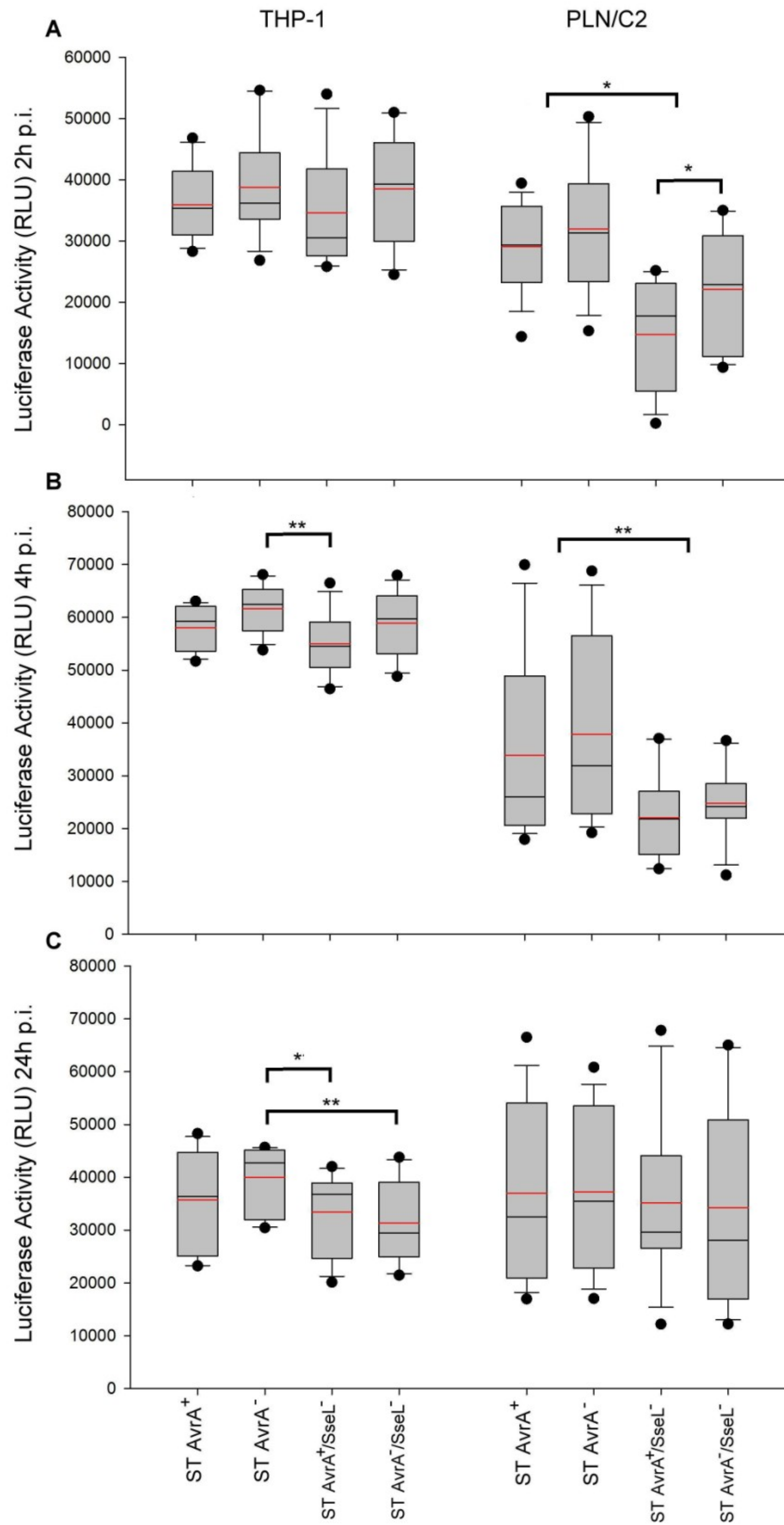
The complete deletion of the *sseL* gene in *S.* Typhimurium strains appeared to affect NFκB activation in the macrophage cell lines tested. However, contrary to expectations, the *S.* Typhimurium strains without the *sseL* gene showed significantly lower NFκB activation than the *S.* Typhimurium strains with the *sseL* gene, independent of the presence or absence of the *avrA* gene at 2 and 4 hours post-infection in porcine macrophages (Figures 15A, $P < 0,05$ and 15B; $P \leq 0,001$). Additionally, *S.* Typhimurium Δ*avrA* mutant presented higher NFκB activation than the *S.* Typhimurium Δ*avrA* Δ*sseL* double mutant (Figure 15C; $P \leq 0,001$) and the *S.* Typhimurium *avrA*+Δ*sseL* mutant in human macrophage cell line 24 hours post-infection. These results were somewhat puzzling, as SseL has been reported to inhibit NFκB activation during *Salmonella* infection (Le Negrate *et al*., 2008).

In order to investigate further the effect of the deletion of the *sseL* gene in *S.* Typhimurium on host immune response and the possible overlapping function with the *avrA* gene, porcine and human epithelial and macrophage cell lines (LoVo, IPEC-J2, PLN/C2 and THP-1) were infected with the strains *S.* Typhimurium SL1344 *avrA*+ and the strains harbouring deletion mutations of Δ*avrA*, Δ*sseL* or deletions of both genes (Δ*avrA*

Δ*sseL*) at MOI of approximately 10. Eukaryotic RNA was extracted 4 and 24 hours post infection and the expression levels of interleukins and the JNK pathway-related gene JUN were determined by means of qRT-PCR.

A possible effect of the presence of the *avrA* gene was observed on the level of expression of JUN, where infection with the *S.* Typhimurium Δ*avrA* Δ*sseL* double mutant showed higher JUN mRNA expression in human intestinal epithelial 24 hours after infection compared to the Δ*sseL* mutant strain (Figure 16B; $P \leq 0{,}001$). This higher level of JUN mRNA generated by the Δ*avrA* mutant was independent on the presence of the *sseL* gene since this difference was also observed in the strains harbouring the *sseL* gene (Figure 16B; $P \leq 0{,}001$).

The IL-8 mRNA expression level was determined in LoVo, IPEC-J2, PLN/C2 and THP-1cell lines 4 and 24 hours post-infection and showed no evidence of effects of the presence or absence of *avrA* and *sseL* genes (Figure 17). However, in contrast to IL-8 determinations, the level of IL-1β expression in human macrophage cell lines infected with the *S.* Typhimurium Δ*avrA* Δ*sseL* double mutant lead to higher IL-1β expression 4 hours post-infection than the Δ*sseL* mutant strain (Figure 18C; $P < 0{,}05$), which suggests an effect of the presence of the *avrA* gene on expression. However, this difference was not observed in the *sseL*+ strains, indicating a possible compensatory or overlapping effect of SseL. Nevertheless, 24 hours post-infection the *S.* Typhimurium Δ*avrA* strain generated higher IL-1β expression than the *avrA*+ strain despite the presence of the *sseL* gene (Figure 18D; $P < 0{,}05$). Additionally, in human macrophages the *S.* Typhimurium Δ*avrA* Δ*sseL* double mutant showed higher levels of IL-1β mRNA than the *S.* Typhimurium strain harbouring both genes (Figures 18C and 18D; $P < 0{,}05$), suggesting a role of one or both of these effector proteins in counteracting the host immune response.



