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**School of Veterinary Medicine and Science**

**Immune Response of the Chicken in  
Determination of Virulence Profiles of  
*Salmonella enterica***

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## Abstract

*Salmonella enterica* subspecies *enterica* (*S. enterica*) infection remains a global problem in a wide range of animals and in man. Poultry-derived food is a common source of human infection with the non-host-adapted *Salmonella* strains while fowl typhoid and pullorum disease are serious diseases in poultry. Development of novel immune-based control strategies against *Salmonella* infection necessitates a better understanding of the host-pathogen interactions at the cellular level. This study characterizes, *in vitro* and *in vivo*, the immune responses that develop following infection of avian species with typhoid and non-typhoid *Salmonella* serotypes. *Salmonella* serovars Typhimurium, Enteritidis, Hadar and Infantis showed a greater level of invasion and/or uptake characters to both chicken macrophages (HD11) and chicken kidney epithelial cells (CKC), when compared with *S. Pullorum* or *S. Gallinarum*. Nitrate and reactive oxygen species were greater in *Salmonella*-infected HD11 cells compared with the non-infected controls. HD11 cells revealed higher mRNA gene expression for CXCLi2 (IL-8), IL-6 and iNOS genes in response to *S. Enteritidis* infection when compared to *S. Pullorum*-infected cells. *S. Typhimurium*- and *S. Hadar*-infected HD11 showed higher gene expression for CXCLi2 versus *S. Pullorum*-infected cells. Higher mRNA gene expression levels of pro-inflammatory cytokine IL-6, chemokines CXCLi1 (K60) and CXCLi2 and iNOS genes were detected in *S. Typhimurium*- and *S. Enteritidis*-infected CKC followed by *S. Hadar* and *S. Infantis* while no significant changes were observed in *S. Pullorum* or *S. Gallinarum*-infected CKC. Epithelial cell response and production of pro-inflammatory cytokines and chemokines were greatly influenced by *Salmonella* virulence markers,

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including *Salmonella* pathogenicity island type-1 (SPI-1), SPI-2 and bacterial flagella. In chicken infections, *S. Enteritidis* and *S. Infantis* colonized the caeca more efficiently than *S. Gallinarum* and *S. Pullorum*. High numbers of B-lymphocytes and macrophages were observed in the caecal tonsils of infected birds. *S. Enteritidis* infection in newly hatched birds elicited the expression of CXCLi1 and CXCLi2 chemokines in the caecal tonsils, while *S. Gallinarum* up-regulated the expression of LITAF. In older chickens, *S. Enteritidis* infection resulted in a significantly higher expression of CXCLi2, iNOS, LITAF and IL-10 while *S. Pullorum* appeared to down-regulate CXCLi1 expression in the caecal tonsils. Data from spleens showed either no expression or down-regulation of the tested genes. In conclusion, data from the present study provide further insights on the interaction of *Salmonella* with poultry, and while both *S. Typhimurium* and *S. Enteritidis* are strong inflammatory serotypes, *S. Pullorum* and *S. Gallinarum* are not.

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## **Declaration**

Unless otherwise acknowledged, the work presented in this thesis is my own.

No part has been submitted for another degree at the University of Nottingham or elsewhere.

Ahmed Setta

October 2011



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**List of abbreviations**

APCs	Antigen-presenting cells
AvBD	Avian $\beta$ -defensins
CKC	Chicken kidney cells
CSF	Colony-stimulating factor
CpG-ODN (DNA)	Oligodeoxynucleotide containing unmethylated CpG-dinucleotides
CFU	Colony forming unit
COEC	Chicken oviduct epithelial cells
CT	Caecal tonsil
DCs	Dendritic cells
DCFH-DA	2',7' dichlorofluorescein-diacetate
EC	<i>E. coli</i>
eNOS	Endothelial NOS
FAE	Follicle-associated epithelium
FBS	Foetal bovine serum
GALT	Gut-associated lymphoid tissues
GM-CSF	Granulocyte macrophage-CSF
HG	Harderian gland
IEL	Intraepithelial lymphocytes
IFN	Interferon
I $\kappa$ B	Inhibitor $\kappa$ B
IL	interleukin
iNOS	Inducible NOS
LITAF	lipopolysaccharide-induced tumour necrosis alpha factor
LPS	lipopolysaccharide
M cell	Microfold cell
MALT	Mucosa-associated lymphoid tissues
MAPK	Mitogen-activated protein kinase
MD	Meckel's diverticulum
MHC	Major histocompatibility complex
MIP	Macrophages inhibitory protein

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MØ	Macrophage
MOI	Multiplicity of infection
Nal <sup>r</sup>	Nalidixic acid resistance
NBF	Neutral buffered formalin
NF-κB	Nuclear factor- κB
NK cell	Natural killer cell
NLRs	Nucleotide-binding oligomerization domain (NOD)- like receptors
NOD	Nucleotide-binding oligomerization domain
NO	Nitric oxide
NOS	Nitric oxide synthase
nNOS	Neuronal NOS
Nramp-1	Natural resistance associated macrophage protein 1
OD	Optical density
OT	Oesophageal tonsil
PAMs	Pulmonary alveolar macrophages
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
PMNs	Polymorph nuclear cells
PP	Peyer's patches
PRRs	Pattern recognition receptors
P/S	Penicillin/Streptomycin
PT	Pyloric tonsil
QRT-PCR	Quantitative real-time polymerase chain reaction
rChIFN-γ	Recombinant chicken IFN-γ
RFU	Relative fluorescent units
ROS	Reactive oxygen species
<i>S</i>	<i>Salmonella</i>
SE	<i>S. Enteritidis</i>
SG	<i>S. Gallinarum</i>
SH	<i>S. Hadar</i>

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SI	<i>S. Infantis</i>
SP	<i>S. Pullorum</i>
ST	<i>S. Typhimurium</i>
SCV	<i>Salmonella</i> -containing vacuole
SPF	Specific pathogen free
SPI	<i>Salmonella</i> pathogenicity island
TBS	Tris buffered saline
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPB	Tryptose phosphate broth
TTSS	Type three secretion system
VCC	Viable colony count
wt	Wild type

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## 1 Introduction

### 1.1 General introduction

The family *Enterobacteriaceae* consists of a large number of Gram-negative aerobic or facultatively anaerobic rods. The family includes a group of bacterial genera which share antigenic and biochemical similarities. These genera are *Salmonella*, *Escherichia*, *Shigella*, *Citrobacter*, *Klebsiella*, *Proteus* and *Yersinia*. *Salmonella* is an important genus of the family *Enterobacteriaceae* which contains a vast range of serologically distinguishable serotypes (serovars). Serotypes of genus *Salmonella* are well-known for their impact on human and animal health as they are associated with a wide range of clinical problems, including typhoidal and non-typhoidal infections.

Infections with *Salmonella* in poultry usually cause a variety of acute and chronic diseases. These diseases have been associated with significant economic losses to poultry producers and have been addressed by the application of various testing and control programmes (Gast, 2003). Infected poultry flocks are considered as the most important reservoir of *Salmonellae* which can be transmitted to human, perhaps as a result of the high prevalence of *Salmonella* infections in poultry (Gast, 1997). While recent reports have shown a decline in the prevalence of *Salmonella* in UK layer flock holdings, recent outbreaks in the UK have been linked to imported eggs (FSA, 2006). Poultry meat and eggs contaminated with *Salmonella* remain among the leading causes of human food-borne infections (Rabsch et al., 2001). Human food-borne gastroenteritis remains a critical issue that receives more attention

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by the regulatory authorities to prevent or minimize the risk of transmission of *Salmonella* from poultry and poultry products.

## 1.2 Taxonomy

The classification of *Salmonella* is a complex subject which has passed through several different stages of refinement. The genus *Salmonella* contains a large number of serotypes and nomenclature is mainly based on the somatic (O) and flagellar (H) antigens according to Kaufmann and White scheme (Quinn et al., 2002, Heyndrickx et al., 2005). It is thought that *Salmonella* and *E. coli* might have originally diverged from a common ancestor 120-160 million years ago (Ochman and Wilson, 1987). Genus *Salmonella* can be classified into two main species; *Salmonella enterica* and *Salmonella bongori* (Crosa et al., 1973), although a new species, *Salmonella subterranea*, has recently been identified and defined according to the morphological, biochemical and genetic similarities (Shelobolina et al., 2004, Su and Chiu, 2007). *Salmonella bongori* is mainly associated with diseases of cold blooded animals and was classified as a member of *Salmonella enterica* before its definition as a separate species (Reeves et al., 1989). *Salmonella* pathogenicity island (SPI) type 1 (SPI-1) occurs in both *Salmonella enterica* and *Salmonella bongori* while SPI-2 is only present in *Salmonella enterica*. It is highly suggested that the presence of both SPI in *Salmonella enterica* would enable these bacteria to penetrate epithelial cells, survive within macrophages and cause systemic infections in mammals while *Salmonella bongori* is commonly isolated from non mammalian hosts, including frogs (Ochman and Groisman, 1996). Based on DNA sequence analysis, *Salmonella enterica* is now subdivided into six subspecies: *Salmonella enterica* subspecies *enterica* (*S. enterica*), *salamae*, *arizonae*,

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*diarizonae*, *houtenae* and *indica* (Brenner et al., 2000, Heyndrickx et al., 2005).

Genus *Salmonella* contains more than 2400 serotypes which contribute to most cases of infection in human and animal hosts (Old, 1990, Su and Chiu, 2007).

### **1.3 *Salmonella* serotypes**

The genus *Salmonella* contains a large number of serotypes, with more than 2400 known serovars (Brenner et al., 2000, Su and Chiu, 2007). The antigenic formula of these serovars are listed in the Kauffmann-White Scheme and maintained and annually updated by World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* (Brenner et al., 2000, Popoff et al., 2000).

### **1.4 Zoonotic infections**

Human food-borne zoonosis is a global public health problem. Farm animals are considered as an important source of human food-borne gastroenteritis, including poultry, as exemplified by *Salmonella* and *Campylobacter* (C) (Thorns, 2000, Esteban et al., 2008), and pigs as *Salmonella enterica* subspecies *enterica* serovar Typhimurium (*S. Typhimurium*) infection in pigs is an important public health burden (Boyen et al., 2008a, Boyen et al., 2009). Non-typhoidal *Salmonella* infections, mainly resulting from *S. Enteritidis* and *S. Typhimurium*, are commonly the result of the consumption of poultry-derived food, mainly meat, eggs and egg products (Burr et al., 2005, Schroeder et al., 2005, Zaidi et al., 2006, Much et al., 2007, Stephens et al., 2007). At the processing plants, potential risk of contamination of chicken carcasses with faecal material is very common especially during the process of evisceration (Wilson et al., 1996, Humphrey, 2000). Disease in man occurs in two main

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forms; typhoid fever, a systemic disease caused by *S. Typhi*, or gastroenteritis which is caused by a range of *S. enterica* serovars.