THP-1
PLN/C2
A
B
C
Luciferase Activity (RLU) 2h p.i.
Luciferase Activity (RLU) 4h p.i.
Luciferase Activity (RLU) 24h p.i.
ST AvrA+
ST AvrA-
ST AvrA+/SseL-
ST AvrA-/SseL-

Figure 15: Effect of *avrA* and *sseL* on NFκB activation during *S.* Typhimurium infection. Human macrophage (THP-1) and porcine macrophage (PLN/C2) cell lines containing chromosomally-integrated, NFκB dependent reporter luciferase fusions were infected with the strains: ST AVRA+: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2), ST AVRA-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29), ST AVRA+/SseL-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2/ ΔSseL) and ST AVRA-/SseL-: *S.* Typhimurium SL1344 (ΔavrA/ ΔSseL /pWSK29) at a MOI of approximately 5. At **A**.2h, **B**. 4h and **C.** 24 h p. i. the expression of the NFκB-dependent luciferase enzyme was monitored by measured of luminescence intensity with a Bright-Glo™ Luciferase Assay System (Promega) using a Synergy™ HT Microplate Reader (BIO-TEK). The diagram represent the distribution of Data for at least three independent determinations, the bottom and the top of the box represent the 25th percentile and the 75th percentile, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black and red lines inside the box represent the median and the mean respectively, and the ends of the whiskers show minimum and maximum values. Statistic test: Bonferroni.** = $P \leq 0{,}001$; * = $P < 0{,}05$.

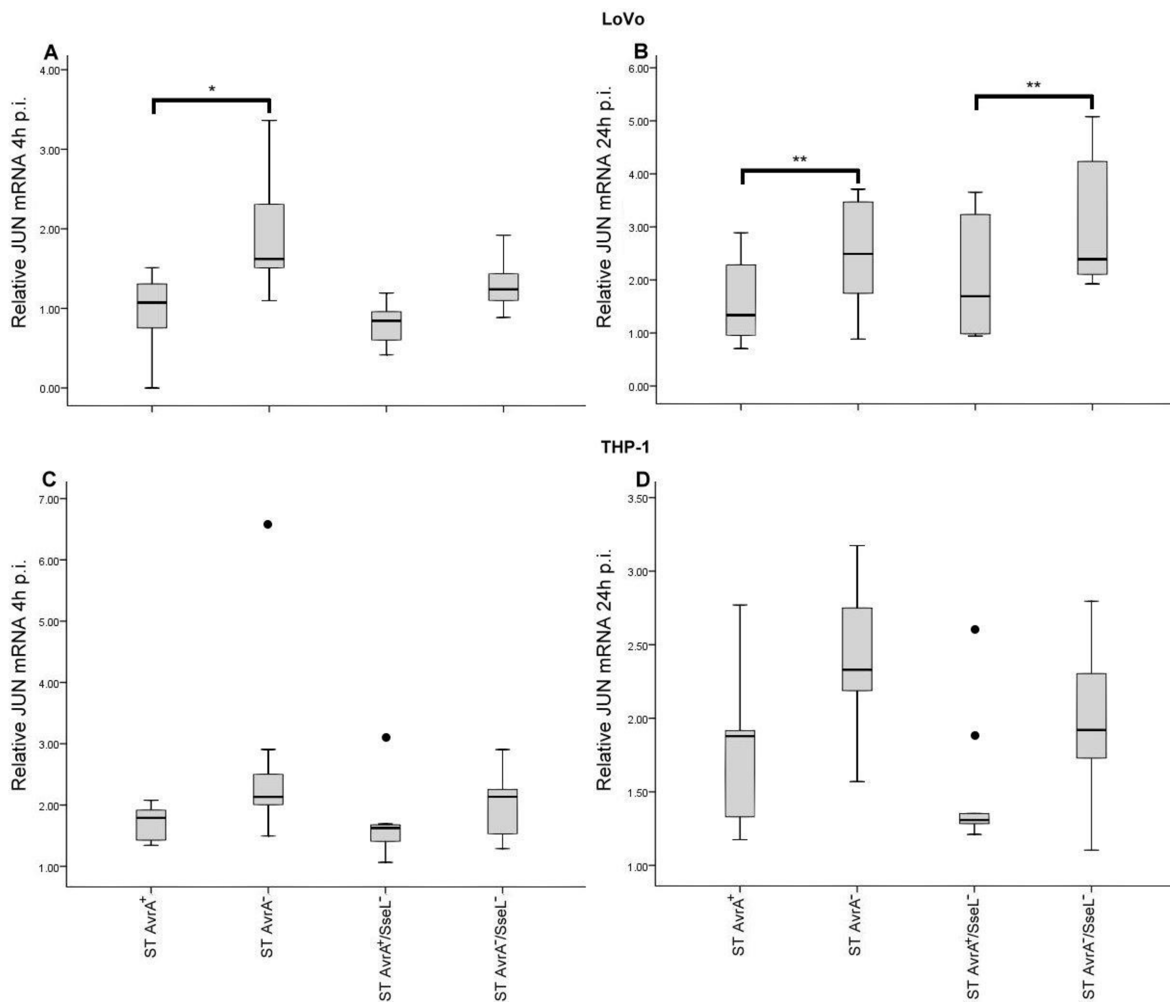
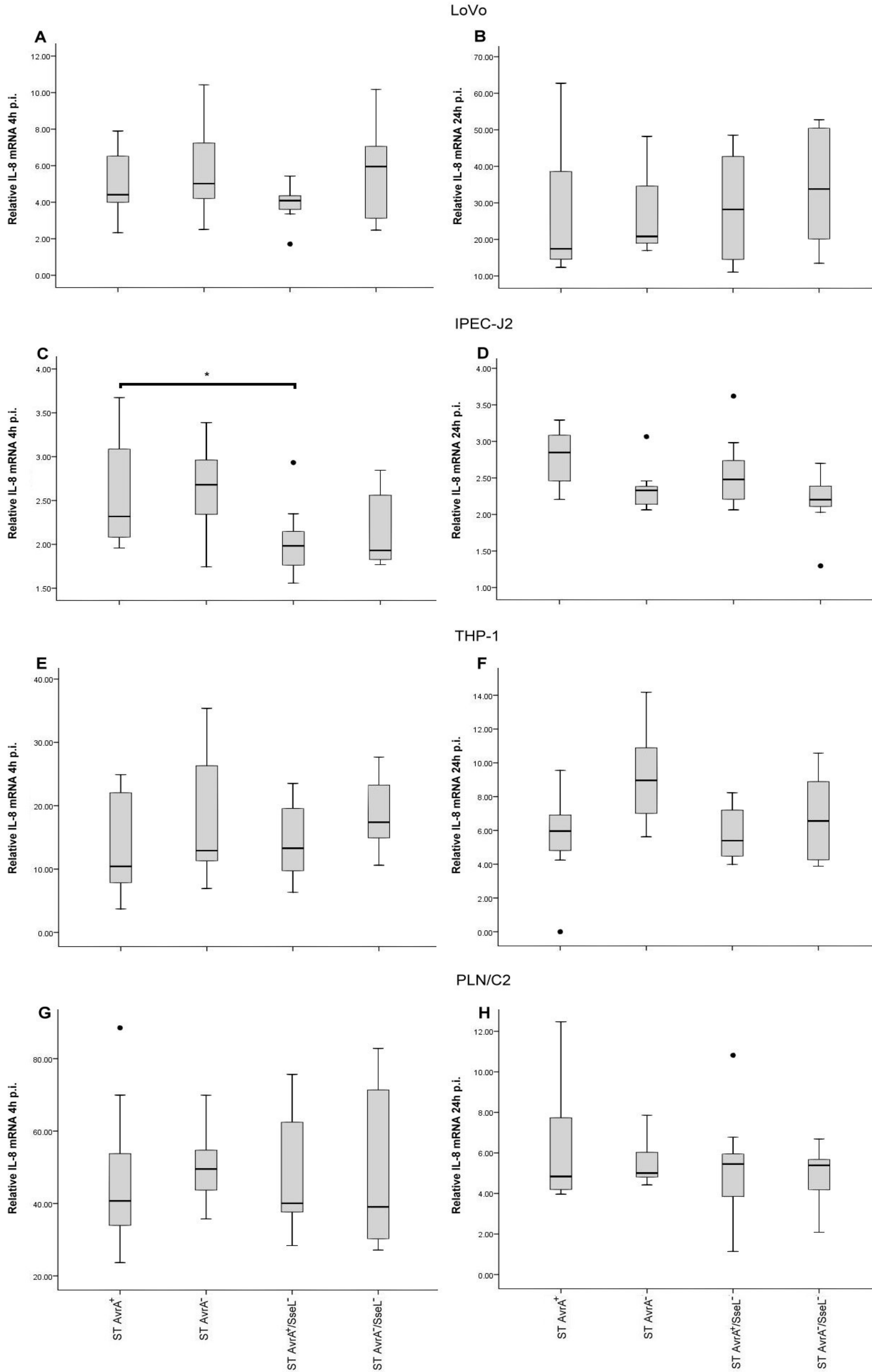