*Salmonella*-associated human infections remain an important health problem and an economic burden world-wide. Transmission usually occurs after the consumption of contaminated materials, including meat, eggs and milk (Much et al., 2007). Around 30,000 human cases were reported in 1990 in the UK, with *S. Typhimurium* and *S. Enteritidis* being the most frequently isolated serotypes which were responsible for around 80% of infections in England and Wales (Humphrey, 2000). In England and Wales, an estimate of 102,227 of non-typhoidal *Salmonella* food-borne cases were also reported in 1995, with 3,412 hospital admissions and 268 deaths (Adak et al., 2002). Approximately 13,000 cases were reported in Great Britain due to *Salmonella* infections in 2007 (Fraser et al., 2009). Furthermore, a total of 9,079 human *Salmonella* isolates were also detected in England and Wales in 2009, where *S. Enteritidis* and *S. Typhimurium* were the most frequently isolated serovars (<http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/Salmonella>).

Historically, *S. Typhimurium* was the most frequently isolated serovar until the 1980s when *S. Enteritidis* was emerged as a serious food-borne pathogen (Cogan and Humphrey, 2003). *S. Enteritidis* phage type 4 (PT4) is the commonly isolated egg-borne *Salmonella* PT across the UK and Europe (Fisher, 2004, Gillespie et al., 2005). Infections with these serotypes have been gradually controlled through introduction of hygienic and management measures and vaccination across Europe, resulting in a significant decrease in

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the rate of *Salmonella* contamination in the UK-produced eggs, and relative increase in importance of other serovars such as *S. Infantis*, which has always been historically important in Eastern Europe (Zhang-Barber et al., 1999, EFSA, 2004a, Elson et al., 2005). Indeed, recent reports have shown increased levels of *Salmonella* infection and egg contamination in some EU countries, compared to the UK (Elson et al., 2005, FSA, 2006, Little et al., 2006). Moreover, a number of investigations and epidemiological studies have been carried out to study the source and pattern of egg contamination in the UK and has been linked to the introduction of eggs from outside the UK, such as Spain (FSA, 2006, Little et al., 2006, Little et al., 2007). Other *Salmonella* serotypes such as *S. Hadar* and *S. Infantis* can also be transmitted to human and are capable of causing food poisoning and, indeed, human cases have been recorded after the consumption of poultry meat contaminated with these serovars (Mochizuki et al., 1992, Wilkins et al., 2002).

Around 2,138 cases of human gastroenteritis were reported in Spain following ingestion of undercooked chickens, where *S. Hadar* was isolated and identified (Lenglet, 2005). In 2006, a European surveillance study conducted by the European Union (EU) on commercial laying flocks showed that more than 30% of the tested layer flocks (5,310) were positive for *Salmonella*, with *S. Enteritidis* as the frequently isolated serovar (EFSA, 2007b). A total of 165,023 of human cases were reported in the EU in 2006, with 62.5% and 12.9% of the cases were due to *S. Enteritidis* and *S. Typhimurium*, respectively (EFSA, 2007a). Also, *S. enterica* is one of the leading causes of food-borne infections, with 151,995 human cases and 4.3% prevalence in examined laying

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flocks in the EU in 2007 (Westrell et al., 2009). In Europe, the highest numbers of cases of travel-associated salmonellosis were reported in Bulgaria followed by Turkey and Malta, where *S. Enteritidis* was the most commonly isolated *Salmonella* strain (de Jong and Ekdahl, 2006). The total costs resulting from food-borne *Salmonella* infections of humans in the United States were estimated at up to 3.5 billion dollars for 1993 (Gast, 1997). About 80% (298 out of 371) of *S. Enteritidis* outbreaks recorded in the United States between 1985 and 1999 were egg associated (Patrick et al., 2004). Moreover, it is also estimated that 1.4 million non-typhoidal *Salmonella* infections with 400 deaths occurs yearly in USA (Voetsch et al., 2004).

## 1.5 Epidemiology

*Salmonella* is an intracellular bacterial pathogen capable of infecting a wide diversity of hosts, causing different forms of disease syndromes, including gastroenteritis, enteric fever, bacteraemia and asymptomatic carriage (Goldberg and Rubin, 1988). Many hosts are susceptible and including cold and warm blooded animals, insects, reptiles, rodents, poultry species and mammals, including man (Murray, 1998).

Clinical outcomes vary from septicaemia to acute and chronic enteritis. Table 1.1 shows *Salmonella* serotypes of clinical importance and the consequences of infections in different hosts (adapted from Quinn et al., 2002). In some cases, abortion may occur in pregnant animals. The development of asymptomatic carriers is also seen in different host species. Septicaemia is the common syndrome in young animals, such as lambs, calves, foals and piglets, with high mortality which may reach 100%, mainly due to the immaturity of the immune

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system of young animals, as also in newly hatched chicks. While acute enteritis is frequently seen in adult cattle, sheep and horses, chronic enteritis is commonly seen in growing pigs but rarely in cattle (Kahn, 2005). The clinical manifestations depend upon the number of micro-organisms being ingested, virulence of infected serotypes and the susceptibility and the immune status of the infected host. Infection in cattle and sheep is commonly endemic on a certain farm with sporadic outbreaks. Septicaemic salmonellosis is common in foals while rare in pigs. Adult horses, dogs and cats also develop the asymptomatic carrier form of infection. In equines, most cases develop after exposure to stressful conditions such as surgery or transport (Kahn, 2005).

**Table 1.1:** *Salmonella* infections in different hosts

Serotype	Hosts	Consequences of infection
<i>Salmonella</i> Typhimurium	Many animals Humans Poultry Mice	Enteritis Food poisoning Asymptomatic carriage Systemic (typhoid) infection
<i>Salmonella</i> Dublin	Cattle, sheep, horses, dogs	Systemic infection Enterocolitis and septicaemia
<i>Salmonella</i> Choleraesuis	Pigs	Enterocolitis and septicaemia
<i>Salmonella</i> Pullorum	Chicks	Pullorum disease, systemic infection (bacillary white diarrhoea)
<i>Salmonella</i> Gallinarum	Adult birds	Fowl typhoid (systemic infection)
<i>Salmonella arizonae</i>	Turkeys	Enterocolitis
<i>Salmonella</i> Enteritidis	Poultry Many other species Human	Often asymptomatic carriage Clinical disease in mammals Food poisoning
<i>Salmonella</i> Brandenburg	Sheep	Abortion
<i>S. Hadar</i> and <i>S. Infantis</i>	Many hosts Poultry	Gastroenteritis in human Asymptomatic carriage

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Infection of farm animals with *Salmonella* usually occurs after ingestion of contaminated feed, with faecal shedding into the environment (Daniels et al., 2003, Davies et al., 2004). Contaminated faeces of rodents and free living birds are potential sources of infection (Daniels et al., 2003). The severity of infection may be increased by the presence of stress factors such as transportation, overcrowding and improper nutrition (Hollinger, 2000). Moulting in poultry, for instance, has been found to be associated with severe forms of *S. Enteritidis* infection (Seo et al., 2001). Prevalence of *Salmonella* infection in poultry is also influenced by other risk factors, including the housing system, cleaning procedure and type of diet (De Vylder et al., 2009, Teirlynck et al., 2009, Van Hoorebeke et al., 2009, Van Hoorebeke et al., 2010a, Van Hoorebeke et al., 2010b). Introduction of newly purchased cattle has been associated with increased incidence of *S. Typhimurium* infection (Hollinger, 2000). The latter finding has been correlated with the replacement of culled animals with those from high risk sources, in which large herds are more susceptible to *Salmonella* infection than small herds. In a survey study of *Salmonella* infection conducted on 454 UK commercial laying farms, high prevalence was associated with *S. Enteritidis* (11.7 %) followed by *S. Typhimurium* (1.8 %), where *S. Infantis* was also isolated but not *S. Hadar* (Snow et al., 2007). The above mentioned predisposing factors should be seriously considered as potential sources of infection to livestock.

From the point of infection biology, serotypes of *S. enterica* can be divided into two main classes (reviewed by Barrow, 2007). A small number of serovars produce typhoid-like infection in a restricted number of host species. These

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pathogens include *S. Typhi* in man, *S. Dublin* in cattle, *S. Choleraesuis* in pigs and *S. Gallinarum* and *S. Pullorum* in poultry. These serovars are transmitted via the faecal-oral route but colonise the gut poorly in the absence of clinical disease and invade with bacterial multiplication in the spleen, liver and other organs. They only re-enter the gut in the later stages of disease and, therefore, rarely enter the human food chain (Uzzau et al., 2000, Barrow, 2007). The second class contains the vast majority of the remaining serovars. These rarely produce systemic disease in normal healthy, adult animals but colonise the gut without disease and are thus able to enter the human food chain producing food poisoning. They include *S. Enteritidis*, *S. Typhimurium*, *S. Infantis*, *S. Montevideo* and many others. Consumption of poultry meat or eggs contaminated with these organisms has been correlated with multiple cases of human infections (Burr et al., 2005, McPherson et al., 2006, Noda et al., 2010).

### **1.6 *Salmonella* infections in poultry**

*Salmonella* species are responsible for a variety of acute and chronic diseases in poultry. Avian salmonellosis can develop as a result of infection with poultry-specific serovars, *S. Gallinarum* and *S. Pullorum*, causing systemic illness in birds as well as other *Salmonella* serotypes, including *S. Typhimurium* and *S. Enteritidis* and many others, which contribute to paratyphoid infections (Gast, 2003). *Salmonella* serotypes which are specific for poultry are usually avirulent in human while serovars of broad host range are usually commensal in adult poultry. Avian salmonellosis is well-described and documented in many published textbooks, including poultry diseases (Lister and Barrow, 2008), diseases of poultry (Shivaprasad and Barrow, 2008) and *Salmonella* in domestic animals (Humphrey, 2000), and can be classified

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into three main groups; (i) fowl typhoid and pullorum disease, (ii) paratyphoid infections and (iii) arizonosis. Here is an overview of the disease conditions associated with *Salmonella* infection in poultry.

### **1.6.1 Fowl typhoid and pullorum disease**

Fowl typhoid and pullorum disease are severe systemic diseases caused by infections with two non-motile *Salmonella* serotypes, *S. Pullorum* and *S. Gallinarum*, respectively. These diseases are typically seen in chickens and turkeys causing severe clinical signs and considerable economic losses. Other birds such as quails, pheasants, ducks and peacocks are also susceptible to infection. Both diseases could be seen in birds in all ages. However, fowl typhoid frequently affects adult and growing birds while pullorum disease is commonly observed in young birds (Lister and Barrow, 2008, Shivaprasad and Barrow, 2008). Fowl typhoid and pullorum disease have been largely eradicated in the Western countries and USA. However, evidence of the re-current infection with these serotypes is beginning to occur since the appearance of fowl typhoid in cage layers and free range birds (Cobb et al., 2005, Parmar and Davies, 2007). Fowl typhoid is still a major disease in the developing countries (Shivaprasad, 2000).