Figure 16: Relative JUN mRNA levels in human intestinal epithelial and macrophages in response to *S.* Typhimurium and effect of *avrA* and *sseL*. A-B Human intestinal epithelial (LoVo) and **C-D** macrophage (THP-1) cell lines were infected with the strains: ST AVRA+: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2), ST AVRA-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29), ST AVRA+/SseL-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2/ ΔSseL) and ST AVRA-/SseL-: *S.* Typhimurium SL1344 (ΔavrA/ ΔSseL /pWSK29) at a MOI of approximately 10. The total RNA of these cells was extracted **A-C** at 4 hours and **B-D** at 24 hours p.i., reverse transcribed and quantify by qRT-PCR. The threshold cycles of the *jun* gen were normalized again the reference gene GADPH. The data represent JUN mRNA fold induction in infected cells relative to that in uninfected cells. The diagram represent the distribution of Data for at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentiles, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line inside the box represents the median, and the ends of the whiskers show minimum and maximum values, the circles represent the Outliers, values greater than 1.5 times the IQR. Transformation: log(10x+1). Statistic test: Tukey-HSD.** = P ≤ 0,001; * = P < 0,05.



LoVo
A
B
Relative IL-8 mRNA 4h p.i.
Relative IL-8 mRNA 24h p.i.
IPEC-J2
C
D
*
THP-1
E
F
PLN/C2
G
H
ST AvrA+
ST AvrA-
ST AvrA+/SseL-
ST AvrA-/SseL-

Figure 17: Relative IL-8 mRNA levels in human and porcine intestinal epithelial and macrophages in response to *S.* Typhimurium and effect of *avrA* and *sseL*. Human and porcine intestinal epithelial (LoVo, IPEC-J2) and macrophages (THP-1, PLN/C2) cell lines were infected with the strains: ST AVRA+: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2), ST AVRA-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29), ST AVRA+/SseL-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2/ ΔSseL) and ST AVRA-/SseL-: *S.* Typhimurium SL1344 (ΔavrA/ ΔSseL /pWSK29) at a MOI of approximately 10. The total RNA of these cells was extracted **A, C, E, G** at 4 hours and **B, D, F, H** at 24 p.i., was reverse transcribed and quantify by qRT-PCR. The threshold cycles of the porcine and human IL-8 gen were normalized again the reference gene GADPH (human and porcine). The data represent IL-8 mRNA fold induction in infected cells relative to that in uninfected cells. The diagram represent the distribution of Data for at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentiles, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line inside the box represents the median, and the ends of the whiskers show minimum and maximum values, the circles represent the Outliers, values greater than 1.5 times the IQR. Transformation: log(10x+1). Statistic test: Tukey-HSD.** = $P \leq 0{,}001$; * = $P < 0{,}05$.

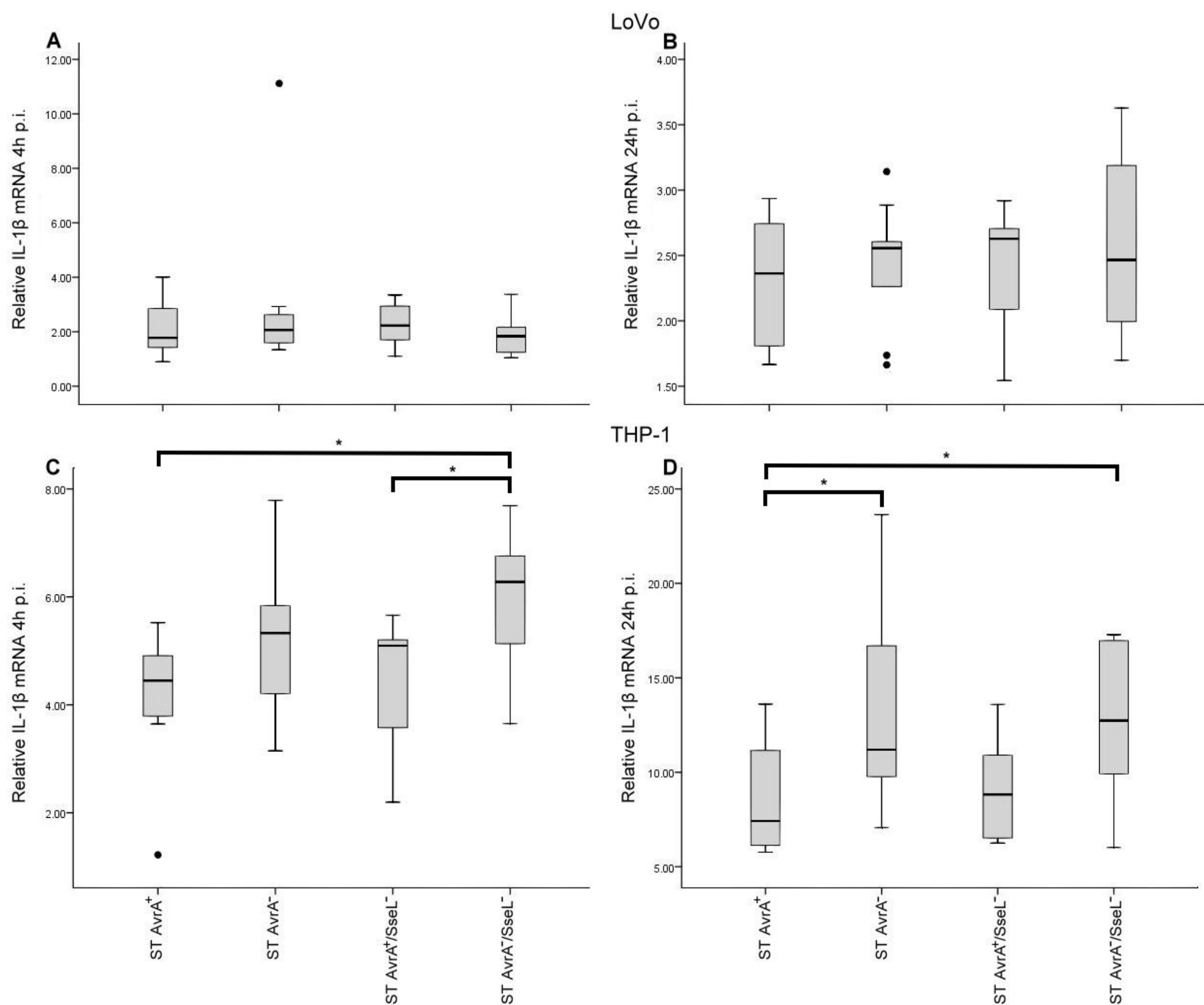


Figure 18: Relative IL-1β mRNA levels in human intestinal epithelial and macrophages in response to *S.* Typhimurium and effect of *avrA* and *sseL*. A-B Human intestinal epithelial (LoVo) and **C-D** macrophages (THP-1) cell lines were infected with the strains: ST AVRA+: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2), ST AVRA-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29), ST AVRA+/SseL-: *S.* Typhimurium SL1344 (ΔavrA/pWSK29-avrALT2/ ΔSseL) and ST AVRA-/SseL-: *S.* Typhimurium SL1344 (ΔavrA/ ΔSseL /pWSK29) at a MOI of approximately 10. The total RNA of these cells was extracted **A-C** at 4 hours and **B-D** at 24 hours p.i., reverse transcribed and quantify by qRT-PCR. The threshold cycles of the porcine and human *IL-1β* gen were normalized again the reference gene GADPH. The data represent IL-1β mRNA fold induction in infected cells relative to that in uninfected cells. The diagram represent the data for at least three independent determinations, the bottom and the top of the box represent the 25th and 75th percentile, the box is the Interquartile Range (IQR) between percentile 75th and 25th, the black line inside the box represents the median, and the ends of the whiskers show minimum and maximum values, the circles represent the Outliers, values greater than 1.5 times the IQR. Transformation: log(10x+1). Statistic test: Tukey-HSD.** = $P \leq 0,001$; * = $P < 0,05$.

5 DISCUSSION

5.1 Comparison of the invasiveness and intracellular persistence of *S.* Choleraesuis and *S.* Typhimurium

S. Choleraesuis and *S*. Typhimurium are the most frequently *Salmonella* serovars associated with porcine natural infections in the field (Chiu *et al*., 2004; Uthe *et al*., 2007; EFSA and ECDC, 2013 Summary Report on Zoonoses in 2011). Both serovars differ in the host range and the diseases they cause. *S.* Choleraesuis is adapted to swine and is frequently associated with systemic infections often leading to pneumonia and hepatitis (Chiu *et al*., 2005; Wollin, 2007; Shinkai *et al*., 2011). *S.* Choleraesuis is also able to infect humans with a higher rate of hospitalization and systemic disease compared to the infections caused by *S.* Typhimurium (Wollin, 2007; Jones *et al*., 2008a). In contrast to *S.* Choleraesuis, *S.* Typhimurium is a broad host range pathogen infecting a wide range of species including swine and humans, causing mainly enterocolitis but also asymptomatic infections which favor the spread of *Salmonella* into the environment (EFSA and ECDC, 2013-Summary Report on Zoonoses in 2011; Ruby *et al*., 2012). The differences in the host range and disease outcomes between these two *Salmonella* serovars are seen in both epidemiological studies and in experimental studies (Bolton *et al*., 1999; Skjolaas *et al*., 2006; Uthe *et al*., 2007; Jones *et al*., 2008a). A better understanding of the factors that regulate the ability to invade and proliferate within different hosts and different cell types is particularly important in the case of food-producing animals such as swine where contaminated meat products play an important role as a source of infection for humans by both *Salmonella* serovars Typhimurium and Choleraesuis.

One aim of this study was to systematically compare the invasiveness and intracellular persistence of the broad host-range *S.* Typhimurium and the swine-adapted *S.* Choleraesuis in intestinal epithelial cells and macrophages taking into account the relevant serovar-host combination. Given the relevance of *S.* Choleraesuis and *S.* Typhimurium in humans and swine, cell lines from both species were used in this study in order to analyze the performance of both serovars in intestinal epithelial and blood macrophage cells. These *in vitro* models were selected since they permit a systematic approach comparing different host cell types and strains. The invasiveness and intracellular persistence of *S.* Choleraesuis and *S.* Typhimurium were compared in both intestinal epithelial and macrophage cells of porcine and human origins, and also the effect of the presence or absence of the *avrA* gen in these *Salmonella* serovars on both parameters was tested in each cell line. Despite the advantage of the cell culture infection models and the possibility to assay bacteria-host cell interactions, it should be taking into consideration that this is an *in vitro* model and the results obtained may not necessarily be directly extrapolated to the *in vivo* situation

(Cencic and Langerholc, 2010; Langerholc *et al*., 2011). Nevertheless, such *in vitro* cell culture infection models can provide valuable information which may be tested in animal studies.

In order to reduce the gap between the *in vitro* model and natural infections, a low infection dose (MOI ≤ 10) was used throughout in this study, since *Salmonella* normally cause infections with less than 10^3 bacteria depending on the strain, host status and vehicle of infection (Foley and Lynne, 2008). In the gentamicin protection/invasion assays applied in this study, the *S*. Choleraesuis A50 strain was less invasive than *S*. Typhimurium SL1344 in intestinal epithelial cells of both human (LoVo) and porcine origin (IPEC-J2) and also porcine macrophages (PLN/C2). This apparent advantage of *S*. Typhimurium in invasion compared with *S*. Choleraesuis showed no significant serovar-specific host or cell type preference in the cell line models used (see Figure 5).

The higher invasiveness of *S*. Typhimurium compared with host-adapted and host-restricted serovars has also been reported in *in vivo* studies with *S*. Choleraesuis in swine (Paulin *et al*., 2007) and in a study with serovar Gallinarum (Chadfield *et al*., 2003) in the avian host. This apparent increased ability of the *S*. Typhimurium to invade host cells relative to *S*. Choleraesuis has been also seen in porcine alveolar macrophages *in vitro* (Watson *et al*., 2000). It has been suggested that this was related to the ability of *S*. Typhimurium to infect a broad range of different hosts (Watson *et al*., 2000; Chadfield *et al*., 2003; Paulin *et al*., 2007). These results indicate that the higher persistence or association of *S*. Choleraesuis in swine (host-adaptation) and the high levels of systemic infections in humans (Wollin, 2007) is not directly associated with enhanced invasiveness in human and porcine intestinal epithelial cells and porcine macrophages by this serovar compared to *S*. Typhimurium.

This higher invasion of *S*. Typhimurium compared with *S*. Choleraesuis was also seen in the present study using the *S*. Choleraesuis SARB4 wild type strain and an *avrA*+ derivative (see Figures 9A and 9B). This difference in invasion between *Salmonella* serovars Typhimurium and Choleraesuis is not due to less bacterial recovery due to cell damage or gentamicin sensitivity, since no cytotoxicity effects were observed in these cell lines infected with these *Salmonella* serovars at MOIs of 1 to 10, as shown in previous studies in our laboratory (Maurischat, 2013).

In contrast to the results obtained for the invasiveness comparison, the differences in the capacity to persist into host cells between *S*. Typhimurium and *S*. Choleraesuis were host cell-specific. The *S*. Choleraesuis strain A50 showed significantly less intracellular persistence than the strain *S*. Typhimurium in porcine intestinal epithelial cells (IPEC-J2) and significantly higher capacity to persist within the human intestinal epithelial cells (LoVo) 24 hours p.i. Additionally, both *Salmonella* serovars showed similar persistence within porcine macrophages (PLN/C2) (see Figure 6). This lower intracellular proliferation

of serovar Choleraesuis within porcine intestinal epithelial cells could be a strategy to avoid the exacerbation of the porcine immune response and may play a role in the *S.* Choleraesuis host adaptation to swine (Cano *et al.*, 2001; Tierrez and García-del Portillo, 2005; Grant *et al*., 2009).

5.2 Effects of the presence or lack of the *avrA* gene in *S.* Typhimurium and *S.* Choleraesuis on serovar-specific host response

The *Salmonella* pathogenicity islands and the effector proteins they encoded are important in modulation of the host cell responses and vital for *Salmonella* intestinal invasion, intracellular persistence and eventual dissemination to host tissues. The absence of the *avrA* gene in *S.* Choleraesuis is one of the major differences between the serovars Choleraesuis and Typhimurium with respect to the genes encoded within the *Salmonella* pathogenicity island 1 (Amavisit *et al*., 2003; Chiu *et al*., 2005; Suez et al., 2013).