Fowl typhoid and pullorum disease are transmitted by vertical (transovarian) or horizontal (lateral) spread, through the faecal-oral route. Vertical transmission is an important mode of infection for both *S. Gallinarum* and *S. Pullorum*. However, little is known about the relative contribution of transovarian transmission for *S. Gallinarum* infection as the outcomes of experimental infection with *S. Gallinarum* depend primarily on the genetic

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background of the infected birds (Lister and Barrow, 2008, Shivaprasad and Barrow, 2008).

Fowl typhoid is an acute or chronic systemic disease affecting mature birds. Clinical signs include high mortality, lack of appetite, decreased egg production and reduced fertility and hatchability. Post-mortem lesions include inflammation of reproductive organs (salpingitis and orchitis), hepatosplenomegaly, peritonitis and perihepatitis. Experimental infection of chickens with *S. Gallinarum* can result in a mortality rate of more than 60% (Jones et al., 2001). Pullorum disease is a septicaemic disease of chicks and turkey poults. The disease is characterized clinically by increased morbidity and mortality, depressed appetite or anorexia, white viscous droppings (diarrhoea) and dehydration. Macroscopic and microscopic lesions demonstrate the involvement of systemic organs and include hepatitis, splenitis, typhlitis, myocarditis, ventriculitis, pneumonia, peritonitis and omphalitis and unabsorbed yolk sac. Synovitis and ophthalmitis have been also observed in the sub-acute form of the disease (Shivaprasad, 2000, Lister and Barrow, 2008, Shivaprasad and Barrow, 2008).

### **1.6.2 Paratyphoid infections**

Infections with the abundant number of motile and non-host-specific *Salmonella* serotypes are collectively referred to paratyphoid infections. Paratyphoid *Salmonellae* comprise more than 2400 serotypes but few of them are extremely important from both economic and public health perspectives (Barrow, 2000, Gast, 2003). The most important human food-borne *Salmonella* serovars are *S. Enteritidis* and *S. Typhimurium* (Gast, 2008).

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Paratyphoid infections can be acquired following either vertical transmission from the infected breeder flocks to the progeny or horizontal transmission between the flocks (Gast, 2008, De Vyllder et al., 2011). Egg transmission of paratyphoid *Salmonellae*, particularly with *S. Enteritidis*, and consequent contamination of the internal egg contents are important aspects of the epidemiology of *S. Enteritidis* in chickens (De Buck et al., 2004b). Two possible mechanisms are common for egg contamination with paratyphoid *Salmonellae* (Gantois et al., 2009a). Infection of eggs can occur following the penetration of egg shells by paratyphoid *Salmonellae* from the infected gut or the contaminated faeces during or after the egg laying (oviposition). The second possible way is by direct contamination of the egg contents or egg shells by paratyphoid *Salmonellae* from infected reproductive organs before oviposition.

With the exception of very young chicks that often develop the acute systemic form of the disease, infections with paratyphoid *Salmonellae* usually result in asymptomatic carriage, with intestinal and internal organs colonization, and potential contamination of the finished carcasses, and consequently *Salmonellae* enter the human food chain. Indeed, *S. Typhimurium* infection of 1-day old chicks results in a severe systemic infection with a high rate of mortality (Barrow et al., 1987a) while infection with *S. Typhimurium* or *S. Enteritidis* in older chicks results in asymptomatic caecal colonization, with persistent shedding of the organisms in faeces (Barrow et al., 1987b, Humphrey et al., 1989). Clinical signs are uncommon in

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growing and mature birds over four weeks of age. Clinical manifestations are often observed in young birds as chicks, poults and ducklings. These include mortality, depression, anorexia, diarrhoea with pasting of the feather around the vent. Macroscopic lesions vary from complete absence of visible gross lesions to a septicaemic picture with congestion of the internal organs, including liver, spleen, lungs and kidneys. Unabsorbed yolk sac, typhlitis, pericarditis and perihepatitis are commonly observed (Lister and Barrow, 2008).

### **1.6.3 Arizonosis**

Avian arizonosis is an acute septicaemic disease of young turkey poults caused by *S. arizonae*. Transmission occurs by both lateral and vertical spread. Clinical signs include depression, depressed appetite, diarrhoea and pasting of the vent feathers, in addition to nervous signs and the eye abnormalities. Gross lesions include peritonitis, omphalitis, airsacculitis, hepatitis, typhlitis and eye lesions (Shivaprasad and Barrow, 2008).

## **1.7 Pathogenesis and virulence factors**

Pathogenesis and immune responses surrounding *Salmonella*-associated infections depend on the infecting *Salmonella* serotype, virulence of the infected serovar and infected hosts, including breed, genetics and immune responses. Most of our knowledge about the pathogenesis and immune responses surrounding *Salmonella*-associated infections are derived from experimental infection of mice, mainly with *S. Typhimurium*. Infection in mice is usually systemic and the severity of infection in mice depends on the virulence of the infecting serotype, route of infection and genetic makeup of

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infected mice (Hormaeche, 1979a, Hormaeche, 1979b, Khan et al., 2001). The different stages developed during the course of *Salmonella* infection will be discussed, including those gained from work with the mouse model with the information available in poultry where appropriate.

### **1.7.1 Attachment and colonization**

Infection with *Salmonella* is acquired by oral ingestion of contaminated food or water, via the faecal oral route. Following ingestion of contaminated material in mammals, a proportion of the bacterial inoculum survives the gastric acidic environment and then reaches the intestinal tract where infection can be established. Prior to invasion, efficient adhesion to the epithelial layer is required, which is mediated by adhesions, including fimbriae (Baumler et al., 1997, Bishop et al., 2008). Whole-genome sequencing has identified 13 fimbrial loci to be encoded by *S. Typhimurium* (McClelland et al., 2001). Fimbrial operons *fim*, *pef*, *lpf*, *agf* (*csg*), *bcf*, *stb*, *stc*, *std* and *sth* have been found to be required for virulence in mice (van der Velden et al., 1998, Weening et al., 2005). It is suggested that fimbriae might allow *Salmonella* to achieve a very close contact with the host epithelial cells and, hence, the involvement of other contributing factors that help in the recruitment of polymorph nuclear cells (PMNs) to the site of infection (Darwin and Miller, 1999).

In poultry, infecting *Salmonella* pass through the crop, proventriculus and gizzard to the intestinal tract. The main site of *Salmonella* adherence and colonization are the blind caeca, although systemic serovars, *S. Gallinarum* and *S. Pullorum*, seem not to colonize the caeca in sufficient numbers (poor

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colonizers). While 13 fimbrial loci are predicted to be encoded by the *S. Enteritidis* genome, 12 fimbrial operons are predicted for *S. Gallinarum* genome, with 8 subunits as pseudogenes (Clayton et al., 2008, Thomson et al., 2008). *S. Enteritidis* mutants lacking fimbrial operon *sefA*, *agfA* or *fimA* colonized the chicken caeca and were excreted in the faeces as the same as the wild-type strain (Thorns et al., 1996, Rajashekara et al., 2000). Studies have also shown that *S. Enteritidis* mutant lacking fimbrial operons *fimD*, *lpfC*, *pefC*, *agfA* and *sefA* colonized the caeca of orally-infected one or five-day-old chicks at the same level as the wild-type strain and was similarly invasive and adherent to chicken gut explants (Allen-Vercoe and Woodward, 1999a, Allen-Vercoe and Woodward, 1999b, Allen-Vercoe et al., 1999). With the exception of *pegA* fimbrial operon, most of the *S. Enteritidis* fimbrial loci are not essential for colonization of the avian gut (Clayton et al., 2008).

Infection of newly hatched chicks with *S. Enteritidis* may result in persistent infection with faecal shedding which may persist till the onset of lay (Van Immerseel et al., 2004b). Several genes, particularly regulatory genes (including *rfaY*, *dksA*, *clpB*, *hupA* and *sipC*) and those required for lipopolysaccharide (LPS) synthesis (including O-antigen ligase, *waaL*) have been identified and correlated with colonization of *S. Typhimurium* and *S. Enteritidis* of chicken gut (Turner et al., 1998, Carroll et al., 2004). As already mentioned, *S. Enteritidis* is also colonizing the reproductive tract of laying hens and the organism has been isolated from the oviduct epithelial cells after both *in vivo* and *in vitro* infections (Hoop and Pospischil, 1993, De Buck et al., 2004a), leading to egg contamination, which is a public health hazard (Gantois

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et al., 2008b). Studies have shown that several genes are expressed in the reproductive tract of chickens infected with *S. Enteritidis*, including genes required for metabolic pathways, motility, cell membrane and cell wall structure and integrity and stress responses, the *rfbH* gene, involved in LPS synthesis, and important for survival in the egg white and resistance to egg antimicrobial components (Gantois et al., 2008a, Gantois et al., 2009b).

### 1.7.2 *Salmonella* invasion

*Salmonellae* are invasive bacteria and harbour multiple systems for interacting with and penetrating the mucosal epithelium for systemic invasion. Indeed, a number of virulence-associated genetic regions, termed *Salmonella* pathogenicity islands (SPI), have been identified. The most two important SPI are SPI-1 and SPI-2 which encode the type three secretion systems, TTSS-1 and TTSS-2, respectively. These two SPI encode structural proteins that form needle-like complexes allowing the insertion of the bacterial proteins into the host cells that modulate the cellular functions and immune pathways (Galan, 2001). It is widely accepted that SPI-1 plays an important role in the invasion of *Salmonella* into epithelial cells (intestinal phase of infection) while SPI-2 mediates the survival and persistence in the infected host (monocytic) cells (systemic phase), including macrophages (Jones et al., 2001, Vazquez-Torres and Fang, 2001b, Chakravorty et al., 2002, Wigley et al., 2002b). To infect systemic organs, it is thought that *Salmonella* can invade the intestinal lymphoid tissues, as observed with *S. Typhimurium* infection in mice (Chen et al., 2009, Dan et al., 2007). In mammals, there is evidence that *Salmonella* can invade the specialized epithelial cells, microfold (M) cells, that are present on the epithelial lining of the gut-associated lymphoid tissues, such as the Peyer's

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patches, which sample and transport the luminal antigens into the subepithelial lymphoid tissues (Clark et al., 1994, Pascopella et al., 1995, Frost et al., 1997). Other mechanisms of *Salmonella* systemic transport may involve uptake by CD18<sup>+</sup> phagocytic cells of the epithelial lining into the subepithelial zone (Vazquez-Torres et al., 1999) and the ability of *Salmonella* to induce disruption of the intestinal tight junctions (Hapfelmeier et al., 2005). Intestinal infection with *Salmonella* induces enteritis through the activity of bacterial effector proteins encoded by SPI-1 and recognition of microbial components, such as flagella and LPS, which mediate a pro-inflammatory cytokine response and neutrophil influx (Eckmann and Kagnoff, 2001, Gewirtz et al., 2001).