It has been suggested that the AvrA effector protein may play a role in *Salmonella* serovar host-specificity and serovar-specific disease outcomes, due to its modulatory effect on the host immune response and its enhanced expression in *Salmonella* serovars with restricted host range which show higher rates of systemic disease (Hardt and Galán, 1997; Streckel *et al*., 2004). However, this proposition has not been confirmed and has not been previously analyzed using *in vitro* infection models of different serovar-relevant host and pathogenicity-relevant cell types. In an effort to determine the influence of the presence or absence of the *avrA* gene in *S*. Typhimurium and *S*. Choleraesuis in serovar-specific host range and serovar-specific pathogenesis, human (LoVo) and porcine (IPEC-J2) intestinal epithelial and macrophage (PLN/C2, THP-1) cell lines were infected with *Salmonella* serovars Typhimurium and Choleraesuis and derivatives of both serovars in the presence or absence of the *avrA* gene. The *Salmonella* AvrA effector protein has been proposed to modulate the host immune response against *Salmonella* infection and counteract inflammation. AvrA has also been suggested to enhance bacterial intracellular survival due to inhibition of apoptosis in infected macrophages (Wu *et al*., 2011).

S. Choleraesuis naturally harbours a deletion of the *avrA* gene and is adapted to swine but can also infect humans, where it causes frequent systemic disease at rates approaching that of the human-restricted serovar *S*. Typhi (Wollin, 2007). In contrast, *Salmonella* serovar Typhimurium has a broad host range including humans and swine as well as many other species, and is normally associated with gastrointestinal pathogenesis. *Salmonella* serovars Typhimurium and Choleraesuis therefore differ in both the host range and in the disease manifestation and outcomes of infections in swine and humans, making these serovars useful for study host-specificity factors in the context of humans and swine *Salmonella* infections.

In order to analyse the possible effect of the presence or absence of the *avrA* gene in host range and disease outcome, the *avrA* gene from *S.* Typhimurium strain LT2 was cloned into the *S.* Choleraesuis strain SARB4. In addition, the *avrA* gene from the strain *S.* Typhimurium SL1344 was deleted and also complemented with the *avrA* gene from *S.* Typhimurium strain LT2. The *avrA* gene from serovar Typhimurium SL1344 harbours a three nucleotide deletion compared to the *avrA* gene from the strain LT2, leading to the absence of a Leucine residue at position 139 in the amino acid sequence of the encoded protein and which has been reported to affect the activity of the AvrA protein (Du and Galán, 2009). On the other hand, the strain *Salmonella* serovar Typhimurium LT2 had been found to be attenuated for virulence during infection (Jarvik *et al*., 2010). For these reasons, in this study the *avrA* gene from the strain *Salmonella* serovar Typhimurium LT2 was used to test the function of AvrA on host-specificity and the strain SL1344 was used in the infection assays. In addition, the *avrA* gene was cloned into both the SL1344 strain or the *S.* Choleraesuis strain SARB4 under control of its own promoter to achieve natural levels of expression of the AvrA protein and experimental conditions more related to natural infections.

The expression of the cloned *avrA* gene was confirmed by PCR of cDNA of total RNA in both the complemented strain *S.* Typhimurium SL1344 $\Delta avrA/avrA_{LT2}^{+}$ harbouring the *avrA* gene from the LT2 strain and also in the *S.* Choleraesuis strain $avrA_{LT2}^{+}$. The lack of mRNA expression of the *avrA* gene in the *S.* Choleraesuis SARB4 wild type strain and the *S.* Typhimurium SL1344 $\Delta avrA$ mutant strain were also confirmed. Possible differences between the serovars are therefore not due to differences in transcription of the cloned *avrA* gene. The *avrA* gene from LT2 used to transform the *Salmonella* strains, has been reported to constitutive synthesize the AvrA protein (Ben-Barak *et al*., 2006). However, the production of the AvrA protein in the transformed strains in the present study has not been able to be tested due to problems with the specificity of the used Anti-AvrA antibody. These transformed strains could be used in future studies of *Salmonella* serovar-specific host interaction. However, to further investigate the effects of the AvrA protein and not only of the presence and transcription of the *avrA* gene in *Salmonella* serovars, the production of the AvrA protein in each strain should be confirmed.

In this study, the presence or absence of the *avrA* gene in *S.* Typhimurium and *S.* Choleraesuis had no significant effect on invasion and intracellular persistence in porcine and human epithelial and macrophage cell lines (see Figures 9 and 10). The previously described effect of AvrA on bacterial intracellular survival (Wu *et al*., 2011) was not observed in the intracellular persistence determined in the present study, possibly due to differences in the experimental model and conditions. For example, in the present study cell lines were used as experimental model and the intracellular persistence was determined

24 hours post-infection relative to the intracellular bacteria 2 hours after infection. In the study of Wu and co-workers, they used an *in vivo* murine model and the intracellular survival was measured 6 days after infection. It is therefore possible that 24 hours post infection was not sufficient to observe the inhibition of apoptosis by AvrA reflected in the bacterial intracellular persistence measured by gentamicin protection assays. One of the disadvantages of using cell lines as experimental models is the difficulty in analyzing effects after 24 or 48 hours post-infection since a longer incubation time and bacterial growth leads to cell culture acidification causing cell death (Bolton *et al*., 1999). The reported effects of AvrA have been principally associated with modulation of host immune response rather than intracellular invasion or growth (see Table 9), which is consistent with the lack of effects observed in the present study.

Despite the absence of effects of the presence or lack of the *avrA* gene in the tested strains on invasion and intracellular persistence in this study, a possible serovar-specific difference in both parameters was observed in all cell lines tested. The *Salmonella* serovar Choleraesuis strains SARB4 wild type and the isogenic strain harbouring the *avrA* gene from the strain serovar Typhimurium LT2 (pWSK29-$avrA_{LT2}$) showed lower invasiveness compared with serovar Typhimurium strains in all the cell lines tested independent of the presence or absence of the *avrA* gene, which suggest that this lower invasiveness of the human and porcine intestinal epithelial and macrophage cell lines is likely due to a serovar-specific feature rather than the action/effect of the presence of the *avrA* gene (see Figures 9A and 9B). This lower invasiveness of *S.* Choleraesuis was also observed in this study using the strains *S.* Choleraesuis A50 wild type and *S.* Typhimurium SL1344 wild type (see Figure 5) despite the different bacterial concentration used to infect the cells (MOI of 1 instead of 5).

The lower capacity to invade host cells by *Salmonella* host adapted serovars compared to serovars with a broad host range has been also observed in others studies using different *Salmonella* host-adapted serovars and different experimental models. The avian host-adapted serovar *S.* Gallinarum showed lower invasiveness than *S.* Typhimurium SL1344 in chicken intestine (Chadfield *et al*., 2003). Likewise, the swine-adapted *S.* Choleraesuis strain A50 also showed lower invasiveness than *S*. Typhimurium strain 4/74 in porcine intestinal mucosa during *in vivo* studies (Paulin *et al*., 2007) and in porcine ileal loop mucosae (Bolton *et al*., 1999). The lower invasiveness by host-adapted serovars could be a strategy to avoid the exacerbation of the host immune response and assure an intracellular niche.

Despite the low invasiveness, *S.* Choleraesuis showed higher intracellular persistence than *S*. Typhimurium in all the cell lines tested, but independent of the presence or absence of the *avrA* gene (see Figures 10A and 10B). This indicates once again that the presence and transcription of the *avrA* gene in the tested strains do not affect in a

significant manner the bacterial intracellular persistence 24 hours after infection in the human and porcine cell lines tested. The higher persistence of *S.* Choleraesuis 24 hours p.i. despite the lower invasiveness compared with *S.* Typhimurium contrast with another study where *S.* Typhimurium showed both higher invasion and more rapid replication than *S.* Choleraesuis in porcine intestinal mucosa (Paulin *et al*., 2007). The difference in the results between Paulin and co-worker's research and the present study could be due to the experimental differences; Paulin and coworkers used *in vivo* porcine models and the strain *S.* Choleraesuis A50. Furthermore, the intracellular persistence in the present study was calculated as the ratio of the intracellular cfu at 2 hours and 24 hours post infection. There are no studies which compare intracellular persistence of *S.* Typhimurium and *S.* Choleraesuis under similar experimental conditions and using different cell types from swine and human origins which makes it difficult to compare the results obtained in the present study. Notably however, both the lower invasion and higher intracellular persistence of *S.* Choleraesuis respect to *S.* Typhimurium were independent of the cell type or host species origin.

The eukaryotic transcription factor, NFκB, is a key factor involved in host immune responses against pathogens. This transcription factor stimulates the transcription of pro-inflammatory genes such as interleukin 6 (IL-6) and IL-8, TNF-α and interferons (*e.g.* IFNα and IFNγ) required for controlling *Salmonella* infection. The activation of NFκB and the host immune response is mediated not only by the host as a defense mechanism, but also by *Salmonella* which exploits the host immune mechanisms leading to inflammation. *Salmonella* can exacerbate or support inflammation through the effects of secreted virulence proteins such as SopE, SopE2 and SopB (Bruno *et al*., 2009) in order to compete with commensal bacteria in the intestinal gut and to access nutrients (Coburn *et al*., 2007; Stecher *et al*., 2010; Kaiser and Hardt, 2011).

Intracellular microorganisms have developed mechanisms to counteract the inflammation and the immune response they induce, in order to maintain their niche. AvrA effector protein has been proposed to exert this function in the context of *Salmonella* host infection by the inhibition of important transcription factors and host signalling pathways such as NFκB and the AP-1/JNK pathway. This mechanism has also been found in other intracellular pathogens such as *Shigella flexneri* which use the virulence factor OspF to counteract the host immune response (Kaiser and Hardt, 2011), and the effector protein YopJ/P from *Yersinia pseudotubercolosis* (Orth *et al*., 1999; Mukherjee *et al*., 2006).

The effect of AvrA on the host immune response has been examined in previous studies in human and mouse cell lines and *in vivo* mouse models, but leading to different conclusions with respect to the effects of AvrA on NFκB activation (Collier-Hyams *et al*., 2002; Ye *et al*., 2007; Du and Galán, 2009; Liu *et al*., 2010). However, the effect of AvrA has not been previously investigated in other relevant *Salmonella* hosts, such as food

producing animals. *Salmonella* is one of the major causes of foodborne disease worldwide, a situation aided in part by the ability of some *Salmonella* serovars to infect both animals and humans which facilitates the transmission of *Salmonella* trough the food chain (EFSA and ECDC, 2013-Summary Report on Zoonoses in 2011). In this study, the effect of the presence or lack of the *avrA* gen in *Salmonella* serovars Typhimurium and Choleraesuis on NFκB activation was analysed in cell lines of both human and porcine origin. Furthermore, the activation of this transcription factor was analysed in both intestinal epithelial cells and blood macrophages in order to analyse the possible roles of AvrA in both enteric and systemic disease, since the *avrA* gene is frequently not present in host-adapted or host-restricted serovars that frequently cause systemic disease instead enteric pathogenesis (Streckel *et al*., 2004). The host:pathogen interaction and the equilibrium in the immune response are decisive in the disease manifestation (systemic versus enteric) and outcome of infection (de Jong *et al*., 2012; Srikanth *et al*., 2011). In this study, the effect of AvrA in *Salmonella* infection in NFκB activation was determined using human and porcine epithelial and macrophage cell lines containing chromosomally-integrated, NFκB-dependent reporter luciferase fusions. This *in vitro* cell model permitted a systematic analyse of NFκB activation in cells infected with both *Salmonella* serovars with and without the *avrA* gene.

In this study, the level of NFκB activation was significantly different in the LoVo human intestinal epithelial cell line infected with *Salmonella* strains harbouring the *avrA* gene compared to *Salmonella* strains which did not have this gene. However, the effect of the *avrA* gene on the regulation of NFκB activation was different depending on the infecting serovar. In serovar Choleraesuis, the presence of the plasmid harbouring the cloned *avrA* gene from *Salmonella* Typhimurium LT2 appeared to counteract the activation of NFκB. On the other hand, LoVo human intestinal epithelial cells infected with the *S*. Typhimurium Δ*avrA* mutant showed significantly lower NFκB activation than cells infected with the wild type strain. The presence of the *avrA* gene in *S*. Typhimurium and *S*. Choleraesuis show to have an effect on NFκB activation in the LoVo cell line 4 hours post-infection, but the role in the inhibition or activation of this transcription factor is not clear (see Figure 11C). In the study of Liu and co-workers, they confirmed that AvrA modulates NFκB activation since genes that are regulated by this transcription factor were significant affected by the presence or absence of the *avrA* gene in the *S.* Typhimurium strain used; however, these genes were both up- and down-regulated, suggesting a complex function of AvrA that was also dependent on the time elapsed after infection (Liu *et al*., 2010). In the present study, a possible effect of the *avrA* gen on NFκB activation was only observed in human intestinal epithelial cells and only 4 hours after infection which is consistent with a complex function of AvrA as suggested by Liu *et al*. (2010). It would therefore appear that elucidation of the role of AvrA may be highly dependent upon how and at which time post-

infection one determines the level of activation of NFκB and NFκB-dependent genes. Currently, the role of AvrA on NFκB activation remains controversial. There are studies showing that the AvrA protein in the context of infection has no effect on the host NFκB activation, whereas other studies show a regulatory effect of AvrA on the NFκB. The following table (Table 9, below) shows a summary of studies about the functions of *Salmonella* AvrA effector protein using different experimental systems. The differences in the experimental procedures and in the strains used in the different publications must be taken into account. With regard to the present study, there are currently no other studies that analyse and compare the effects of the differential presence of the *avrA* gene in *Salmonella* serovars Choleraesuis and Typhimurium on NFκB activation in cells of porcine and human origins. However, as noted above, we have used the cloned *avrA* gene from a strain known to show a constitutive synthesis of AvrA (Ben-Barak *et al*., 2006) and, at least *in vitro*, has been demonstrated to show the proposed activities (Du and Galán, 2009). Furthermore, in the present study cell types which are relevant both for the gastrointestinal/enteric (intestinal epithelial cell lines) and systemic (blood macrophage) forms of *Salmonella* infections were used (Drewinko *et al*., 1976; Tsuchiya *et al*., 1982; Schierack *et al*., 2006; Brosnahan and Brown, 2012).