In poultry, the molecular basis underlying *Salmonella* invasion and pathogenesis is not well-defined. However, it is suggested that systemic *S. Gallinarum* also displays tropism to lymphoid tissues, such as Peyer's patches and caecal tonsils, and can cross the gut during the early stages of fowl typhoid and enter systemic sites via enterocytes and the intestinal lymphoid tissues (Lowry et al., 1999, Barrow et al., 2000). Infection with *S. Typhimurium* in birds, as in mammals, induces intestinal inflammation with recruitment of heterophils (the avian equivalent of mammalian neutrophils), while *S. Pullorum* infection results in little intestinal inflammation and heterophil influx (Henderson et al., 1999). It is more likely that absence of strong heterophil influx in typhoid infection facilitates systemic spread to the internal organs (Kaiser et al., 2000).



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### 1.7.3 TTSS and *Salmonella*-induced enteritis

*Salmonella*-encoded TTSS mediate enteropathogenic responses. It has been shown that TTSS-1-secreted proteins sops (SopA, SopB and SopD) are essential for induction of inflammation, influx of PMNs, fluid secretion and enteritis following infection of bovine ligated ileal loops with *S. Dublin* (Galyov et al., 1997, Jones et al., 1998, Wood et al., 2000). It has been reported that TTSS-1-secreted proteins (SipA, SopA, SopB, SopD and SopE2) play an important role in induction of intestinal pathology and production of CXC chemokines following infection of bovine ligated loops with *S. Typhimurium* (Zhang et al., 2003). Studies have also shown that SPI-1-encoded TTSS (hilA, sipA and sipB) are important for intestinal colonization and migration of neutrophils in pigs infected with *S. Typhimurium* (Boyen et al., 2006b) and are required for invasion and early cytotoxicity of porcine pulmonary alveolar macrophages (PAM) (Boyen et al., 2006a).

### 1.7.4 Persistence of *Salmonella* infection

An overarching feature of *Salmonella* is its capability to survive and persist inside infected host cells, including macrophages (Richter-Dahlfors et al., 1997, Gorvel and Meresse, 2001, Okamura et al., 2005). It has been shown that *Salmonella* mutants that are impaired in their ability to survive within macrophages are avirulent in mice (Fields et al., 1986). In fact, the interaction of *Salmonella* with macrophages is central to the progression of systemic infection in both birds and mammals (Barrow et al., 1994). To infect systemically, it is suggested that *Salmonella* is carried within phagocytes, such as macrophages and dendritic cells (DCs), to the systemic organs, spleen and liver.

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The survival of *Salmonella* inside infected cells, including macrophages and DCs, is mediated through a number of mechanisms, and involving the contribution of SPI-2-encoded proteins, that inhibit the normal maturation of phagosome to form an inhibitory *Salmonella* containing vacuole (SCV) and, therefore, allows bacterial survival, persistence and even replication (Hensel, 2000, Galan, 2001, Salcedo et al., 2001, Cheminay et al., 2005). Recent evidence has also indicated a role for TTSS-1 effector protein sopB in intracellular survival and replication of *S. Typhimurium* (Rodriguez-Escudero et al., 2011). SopB mutants were defective in binding to Cdc42 with reduced localization to SCV. Indeed, *Salmonella* can interfere with phago-lysosomal fusion and delay vacuole acidification (Ishibashi and Arai, 1990, Buchmeier and Heffron, 1991). In the intracellular environment, SCV has the ability to divide into SCVs along with reduction in the number of intracellular acidic lysosomes, which promote the survival and replication of *Salmonella* inside infected host cells (Eswarappa et al., 2010).

Failure of *Salmonella* to survive within the macrophages, leading to full attenuation of systemic infection in chickens, has been linked to absence of a functional SPI-2 system in *S. Typhimurium*, *S. Pullorum*, and *S. Gallinarum* (Jones et al., 2001, Wigley et al., 2002b, Jones et al., 2007). These data show how important the survival within the macrophages is for the establishment of systemic infection in poultry. Studies with genetically *Salmonella* resistant and susceptible chickens have shown the involvement of macrophages in the innate resistance to salmonellosis (Wigley et al., 2002a, Wigley et al., 2006). It has

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been shown that macrophages from resistant chicken lines clear *Salmonella* more effectively than those from susceptible lines, through oxidative burst-mediated killing and production of pro-inflammatory cytokines and chemokines, including IL-18 (Wigley et al., 2006). The interaction of *S. Pullorum* with macrophages is central for persistence and development of the carrier state in chickens. *S. Pullorum* persists in splenic and hepatic macrophages for over 40 weeks following experimental infection of one-week-old laying hens (Wigley et al., 2001). After the initial immune response to infection, a few viable *Salmonella* survive within the systemic organs, liver and spleen, of birds that survive the acute infection (Wigley et al., 2001, Wigley et al., 2005). At the onset of lay (egg production), the high levels of the female sex hormone appear to inhibit the capacity to respond to infection (low immune responsiveness) and, therefore, a recrudescence of systemic infection and spread to the reproductive tract occurs, leading to the shedding of *S. Pullorum* in the laid eggs and infection of the progeny (Wigley et al., 2005). The spread of infection into the reproductive organs at the onset of lay is likely to be as a result of a generalized loss of proliferative T-cells (Wigley et al., 2005).

## **1.8 Avian immune system**

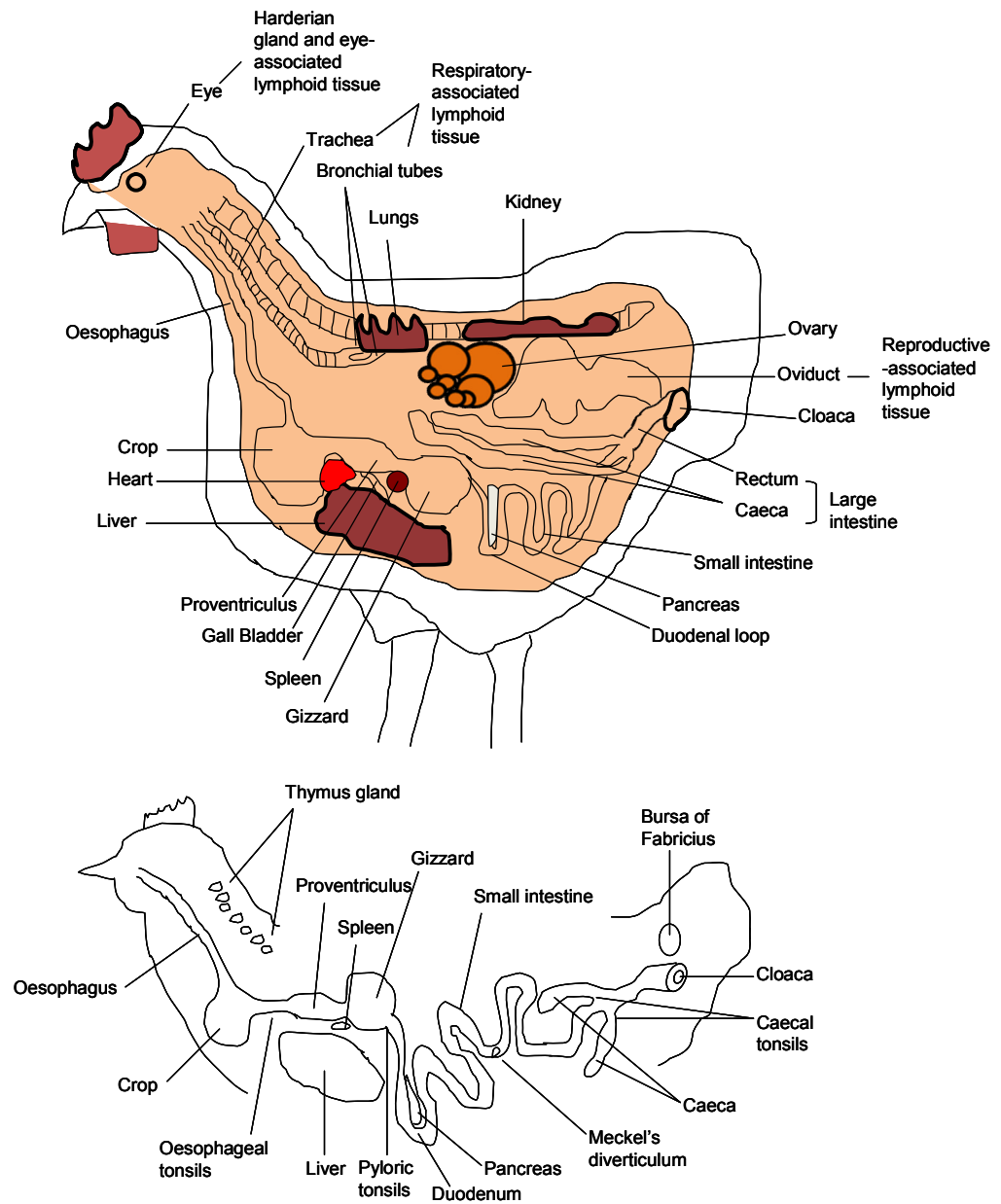
Before reviewing the immune mechanisms underlying *Salmonella* infections, it is important to briefly consider the structure of the avian immune system. From the immunological point of view, the chicken, *Gallus gallus domesticus*, is the best-studied avian species, certainly because of its economic importance and the availability of inbred lines. Birds have evolved unique lymphoid tissues and organs to interact with the various microbial pathogens. These tissues are developed from epithelial or mesenchymal embryonic origins (anlages) which

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are infiltrated by haematopoietic stem cells. The avian immune system consists primarily of the thymus, bursa of Fabricius, spleen, mucosa-associated lymphoid tissues (MALT), bone marrow and blood (reviewed by Olah and Vervelde, 2008).

The thymus and the cloacal bursa are considered the central (primary) lymphoid organs which are the sites of development and presence of immunologically competent T and B lymphocytes, respectively. From these tissues, immunologically mature cells enter the circulation and colonize the peripheral (secondary) lymphoid tissue, such as spleen and other body distributed lymphoid tissues. Figure 1.1 shows a schematic representation of the avian internal organs and the distribution of avian lymphoid organs.



**Figure 1.1:** Schematic representation of the avian internal organs and distribution of avian immune (lymphoid) tissues.

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### **1.8.1 Primary lymphoid organs**

The primary lymphoid organs comprise the thymus and the bursa of Fabricius. The thymus comprises 7-8 separate lobes located on either side of the neck. Microscopically, each thymic lobe is further divided by connective tissue septae into lobules, each lobule consisting of a cortex and medulla (Kendall, 1991). The bursa of Fabricius, a hollow oval chestnut-like sac located dorsally to the cloaca, is the site for B-cell lymphopoiesis, lymphocyte maturation and development of the antibody repertoire. The bursa contains a number of longitudinal (about 15-20) bursal folds, each fold consisting of surface epithelium, bursal follicle (formed of cortex and medulla), in addition to the blood and lymph vessels (Olah and Vervelde, 2008). The bursal follicles contain heterogenous cells populations, including lymphocytes (about 98% are B cells), macrophages, epithelial cells and secretory dendritic cells (Olah and Glick, 1978a, Olah and Glick, 1978b, Olah and Glick, 1992, Olah and Vervelde, 2008). These avian primary immune organs undergo age-related changes in the form of physiological regression (involution) after they reach their maximum size and development, around 3-6 months of age for the thymus and 20-26 weeks of age for the bursa (Ciriaco et al., 2003).

### **1.8.2 Secondary lymphoid organs**

The secondary lymphoid organs comprise the spleen and MALT, including the eye-associated lymphoid tissue (Harderian gland and conjunctival-associated lymphoid tissues), respiratory-associated lymphoid tissues, gut-associated lymphoid tissues (GALT), and genital-, skin- and pineal-associated lymphoid tissues. The chicken spleen is a round or oval organ situated adjacent to the proventriculus. Many cell types can be recognized in the spleen red pulp, such

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as T cells, plasma cells, macrophages and heterophils (Olah and Vervelde, 2008). Chickens have lymphoid aggregations scattered among the mucosal surfaces, including the intestinal and the respiratory tracts, which are the principle targets for various pathogens. The development of these lymphoid tissues occurs during the embryonic and the post-hatch periods, and independent of antigen stimulation, though further development or structural maturation of these tissues is influenced by the intestinal microflora, antigen-driven (Hegde et al., 1982).

#### ***1.8.2.1 Gut-associated lymphoid tissues***

The GALT consist primarily of oesophageal tonsils, pyloric tonsils, Peyer's patches, Meckel's diverticulum and caecal tonsils. The GALT also include the lymphocyte clusters and follicles distributed among the intra-epithelium and lamina propria of the gastrointestinal tract, such as the pharynx, the cervical and thoracic parts of the oesophagus, the proventriculus, caecum, rectum and proctodeum (Kitagawa et al., 1998, Casteleyn et al., 2010). The oesophageal and pyloric tonsils of the chicken were identified in 2003 and 2007, respectively (Olah et al., 2003, Nagy and Olah, 2007). The 6-8 oesophageal tonsils are located at the junction of the oesophagus and the proventriculus. The pyloric tonsil of the chicken is situated at the pyloric region at the beginning of the duodenum forming a complete ring of lymphoid tissues. Both types of lymphocytes (B and T cells) are recognized in the oesophageal and pyloric tonsils. Meckel's diverticulum is situated at the middle of the jejunum of young chicks in the form of a small appendage that represents the remnant of the yolk sac, and contains B, T and plasma cells (Olah et al., 1984). Peyer's patches are defined as lymphoid clusters (5-6 in number) and widely

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distributed among the intestinal tract, except the one that is regularly found anterior to the ileocaecal junction (Befus et al., 1980, Olah and Vervelde, 2008, Casteleyn et al., 2010). The GALT also include two separate lymphoid nodules, the caecal tonsils, located at the proximal end of each caecum at the ileocolonic junction (Befus et al., 1980). The caecal tonsils and only one or two Payer's patches can be identified by the naked eye at 10 days of age (Befus et al., 1980, Lillehoj and Trout, 1996). B and T cells, in addition to macrophages, can be found throughout the Peyer's patches and caecal tonsils.

The GALT in chickens, such as the caecal tonsils and the Peyer's patches, have a well-organised structure consisting of specialized lymphoepithelium (containing the microfold (M) cells), subepithelial zone, follicular structure (follicles and germinal centres) and interfollicular areas (Befus et al., 1980, Burns and Maxwell, 1986). The follicle-associated epithelium (FAE) is characterized by the presence of intraepithelial lymphocytes (IEL) and the pinocytotic M cells (Befus et al., 1980, Jeurissen et al., 1999). They are mainly consisted of T lymphocytes, and to lesser extent, a small number of other immune cells, including the B lymphocytes. CD8<sup>+</sup> T cells constitute the highest population of IEL, increase rapidly after hatch and becoming heavily distributed at 6 weeks of age (Jeurissen et al., 1999). IEL are present not only in the FAE of the GALT but also distributed along the epithelial lining of the intestinal tract. Characterization of the chicken's intestinal leukocytes has revealed about 80% lymphocytes, 10-20% mononuclear cells and less than 1% PMN and plasma cells (Befus et al., 1980, Lillehoj and Trout, 1996). Further studies to identify the intestinal IEL in chickens have shown the presence of

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polypeptides similar to mammalian CD3, CD4 and CD8 (Chan et al., 1988, Chen et al., 1988, Lillehoj et al., 1988). The lymphoid follicles are heavily infiltrated with B cells while T cells are mostly found in the interfollicular space, although CD4<sup>+</sup> T cells have been identified in the follicular medulla of the caecal tonsil (Yasuda et al., 2002).

Since the gut is the site that is frequently challenged by pathogenic micro-organisms, many antigen-presenting cells (APCs) have evolved to present the processed antigens to the effector arms of the adaptive immune system (B and T lymphocytes). These include dendritic cells, macrophages and the M cells, representing a unique morphological cell type in the caecal tonsils and Peyer's patches. Indeed, the M cells possess strong pinocytotic and phagocytotic activities against the antigens that could be present in the intestinal lumen and they are well equipped for the uptake of particular antigens from the gut and transport to the subepithelial lymphoid tissue (Bockman and Cooper, 1973). M cells in caecal tonsils of chickens was firstly described as M cell-like cells in 1992 (Kato et al., 1992). Further studies have elucidated the cellular kinetics and detailed morphology of M cells in the caecal tonsils of chickens. These studies have shown that M cells of the caecal tonsils are typified by short irregular or no microvilli on the apical surface and the presence of numerous small vacuoles in the cell cytoplasm, reflecting active pinocytosis (Takeuchi et al., 1998, Jeurissen et al., 1999, Kitagawa et al., 2000).

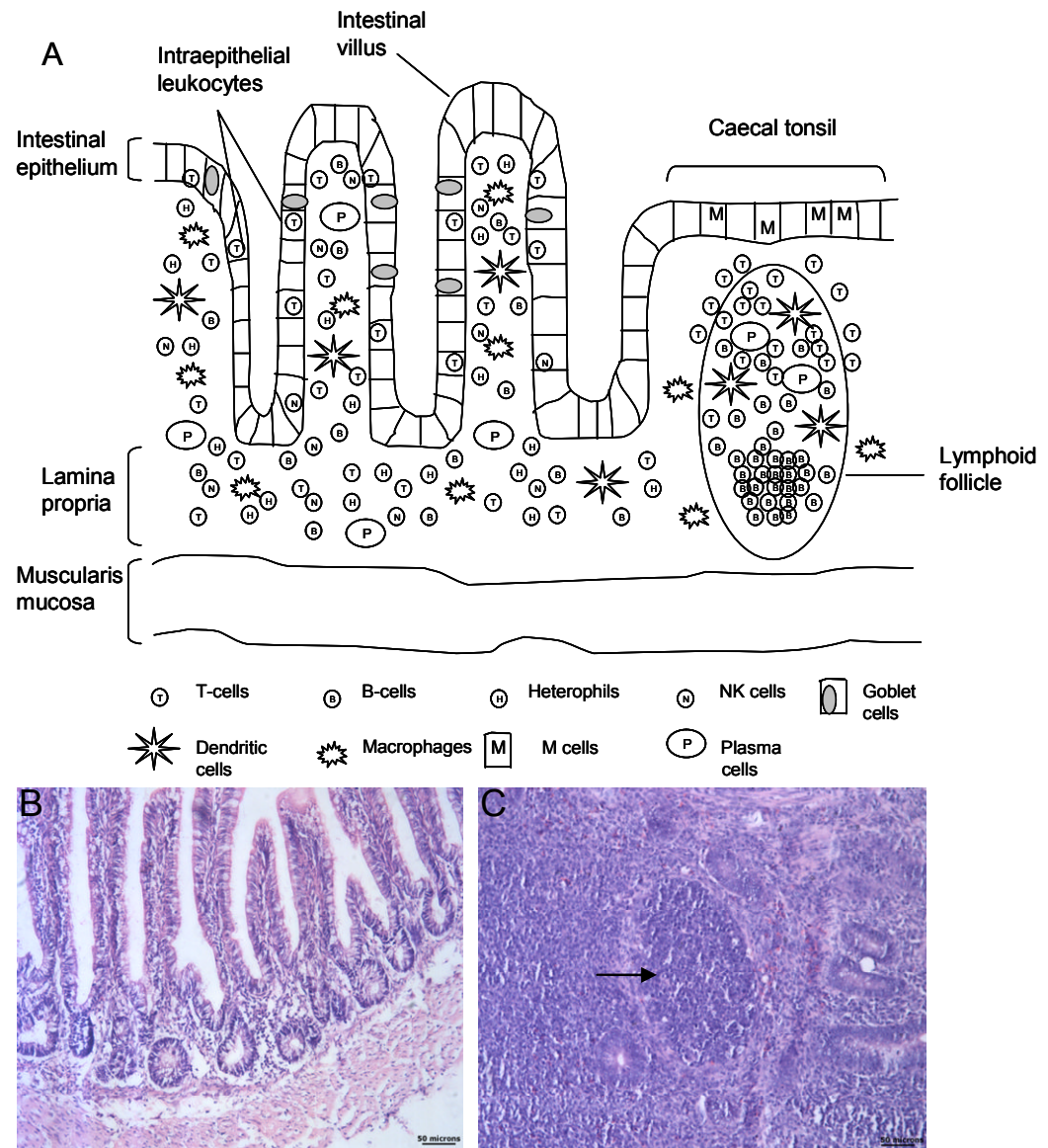
Tissues of the caecal tonsils consist mainly of B lymphocytes, with few plasma cells and CD4<sup>+</sup> and CD8<sup>+</sup> T cells. In contrast to the Peyer's patches

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which are mainly consisted of IgA<sup>+</sup> B cells, lymphocytes of the caecal tonsils consisted primarily of IgM<sup>+</sup> and IgY<sup>+</sup> B cells, with few IgA<sup>+</sup> B cells (Lillehoj and Trout, 1996). Most macrophages and T cells can be found throughout the tonsils especially in the subepithelial and interfollicular areas.

From the above mentioned, it is very clear that the immune system of birds differs from that of mammals, including the enteric immune system. Compared to mammals, although chickens lack some structural components of the immune system, they possess other well-developed immune organs and tissues that play a primary role in protecting against invading pathogens. Figure 1.2 shows a schematic representation of the organization of immune cells in the avian intestinal tract (adapted from Smith and Beal, 2008).



**Figure 1.2:** Avian enteric immune system.

(A) Schematic representation of the organization of immune cells in the intestinal tract and the GALT, such as caecal tonsils (adapted from Smith and Beal, 2008). (B), (C) Representative images of a chicken caecum showing the villus structure of the intestinal epithelium of a newly hatched chick (B) and the follicular structure (arrow) and the interfollicular space of the caecal tonsils of an older chicken (C), magnification X 20, scale bar 50  $\mu$ m.