Table 9: Previous reported functions of *Salmonella* AvrA effector protein in host-bacteria interaction

Experimental model	Strains	AvrA observed effects	References
Cell lines: Henle-407, Macrophages J774A/ BALB/c mice: infected with 10^6 bacteria	*S.* Typhimurium SL1344	No AvrA effect in invasion of Henle cells, toxicity in J774 cells or in mouse virulence.	(Hardt and Galán, 1997)
Hela cell line: cells transfection experiments	plasmid with *avrA* gene from *S.* Typhimurium SL3201	AvrA Inhibits NFκB activation, down regulates IL-8 and activates apoptosis	(Collier-Hyams *et al.*, 2002)
HT-29 cell line	*S.* Typhimurium PhoP^c, SL3201, *S.* Pullorum	AvrA Inhibits NFκB activation, down regulates IL-8 gene expression	(Collier-Hyams et al., 2002)
cell free system/ Human embryonic kidney 293 cells, Hela cell line, mice: infected with 10^7 bacteria	*S.* Typhimurium PhoP^c, SL3201	Inhibit NFκB activity in cell lines and mice/ Cell free system: AvrA removes the ubiquitin moieties from –ub- β-catenin and IκB/ Blocks degradation of IκB and β-catenin IL-6 was down-regulated AvrA expression increased epithelial proliferation and inhibits cell apoptosis	(Ye et al., 2007)
Drosophila 293T human cells transfection/ Streptomycin pre-treated mouse10^8	*S.* Typhimurium SL3201	Down-regulates IL-8 (drosophila)/ Blockade of JNK, MAPKK, MKK4/7 Inhibits JNK mediated apoptosis	(Jones *et al.*, 2008b)

Continuation Table 9: Previous reported functions of *Salmonella* AvrA effector protein in host-bacteria interaction

T84, HT29-CL19A cells, streptomycin pre-treated mouse infected with 10^7 bacteria	*S.* Typhimurium 14028	AvrA stabilizes epithelial tight junctions, down-regulates IL-6	(Liao e*t al.*, 2008)
Henle 407 intestinal epithelial cells MOI=30	*S.* Typhimurium SL1344 ($avrA_{LT2}^{+}$)	No effect in NFκB and p38 while infection Inhibits JNK	(Du and Galán, 2009)
Streptomycin pre-treated mouse Cronical effect 18 h, 1,2,3 weeks p.i.	*S.* Typhimurium ATCC14028 SL1344	AvrA promotes intestinal *Salmonella* invasion in vivo/ No effect in IL-1β	(Lu *et al.*, 2010)
Streptomycin pretreated mouse 7 days p.i. infected with 10^8 bacteria	*S.* Typhimurium SL3201	JNK suppression Survival of macrophages suppression of apoptosis	(Wu e*t al.*, 2011)
Streptomycin pre-treated mouse Microarray/RTPC 8h 4days. Infected with $1x10^7$ bacteria	*S.* Typhimurium SL1344	Involved in mTOR signalling pathway May inhibit activation of JNK at 8 hours post infection/4 days post infection no effect on JNK Modulates NFκB activation	(Liu e*t al.*, 2010)

In the early phase of infection (2 and 4 hours p.i.), infection with *S.* Typhimurium strains generated a higher NFκB activation than the *S*. Choleraesuis in the cell lines of both swine and human origins independent of the cell type (intestinal epithelial or macrophage cells) (see Figures 11A, 11B, 11C and 11D). This lower NFκB activation in cells infected with the *S*. Choleraesuis was also observed 24 hours following infection in porcine epithelial and macrophage cell lines, independent of the presence or lack of the *avrA* gene (see Figures 11E and 11F). In the human macrophage THP-1 cell line, the effect on NFκB activation during infection with the serovar Choleraesuis changed between 4 and 24 hours post infection: in the early infection phase (see Figures 11B and 11D), *S*. Choleraesuis infection showed lower NFκB activation, and 24 hours post infection the level of activation was significantly higher compared to *S*. Typhimurium (see Figure 11F). This difference in the NFκB activation is not directly dependent on intracellular bacterial numbers since this

higher activation by *S*. Choleraesuis in THP-1 cells was observed despite the lower intracellular cfu of this serovar compared with the serovar Typhimurium which despite having a higher intracellular load, did not show a higher activation of NFκB. This lower NFκB activation in human and porcine intestinal epithelial cells and macrophages in the early phase of infection (2-4 h p.i.) by *S*. Choleraesuis followed by a higher NFκB activation in human macrophages 24 hours post infection, could play a role in the higher rate of systemic disease caused by this *Salmonella* serovar in humans. A lower intestinal immune response has been suggested to favour *Salmonella* systemic infections, a strategy described for *Salmonella* serovar Typhi infections which avoid local intestinal inflammation (Mastroeni and Grant, 2011; de Jong *et al*., 2012; Feasey *et al*, 2012). Macrophages have been suggested to contribute to the spread of *Salmonella* to other tissues but the exact role of macrophage and their cell death in *Salmonella* systemic dissemination remains unclear (Watson *et al*., 2000; Guiney, 2005; Mastroeni and Grant, 2011).

Due to the possible effect of the presence and transcription of the *avrA* gene in both *Salmonella* serovars on NFκB activation in the LoVo cell line and the serovar-specific, time-dependent activation of NFκB observed in THP-1 cells, the *Salmonella* serovar-specific activation of the immune response was further tested in human intestinal epithelial and macrophage cell lines by measuring the expression of the *jun* gene which is part of the transcription factor AP-1 and JNK signaling pathway. Like NFκB, the AP-1/JNK pathway is activated by the host immune system as a defense mechanism against *Salmonella* infection, and is also modulated by *Salmonella* effector proteins for survival within the host (Weston and Davis, 2007; Haraga *et al.,* 2008).

The presence of the *avrA* gene and the proved expression of *avrA* mRNA in *S*. Typhimurium appeared to inhibit the expression of the JUN gene in human epithelial cells at both 4 and 24 hours post infection, as the presence of the *S*. Typhimurium LT2 *avrA* gene in the *S*. Typhimurium SL1344 Δ*avrA* strain led to a reduced level of JUN mRNA at 4 and 24 hours following infection of LoVo cell line compared to the SL1344 Δ*avrA* strain harbouring the vector alone (see Figures 12A and 12B). This result suggests that the presence of the *avrA* gene in *S*. Typhimurium modulates the transcription of JUN and subsequently the AP-1 transcription factor which is also known to regulate the transcription of important genes involved in the host immune response against *Salmonella* (Nishina *et al*., 2003; Karin and Gallagher, 2005). This result is in accordance with other studies where the AvrA effector protein counteracted the activation of the JNK pathway during *S*. Typhimurium infection in different experimental models such as human and murine epithelial cell lines and *in vivo* murine models (Jones *et al*., 2008b; Du and Galán, 2009; Liu *et al*., 2010; Wu *et al*., 2011). However, there were no statistically significant effects of the presence or absence of the *avrA* gene in *S*. Choleraesuis or *S*. Typhimurium on JUN mRNA levels in the human macrophage cell line THP-1. As there are no

publications analyzing the differential presence of the *avrA* gene in *S.* Choleraesuis or *S.* Typhimurium infection in human macrophages, it is difficult to directly compare these results with published data. Nevertheless, the results obtained regarding to the effect of the presence of the *avrA* gene on regulation of activation of host transcription factors AP-1 and NFκB suggest that AvrA has the ability to modulate both transcription factors during infection in human intestinal epithelial cells.

Despite the possible effect of harbouring the *avrA* gene in the transcription factors AP-1 and NFκB observed during *Salmonella* infection in the LoVo cell line, in this intestinal epithelial cell line no statistically significant effect of the presence or absence of the *avrA* gene in *S.* Choleraesuis or *S.* Typhimurium was observed on the level of expression of IL-8 and IL-1β, both of which are known to be regulated by these transcription factors (Roman *et al*., 2000; Hoffmann *et al*., 2002; Vitiello *et al*., 2004; Murphy *et al*., 2008). The expression of IL-1β gene was stimulated to significantly higher levels in human macrophage cells infected with the strain *Salmonella* serovar Typhimurium Δ*avrA* mutant than with the *avrA*+ strain, 24 hours post infection, suggesting that the presence of the *avrA* gene inhibited its induction (see Figure 14D). This is according with the proposed AvrA function in modulation of the host immune response suggested in other studies (Table 9; Collier-Hyams *et al*., 2002; Ye *et al*., 2007; Jones *et al*., 2008b; Liao e*t al*., 2008; Du and Galán, 2009). However, the expression levels of IL-8 and IL-1β showed significant variations depending on the serovar causing the infection and the specific cell type infected.