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### 1.8.3 Chickens versus mammals

The immune system of the chicken differs from that of mammals, although some features are shared between the two systems. In contrast to mammals, chickens possess different repertoires of immune tissues, cells, and molecules. One fundamental or major difference between the immune system of chickens and mammals is the lack of highly structured lymph nodes in chickens. However, chickens have the bursa of Fabricius, a primary lymphoid organ which is the site for the maturation of B lymphocytes and the development of B-cell receptor repertoire. They also lack functional eosinophils and neutrophils, yet have a group of PMNs known as heterophils, which are the avian equivalent of mammalian neutrophils. Unlike mammals, chickens possess different repertoires of TLRs (Boyd et al., 2007), cytokines and chemokines (Kaiser et al., 2005), defensins (Lynn et al., 2007) and antibodies and other immune molecules (Kaiser, 2007, Kaiser et al., 2009). Chickens also lack IgE, but express IgA, IgM and IgY, the functional equivalent of mammalian IgG (Kaiser et al., 2009).

#### 1.8.3.1 *Toll-like receptors (TLRs)*

The innate immune system recognizes pathogenic microbes through a wide range of recognition components, which are either soluble molecules, such as LPS-binding protein, or cell-associated components, including the TLRs and scavenger receptors. These pattern recognition receptors recognize pathogen-associated conserved motifs, called pathogen-associated molecular patterns (PAMPs), which are expressed by the invading infectious microbes, including pathogen cell-surface components, such as bacterial LPS and flagellin, or pathogen nucleic acid, including single- and double-stranded RNA and CpG

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DNA (Janeway and Medzhitov, 2002). According to their location, PPRs can be broadly classified into cytoplasmic, such as the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), or membrane-bound, including the TLRs and scavenger receptors (reviewed by Min et al., 2001). Innate immune recognition, which is mediated through the interaction of PAMPs and PRRs, promote intracellular signalling pathways which result in the secretion of a wide range of antimicrobial molecules and immune mediators, including cytokines and chemokines, and activation of the adaptive immune responses. Indeed, innate immune responses involve activation of microbicidal killing mechanisms, including nitric oxide and oxygen production, secretion of cytokines and chemokines, that mediate the inflammatory process, and expression of co-stimulatory molecules, including MHC, required for antigen presentation to, and activation of, the effector cells of the adaptive immune system, B and T lymphocytes (reviewed by Koskela et al., 2004, Schneider et al., 2004, Santos et al., 2006).

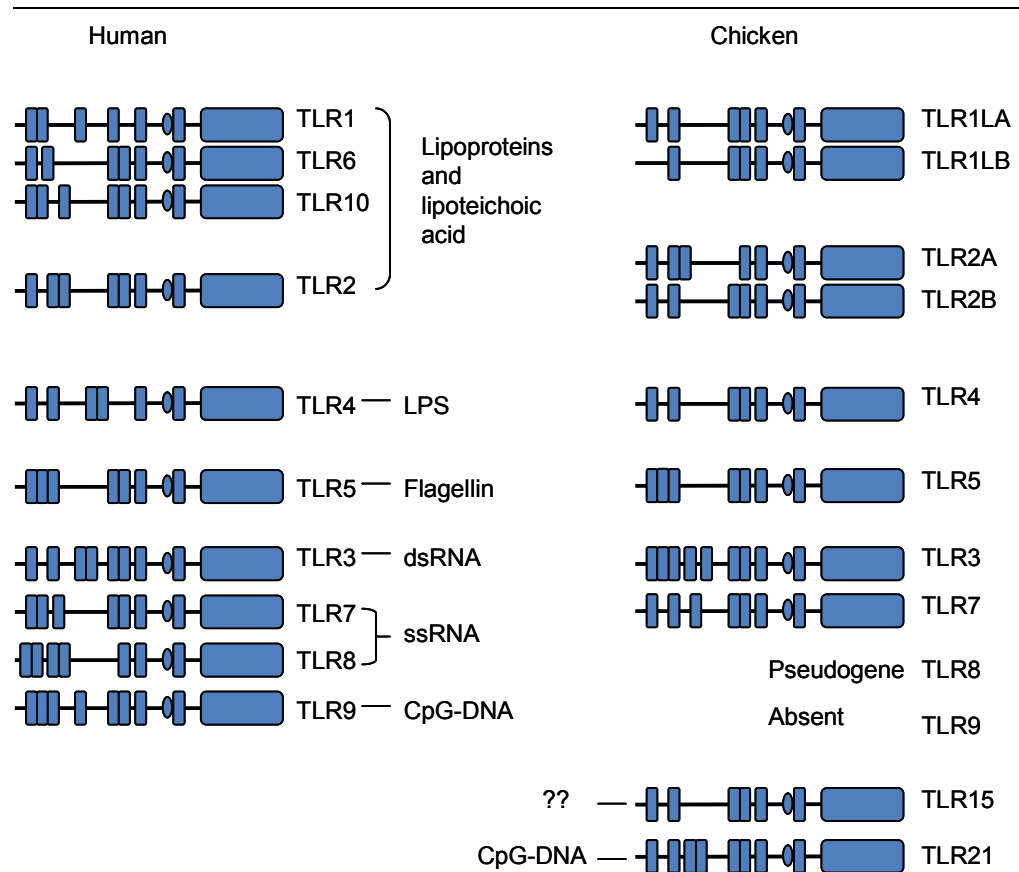
TLRs are highly conserved molecules that play a primary role in pathogen detection and initiation and regulation of host immune responses (reviewed by Aderem and Ulevitch, 2000). TLRs are the best studied pattern recognition receptors (PRRs) in both avian and mammalian hosts and, thus, their interaction with *Salmonella* has been extensively studied. TLRs and interleukin-1 receptors (IL-1Rs) have a conserved cytosolic domain known as Toll/IL-1R (TIR) domain. Generally, each TLR is formed of a TIR domain, a transmembrane domain and an ectodomain which contains leucine rich repeats (LRRs).

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To date, 11 TLRs have been described in human. These include TLR1, TLR6 and TLR10, which are encoded by the same locus (TLR1/6/10), TLR2, TLR3, TLR4, TLR5, TLR7, TLR8, TLR9 and TLR11. Of these TLRs, TLR4 and TLR5 have been extensively studied in mammalian (human or mice) models. Whilst TLR4<sup>+</sup> cells respond to LPS stimulation through production of high levels of NO and secretion of the pro-inflammatory cytokines and chemokines (Royle et al., 2003, Akira and Takeda, 2004), TLR5 recognizes bacterial flagellin, the primary structural component of bacterial flagella (Hayashi et al., 2001).

To date, ten TLRs have been identified in chickens (Temperley et al., 2008), and include TLR1LA, TLR1LB, TLR2A, TLR2B, TLR3, TLR4, TLR5, TLR7, in addition to TLR15 and TLR21, which recognizes DNA containing CpG-dinucleotides (CpG-DNA). Avian TLRs expressed a pattern of gene duplication and gene loss in comparison with mammals. The avian TLR1LA and TLR1LB are the equivalent orthologues to the mammalian TLR1/6/10 (Temperley et al., 2008). Chickens also possess a duplicated TLR2 gene, termed TLR2A and TLR2B (Temperley et al., 2008, Cormican et al., 2009) and also have equivalent orthologues of mammalian TLR3, TLR4, TLR5 and TLR7 (Leveque et al., 2003, Iqbal et al., 2005a, Philbin et al., 2005, Keesstra et al., 2008) while TLR8 is disrupted (pseudogene) and TLR9 is absent (Philbin et al., 2005, Temperley et al., 2008). Although chickens lack the equivalent orthologue of human TLR9, they respond to the mammalian TLR9-agonist, CpG DNA, by the avian-specific TLR-21 (Keesstra et al., 2010). TLR agonists (ligands) and a comparison of the human and chicken TLRs are given in Figure 1.3 (adapted from Kaiser et al., 2009).

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**Figure 1.3:** Comparison of human and chicken TLRs.

TLRs are divided into two main classes according to their interaction with various PAMPs; TLRs that recognize cell-surface components of microbes are expressed on the cell surface, while those that recognize pathogen nucleic acid are primarily expressed in endocytic vesicles. TLR15 and TLR21 are chicken-specific TLRs and while TLR21 recognizes CpG-DNA, TLR15 is thought to recognize surface PAMPs, though it is suggested that this TLR interacts with another cell-associated component away from TLR agonists (Nerren et al., 2010).

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### **1.8.3.2 *Heterophils***

Heterophils, the avian equivalent of mammalian neutrophils, are considered the first line of cellular defence against microbes. Heterophils, the primary innate effector cells, are attracted to the site of infection under the effect of chemokines produced by the other immune and non-immune cells. During the activation process aimed at eradicating the invading pathogen, heterophils possess a wide range of biological functions, including phagocytosis, oxidative burst and degranulation, the process which involves the release of protein granules, such as cathelicidin and defensins, into the phagosome. As in mammalian and fish hosts, it is suggested that avian heterophils may represent a physical barrier against infection, that block, trap and kill pathogens extracellularly via production of a net of extracellular fibres, known as heterophil extracellular traps (Chuammitri et al., 2009).

### **1.8.3.3 *Antimicrobial peptides***

Defensins (gallinacins) are small peptides that contribute to the antimicrobial properties of host cells, particularly leukocytes and epithelial cells. They play a primary role in eradicating microbial pathogens and migration of innate cells to the site of infection (Soruri et al., 2007). In contrast to mammals, that possess three families of defensins ( $\alpha$ -,  $\beta$ - and  $\theta$ -defensins), birds only have  $\beta$ -defensins (Harwig et al., 1994, Lynn et al., 2007). Avian  $\beta$ -defensins (AvBD) are produced by many organ tissues, including the gut and oviduct (Mageed et al., 2008, Ma et al., 2009), and cell types, including epithelial cells, and in response to *Salmonella* infection (Haagsman et al., 2007, Milona et al., 2007, Akbari et al., 2008, Derache et al., 2009, Ebers et al., 2009). Studies have shown that expression of AvBD in chicken gut is important in mediating

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protection against enteric pathogens during the first few days of newly hatched chicks (Bar-Shira and Friedman, 2006, Crhanova et al., 2011).

#### ***1.8.3.4 Avian cytokines***

Cytokines are important proteins secreted by cells that play a central role in the immune and inflammatory responses. They are the effector messengers of the innate and adaptive immune systems that initiate and manipulate the immune responses directed toward eradicating microbial pathogens. Chemokines are a class of cytokines that have chemoattractant activities which control the movement of immune cells (Kaiser and Staheli, 2008). The availability of avian genome sequences, and the recent cloning of avian cytokines and chemokines, has led to a major shift in the ability to understand the host-pathogen interactions in avian hosts, particularly chickens (Hillier et al., 2004, Kaiser et al., 2009). These cytokines are given in (Table 1.2) (adapted from Kaiser, 2010). Chicken equivalent orthologues of interferon (IFN)- $\gamma$ , interleukin (IL)-1 $\beta$ , IL-18, IL-10, IL-12, IL-17, Th2 cytokines (IL-4 and IL-13), IL-6, CCLi2, CXCLi1 (previously called K60) and CXCLi2 (previously called IL-8), transforming growth factors (TGF), tumour necrosis factors (TNF) and colony-stimulating factors (CSF) have been cloned, sequenced and identified (reviewed by Kaiser, 2010).

**Table 1.2:** Chicken cytokine repertoire.

Cytokine family	Chicken orthologues	Reference
<i>Interleukins</i>		
IL-1 family	IL-1 $\beta$ <sup>*</sup> , IL-18 <sup>*</sup> , IL-1RN, IL-1F5	(Weining et al., 1998, Schneider et al., 2000)
IL-10 family	IL-10 <sup>*</sup> , IL-19 <sup>*</sup> , IL-22 <sup>*</sup> , IL-26 <sup>*</sup>	(Rothwell et al., 2004)
IL-17 family	IL-17 <sup>*</sup> (A, B, C, D, F)	(Min and Lillehoj, 2002, Hong et al., 2008)
IL-12 family	IL-12 <sup>*</sup> ( $\alpha$ , $\beta$ ), IL-23	(Balu and Kaiser, 2003, Degen et al., 2004, Kaiser et al., 2005)
Th2 family	IL-4 <sup>*</sup> , IL-5 <sup>*</sup> , IL-13 <sup>*</sup>	(Avery et al., 2004)
T-cell proliferative	IL-2 <sup>*</sup> , IL-15 <sup>*</sup> , IL-21 <sup>*</sup>	(Sundick and Gill-Dixon, 1997, Lillehoj et al., 2001, Kaiser et al., 2005)
Others	IL-3, IL-6 <sup>*</sup> , IL-7 <sup>*</sup> , IL-9 <sup>*</sup> , IL-11, IL-34	(Schneider et al., 2001, Kaiser et al., 2005)
<i>Interferons</i>		
Type I	IFN- $\alpha$ <sup>*</sup> , IFN- $\beta$ <sup>*</sup> , IFN- $\kappa$ , IFN- $\omega$	(Sekellick et al., 1994, Sick et al., 1996)
Type II	IFN- $\gamma$ <sup>*</sup>	(Digby and Lowenthal, 1995)
Type III	IFN- $\lambda$ <sup>*</sup>	(Kaiser et al., 2005)
<i>Chemokines</i>		
XCL	XCL1 <sup>*</sup>	(Rossi et al., 1999)
CCL	CCL1 <sup>*</sup> , CCL2 <sup>*</sup> , CCL3 <sup>*</sup> , CCL4 <sup>*</sup> , CCL5, CCL7, CCL8, CCL11, CCL13, CCL15, CCL16, CCL17 <sup>*</sup> , CCL18, CCL19 <sup>*</sup> , CCL20 <sup>*</sup> , CCL21 <sup>*</sup> , CCL23	(Petrenko et al., 1995, Sick et al., 2000, Hughes et al., 2001, Kaiser et al., 2005, Wang et al., 2005, DeVries et al., 2006, Hughes et al., 2007)
CXCL	CXCL1 <sup>*</sup> , CXCL2 <sup>*</sup> , CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL12, CXCL13, CXCL14	(Bedard et al., 1987, Sick et al., 2000, Kaiser et al., 2005, Poh et al., 2008)
CX3CL	CX3CL1 <sup>*</sup>	(Kaiser et al., 2005)
<i>Transforming growth factors</i>	TGF- $\beta$ 2 <sup>*</sup> , TGF- $\beta$ 3 <sup>*</sup> , TGF- $\beta$ 4 <sup>*</sup>	(Jakowlew et al., 1988, Jakowlew et al., 1990, Burt and Paton, 1991, Jakowlew et al., 1997, Pan and Halper, 2003)
<i>Tumour necrosis factors</i>	TNF- $\alpha$ <sup>*</sup> , OX40L, AITRL, FAST, 4-1BBL, VEGI, CD30L <sup>*</sup> , CD40L, TRAIL <sup>*</sup> , RANKL, BAFF <sup>*</sup>	(Abdalla et al., 2004, Koskela et al., 2004, Schneider et al., 2004, Kaiser et al., 2005, Hong et al., 2006b, Guan et al., 2007, Chen et al., 2009)
<i>Colony-stimulating factors</i>	GM-CSF <sup>*</sup> , G-CSF <sup>*</sup> , M-CSF <sup>*</sup>	(Leutz et al., 1984, Leutz et al., 1989, Avery et al., 2004, Kaiser et al., 2005, Santos et al., 2006, Gibson et al., 2009)

\*Avian cytokines that have been cloned and expressed in avian species.

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## 1.9 Immune responses to *Salmonella*

Immune responses to different pathogenic microbes are complex and encompass many aspects of innate and adaptive immune mechanisms. In general terms, innate immunity involves early and rapid immune mechanisms that mediate the initial protection against invading pathogens. Later, and even more effective, immune responses that develop more slowly against infections are called the adaptive responses.

A number of studies have been carried out to study the immunology of *Salmonella*-associated infections in the mouse model. In contrast to chickens, experimental infection with *S. Typhimurium* in mice results in a systemic illness (Carter and Collins, 1974, Khan et al., 2001). The development of typhoid-like disease following experimental infection of mice with this non-host-adapted serovar makes the mouse a good model for studying the systemic form of *Salmonella* infection. Nevertheless, this form of the disease is not comparable with the rather asymptomatic gut colonization of most non-host-adapted *Salmonella* serotypes in poultry, although systemic infections may develop in the very young birds, leading to increased morbidity and mortality rates (Barrow et al., 1987a, Humphrey et al., 1989, Withanage et al., 2004, Withanage et al., 2005b). Since the use of mice as a fertile model to study the pathogenicity and immunology of *Salmonella*-associated infections, it is worth mentioning the immune responses developed in response to *Salmonella* infection in mammals followed by the information available in poultry.

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### 1.9.1 Mammalian immune responses

Several *in vivo* and *in vitro* studies have been conducted to study and characterize the cellular responses and cytokine expression that accompany *Salmonella* infection in mammals, mainly using the mouse model. Protective roles have been observed for IL-1 $\alpha$ , IL-6, TNF- $\alpha$ , IFN- $\gamma$ , IL-12, IL-15 and IL-18, but not for IL-4 (reviewed by Eckmann and Kagnoff, 2001). The interaction of bacterial components, including LPS, flagellin and other PAMPs with PRRs, including the TLRs, of the host cells, stimulates the production of a strong inflammatory response, typified by the release of pro-inflammatory and Th1 cytokines, such as IL-6, IL-1, IL-12, IL-18, TNF- $\alpha$  and IFN- $\gamma$  and iNOS (McCormick et al., 1993, Jung et al., 1995, Mastroeni et al., 1998, Mastroeni et al., 1999, Khan et al., 2001). Exposure of intestinal epithelial cells and macrophages to *Salmonella* or their products, including flagellin and LPS, has been associated with the release of a wide range of immune mediators, cytokines and chemokines, such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-12, IL-18 (IFN- $\gamma$ -inducer), GM-CSF, IL-8 and MIP (Yamamoto et al., 1996, Wyant et al., 1999, Rosenberger et al., 2000). These cytokine and chemokine responses mediate a robust inflammatory response, and also promote the attraction of immune cells to the site of inflammation, to clear *Salmonella* infection. This is in addition to the release of GM-CSF which helps the maturation of mononuclear cells into active phagocytes/macrophages to help with the killing mechanisms of *Salmonella* (Jung et al., 1995). The release of IFN- $\gamma$  has also been correlated with the ROS-mediated killing of *Salmonella* in murine macrophages (Foster et al., 2003). Up-regulation of IL-8 and MIP-1 $\alpha$  chemokines have been reported in human neutrophils exposed to heat-inactivated *S. Typhimurium* (Hachicha et

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al., 1998). Furthermore, the roles of IL-1 $\alpha$  and TNF- $\alpha$  in *Salmonella* infections have been shown *in vivo*, and since treatment with IL-1 $\alpha$  and TNF- $\alpha$  increases the survival rate following *Salmonella* infection while neutralization of TNF- $\alpha$  increases the severity of infection (Nauciel and Espinasse-Maes, 1992, Morrissey et al., 1995, Gulig et al., 1997). It has been shown that IFN- $\gamma$  plays a central role in the immune responses developed during the course of *Salmonella* infection. Evidence of this comes from the fact that IFN- $\gamma$  is rapidly up-regulated in the infected mice in response to *Salmonella* infection (Ramarathinam et al., 1991). LPS stimulation of IFN- $\gamma$ -primed murine macrophages increases the expression of NOD-2 and NO production, which is a potent antibacterial molecule (Totemeyer et al., 2006). IFN- $\gamma$  receptor knockout mice and mice with neutralizing antibodies to IFN- $\gamma$  were unable to clear the primary infection with *Salmonella* (Hess et al., 1996, Gulig et al., 1997, Bao et al., 2000). Mice with neutralizing antibodies to IL-12, which has IFN- $\gamma$ -inducing features, fail to clear *Salmonella* infection efficiently, with an increase in the hepatic and splenic colonization (Mastroeni et al., 1998) and decrease in survival rates (Kincy-Cain et al., 1996). In both cases, treatment of infected mice with IFN- $\gamma$  or IL-12 reduces the systemic bacterial colonization and increases survival rates (Matsumura et al., 1990, Kincy-Cain et al., 1996). These combined data support the notion that cell-mediated immunity, particularly Th1 immune response, plays a crucial role in immune defences against primary *Salmonella* infection, even much more than the humoral immunity. However, Ig-producing B-cells do contribute to the immune defences against secondary infection in mice (Mastroeni et al., 2000, Mittrucker et al., 2000), although their role in the gastrointestinal clearance is

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limited (Wijburg et al., 2006). In fact, both humoral and cell-mediated immune responses have been triggered in response to intraperitoneal administration of live or heat-inactivated *Salmonella* vaccines in mice (Thatte et al., 1993). Antibodies have been suggested to play a role in protection during the early phases of infection by promoting bacterial killing before reaching the intracellular environment (Collins, 1974). Hence, the development of protective immunity against *Salmonella* infection is bi-directional, linking the cellular and humoral immune responses and relies on a cross-talk between the two components of the adaptive immune system, as also suggested by (Mastroeni, 2002).