The relative IL-8 mRNA expression level was significantly lower in response to *S.* Choleraesuis infection compared to *S.* Typhimurium in all the cell lines tested at 4 hours post-infection, and independent of the presence or absence of the *avrA* gene (see Figures 13A, 13C, 13E and 13G). The lower IL-8 levels corresponded with the lower NFκB activation by serovar Choleraesuis in the present study and also with other studies where this *Salmonella* serovar elicited a later (Wang *et al*., 2008) or lower cytokine induction than serovar Typhimurium in *in vitro* and *in vivo* infections (Skjolaas *et al*., 2006; Paulin *et al*., 2007; Skjolaas *et al*., 2007; Uthe *et al*., 2007). In addition, the infection with *S.* Choleraesuis generated lower expression levels of IL-1β in THP-1 cells compared to *S.* Typhimurium at both 4 and 24 hours post infection (see Figures 14C and 14D).

In contrast, the level of IL-1β expression was similar between LoVo cells infected with *S*. Choleraesuis and cells infected with *S*. Typhimurium 4 hours post infection (see Figure 14A) but 24 after infection *S*. Choleraesuis wild type strain (without *avrA* gene) generated higher expression of IL-1β than *S*. Typhimurium harbouring the cloned *avrA* gene. This differential interleukin response to *Salmonella* serovars Choleraesuis and Typhimurium was also dependent on the presence or absence of the *avrA* gene, and was observed 24 hours post infection for the IL-8 level in the cell lines IPEC-J2, LoVo and

THP-1 which could may be related to an effect of AvrA in host immune response (see Figures 13B, 13D and 13F).

The *Salmonella* effector protein SseL has also been shown to modulate the host immune response during *Salmonella* infection, and the *sseL* gene is present in both *S.* Typhimurium and *S*. Choleraesuis. Like the AvrA protein, SseL has been suggested to possess a deubiquitinase activity (Rytkonen *et al*., 2007; Le Negrate *et al.*, 2010). Also, like AvrA, the effects of SseL are also controversial, with studies suggesting that SseL inhibits NFκB activity (Le Negrate *et al.*, 2010), but with a more recent study finding no effect of this effector protein on NFκB activation (Mesquita *et al*., 2013). In the present study the possible overlapping functions of the effector proteins AvrA and SseL in the host immune response were examined using *S.* Typhimurium strains SL1344 *avrA*+ and the strains harbouring deletion mutations of Δ*avrA*, Δ*sseL* or both genes (Δ*avrA* Δ*sseL*).

A possible overlapping effect of these two effector proteins was suggested by the observation that 2 hours post-infection, the *S.* Typhimurium Δ*avrA* Δ*sseL* double mutant strain showed significantly higher NFκB activation than the Δ*sseL* mutant harbouring the *avrA* gene in porcine macrophage cell line PLN/C2 (see Figure 15A). Likewise, in the THP-1 human macrophage cell line, the *S.* Typhimurium Δ*avrA* Δ*sseL* double mutant showed higher IL-1β expression 4 hours post-infection than the Δ*sseL* mutant strain possessing the *avrA* gene (see Figure 18C). Furthermore, the *S.* Typhimurium Δ*avrA* Δ*sseL* double mutant lead to higher JUN expression in human intestinal epithelial cell line 24 hours after infection compared to the Δ*sseL* mutant strain harbouring the *avrA* gene (see Figure 16B). These differences were generally not present within the *sseL*+ strains. These results suggest a possible compensatory effect of the *sseL* gene in counteracting the host immune response which could compensate for the absence of the *avrA* gene in the *Salmonella* strains and, in addition to the reported complex *Salmonella* AvrA regulation, could explain the absence of difference between the *Salmonella* strains with and without the *avrA* gene during infection of the porcine intestinal epithelial and macrophages cells observed in the present study.

5.3 Conclusions

The comparison of infections of porcine and human intestinal epithelial and macrophage cell lines with *S.* Typhimurium or *S.* Choleraesuis, showed that *S.* Typhimurium was generally more invasive but showed a lower intracellular persistence compared to *S.* Choleraesuis. Secondly, *S.* Typhimurium generated a higher, early (2-4 h p.i.) NFκB activation, elevated transcription of IL-8 mRNA in all cell lines tested and a higher IL-1β response in the THP-1 cell line. The statistically significant higher levels of IL-1β mRNA in response to serovar Typhimurium was different depending on host, time post-infection and the presence of the *avrA* gene. In general however, the higher immune response to *S.* Typhimurium by porcine and human cells was independent on the presence or absence of the *avrA* gene in both *Salmonella* serovars. Generally, the response to *S.* Choleraesuis infection in porcine and human cells was lower in the early phase of infection, but changed during the course of infection, particularly in human macrophages. This later response of the host immune system against infection with *S.* Choleraesuis should be further investigated *in vivo* with longer infection time analysis in relation with the clinic disease manifestation. Together with molecular analyses to elucidate additional, specific determinants in these serovars which lead to the differential host immune response and could improve vaccine strains and further understanding of disease outcome in relation to host responses.

In the present study the possible effect of the presence or lack of the *Salmonella avrA* gen in the differential host response to the serovars Choleraesuis and Typhimurium was evaluated since this virulence effector is – until now – the only known SPI-1 T3SS effector protein which is present in the genome of the serovar Typhimurium but is missing in serovar Choleraesuis (Chiu *et al.*, 2005; Suez *et al.*, 2013). Despite the complex regulation of the *avrA* gene, in the present study, this gene was successfully cloned into *S.* Choleraesuis and was successfully transcribed into mRNA, which provided the possibility of directly comparing the influence of the presence and transcription of the *avrA* gene in both serovars and to further investigate in future studies the possible role of this effector protein in differential pathogenicity and host response with other *Salmonella* serovars and/or different hosts. The differences in invasion and intracellular persistence between *Salmonella* serovar Choleraesuis and Typhimurium observed in this study were not dependent on the presence or absence of the *avrA* gene in either serovar.

The possible effects of harbouring the *avrA* gene on modulation of the host immune response against *Salmonella* infection with serovars Choleraesuis and Typhimurium were statistically significant only in the human LoVo intestinal epithelial cell line and only affected the NFκB activation in the early phase of infection (2-4 h p.i.), and JUN mRNA expression levels in both the early and the late phase of infection (24 h p.i.) only in *S.*

Typhimurium. A possible effect of harbouring the *avrA* gene was also observed in IL-1β mRNA levels in the later phase of *S*. Typhimurium infection in the human macrophage cell line THP-1. The presence or absence of the *avrA* gene in both *Salmonella* serovars had no effect on the immune response in the porcine cell lines tested. Finally, it can be concluded that any differences observed in invasion, intracellular persistence and host immune response between *S.* Choleraesuis and *S*. Typhimurium are not explained by the presence or absence of the *avrA* gene in *S*. Choleraesuis, indicating that the host-adaptation of this serovar for swine, and the high rates of systemic infections in humans is dependent upon other factors. This observation should be the focus of future studies.

6 REFERENCES

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8 SELBSTÄNDIGKEITSERKLÄRUNG

Hiermit ekläre ich, dass ich die vorligende Arbeit selbstständig und nur unter Verwendung der angegebenen Quellen und Hilfen angefertig habe. Diese Arbeit wurde keiner anderen Prüfungsbehörde vorgelegt.

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