Stimulation of TLRs is a key determinant of the initial, innate immune responses to microbial pathogens (reviewed by Aderem and Ulevitch, 2000). Studies have shown that TLR4 activation is required for control of systemic *S. Typhimurium* infection in mice (O'Brien et al., 1980, Royle et al., 2003, Talbot et al., 2009). LPS-resistant mice (C3H/HeJ), which have a TLR4 mutation that impairs its function, are more susceptible to *Salmonella* infection, and showing higher systemic organ invasion and succumb to infection eight days after infection with *S. Typhimurium* (O'Brien et al., 1982, Vogel et al., 1999). *S. Typhimurium*-dependant TLR4 stimulation mediates a pro-inflammatory immune response (nitric oxide (NO) and TNF- $\alpha$ ) in murine systemic organs and macrophages and involve the activation of many signalling pathways, including MAPK, NF- $\kappa$ B, TRIF, TRAM and MyD88 pathways (Royle et al., 2003, Totemeyer et al., 2003, Totemeyer et al., 2005, Cook et al., 2007, Talbot et al., 2009). Microarray analysis of TLR4-inducible genes in *S. Typhimurium*-

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infected murine macrophages has shown that most of these genes are correlated with oxidative burst responsive genes, pointing out the role of TLR4 in regulating NADPH oxidase activity in the intracellular environment (Wright et al., 2009).

As already mentioned, flagellin is a potent stimulator of TLR5 and is a major pro-inflammatory marker of *Salmonella* (Zeng et al., 2003). In mammals, it has been shown that triggering of TLR5 by TLR5-agonist, flagellin, mediate the induction of several signalling pathways, including the NF- $\kappa$ B and MAPK pathways (Hayashi et al., 2001, Okugawa et al., 2006). The stimulation of TLR5 and activation of these signalling pathways can lead to activation of the components of the pro-inflammatory cytokine immune response, such as IL-6 (Hayashi et al., 2001). However, evidence of flagellin-mediated T-cell inhibition, through suppression of cytokine signalling, has been also shown in human T-cells (Okugawa et al., 2006). Activation of human blood mononuclear cells and THP-1 monocytes with the TLR5 agonist, flagellin, has led to activation of Th1 cytokine responses, including the production of IL-18, IL-12 and IFN- $\gamma$  (Bachmann et al., 2006). Evidence of this also comes from experimental infection of mice with non-flagellated *S. Typhimurium* and *S. Enteritidis* (Vijay-Kumar et al., 2006). In this study, infection with non-flagellate mutants of the non-host adapted *Salmonella* serovars failed to stimulate initial intestinal inflammation in infected pathogen-free mice, manifested by reduced neutrophil infiltration in the caecum, and showed an increase in epithelial cell apoptosis, both *in vivo* and *in vitro*, leading to a profound or severe systemic infection in mice (Vijay-Kumar et al., 2006). This

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indicates that stimulation of TLR5 by bacterial flagellin is required for bacterial recognition by the host cells and development of immune responses to clear the primary infection. Moreover, it has been shown that *motA* mutant of *S. Enteritidis* (flagellated and non-motile) is defective in invasion of epithelial cells compared to the parent strain at 1 h post-infection of Caco-2 cells (van Asten et al., 2004), indicating that flagellin-mediated bacterial motility promotes bacterial invasion of epithelial cells. Studies from mammalian models indicate not only the importance of innate responses to *Salmonella* infection but also suggest that the course of infection might be regulated by manipulation of these responses.

### **1.9.2 Avian immune responses**

The relevance of the above data derived from murine typhoid model, is not necessarily comparable to *Salmonella* infection in the avian host. In particular, systemic infection in chickens is usually caused by *S. Pullorum* or *S. Gallinarum*, which seem to have different pathogenesis and immune responses in chickens (Thomson et al., 2008), compared to systemic *S. Typhimurium* infection in mice, while infection with the non-host-adapted serovars, including *S. Typhimurium* and *S. Enteritidis*, often causes disease-free gut colonization, except in very young chicks. This host species-specific difference in the pathogenesis and immune responses could be influenced by multiple factors affecting the host-pathogen interaction, including differences in the interaction with immune components, including macrophages, which play a fundamental role in *Salmonella* infection. Avian immune responses to *Salmonella* infection will now be discussed in more details.



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### **1.9.2.1 Cell culture studies**

Avian *in vitro* models, including epithelial cells, macrophages and heterophils, respond to infection with the non-host-adapted serovars, particularly, *S. Enteritidis* and *S. Typhimurium*, or exposure to TLR agonists through the expression of a wide range of immune responses. The interaction of *Salmonella* with various avian cells will be discussed in more details.

#### **1.9.2.1.1 Epithelial cells**

Specialized epithelial cells represent not only a physical barrier against invading pathogens, through production of mucous and antimicrobial peptides (defensins), but also an initiator of innate immunity. As an important part of the innate immune system, interaction of enterocytes with microbial components, through a group of PRRs, including the TLRs, is crucial for the activation initial innate enteric immune pathways and stimulation of further immune responses, aimed at eliminating the invading pathogen. Pathogen-infected epithelial cells rapidly stimulate the secretion of pro-inflammatory chemokines that recruit innate immune cells, including macrophages, heterophils and dendritic cells, to the site of infection and mediate inflammatory responses (Kaiser et al., 2000, Onai et al., 2002).

The interaction of *Salmonella* with the epithelial cells is very important in the early stages of infection. However, little is known about the role of epithelial cells in cellular responses and mechanisms surrounding *Salmonella*-associated infections in poultry. Nevertheless, in a comparison study to investigate the molecular basis of *Salmonella* infections in poultry, exposure of primary chicken kidney cells (CKC) to *Salmonella* revealed variable degrees of

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invasiveness (Barrow and Lovell, 1989) and induced differential levels of cytokine immune response (Kaiser et al., 2000). In this study, *S. Typhimurium*, *S. Gallinarum*, *S. Enteritidis* and *S. Dublin* were found to be invasive to CKC, with *S. Typhimurium* being more invasive. Both *S. Typhimurium* and *S. Enteritidis*, but not *S. Gallinarum*, were found to stimulate 8 to 10-fold increase in production of the pro-inflammatory cytokine IL-6, while IL-1 $\beta$  was down-regulated in response to *S. Gallinarum* infection (Kaiser et al., 2000). Moreover, *S. Enteritidis*-infected primary chicken oviduct epithelial cells (COEC) elicited the expression of pro-inflammatory immune mediators, including CXCLi1, CXCLi2, CCL chemokines and iNOS, and IL-10 anti-inflammatory cytokine (Li et al., 2009). This study also showed that the production of CXCLi1 and CXCLi2 chemokines was *sipA* dependent.

#### ***1.9.2.1.2 Macrophages***

Macrophages are tissue monocytes which are a group of cells which possess an important role in tissue homeostasis, pathogen recognition and elimination, inflammation and immunity (reviewed by Kaspers et al., 2008). Upon stimulation, they undergo a series of biological responses, including phagocytosis, the production of antimicrobial molecules and expression of cytokine and chemokine immune response (Bliss et al., 2005). Production of NO and oxygen molecules by immunologically activated macrophages is considered as a highly potent microbicidal mechanism, which play a major role in the intracellular microbial killing, such as *Salmonella* (Foster et al., 2003, Withanage et al., 2005a). Moreover, antigen presentation by functional antigen-presenting cells (APCs), including macrophages, is the essential key step toward the activation of the adaptive immune response. Antigen is

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processed into peptides and expressed on the surface of APC bound to either MHCI or MHCII molecules. MHCI is expressed by most cell types while MHCII is expressed on the phagocytes (Kaiser and Staheli, 2008).

Engulfment of microbial pathogens, phagocytosis, is the most conserved and well-known function of macrophages. Active macrophages have been observed in the embryonic liver and spleen as well as very young chicks and turkey poults (Jeurissen and Janse, 1989, Qureshi et al., 2000), indicating that these innate immune cells are active at hatching. Phagocytosis of a number of poultry bacterial pathogens by cultured macrophages, either primary cells or continuous macrophage cell lines such as HD11 and MQ-NCSU cells, has been shown following infection with a range of bacterial pathogens, including *Salmonella* (Okamura et al., 2005, Withanage et al., 2005a, Babu et al., 2006), *Campylobacter* (Smith et al., 2005) and *E.coli* (Miller et al., 1990).

Nitric oxide is an important mediator with diverse physiological and pathological functions, including regulation of blood pressure (vasodilatation), neurotransmission and host defences to infections and tumours, through its antimicrobial and anti-tumour mediated activities (Hibbs et al., 1987, Bredt and Snyder, 1989, Furchgott and Vanhoutte, 1989, Stuehr and Nathan, 1989). Control of NO synthesis is mediated by different enzymes, known as NO synthases (NOS). NOS exist in three distinct isoforms, endothelial (eNOS), neuronal (nNOS) and inducible (iNOS) (Hiki et al., 1991). Stimulation of macrophages results in stimulation of iNOS which leads to production of high quantities of NO (Stuehr and Nathan, 1989, MacMicking et al., 1997). Studies

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have shown that TLR agonists LPS and CpG-DNA are highly potent inducers of NO from chicken macrophages (He et al., 2006). The release of reactive oxygen species (ROS), the oxidative burst, by activated macrophages is an important anti-microbial mechanism of activated macrophages. In this process, and after uptake of the microbial pathogen by the phagocyte into the phagosome, NADPH oxidase is activated, leading to production of high amounts of ROS, superoxide. The superoxide is then converted to hydrogen peroxide which is then converted to hypochlorous acid, which is suggested to have bactericidal activity.

Activated macrophages produce a series of cytokines and chemokines that regulate the innate and adaptive immune responses. Production of inflammatory cytokines IL-6 and IL-1 $\beta$  by chicken macrophages activated by different PAMPs, including LPS and CpG-DNA, has been reported (Weining et al., 1998, Kaiser et al., 2000, Schneider et al., 2001, Xie et al., 2003). *Salmonella* infection of chicken macrophages has been found to up-regulate IL-1 $\beta$ , IL-6, K60 and IL-8 (Wigley et al., 2006). Also, induction of anti-inflammatory cytokine, IL-10, which has an immuno-regulatory function, by LPS-stimulated HD11 has been observed (Rothwell et al., 2004).

The ability of *Salmonella* to survive within infected cells, including macrophages, is central to the pathogenesis and immune responses that develop during the course of infection. In response to *Salmonella* infection or stimulation with *Salmonella* PAMPs, activated macrophages produce significant levels of anti-bacterial and immune mediators, including NO, ROS

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and cytokines and chemokines. Exposure of chicken macrophages-like HD11 to *S. Typhimurium* or *S. Enteritidis* induced higher amounts of NO, and also when compared to murine macrophages (Babu et al., 2006). Exposure of chicken macrophages HD11 cells to TLR agonists, including CpG-DNA and *S. Enteritidis*-derived LPS, has also been correlated with a wide range of immune responses and mechanisms, including increases in NO production and iNOS expression, expression of IL-6 inflammatory cytokine and stimulation of TLR15 gene expression, with decrease in the intracellular survival of *S. Enteritidis* (He and Kogut, 2003, Xie et al., 2003, Ciraci and Lamont, 2011). This includes the induction of several signalling pathways, including protein kinase C, MAP kinase, NF- $\kappa$ B and MyD88 pathways (He and Kogut, 2003, Ciraci and Lamont, 2011). HD11 cells respond to *S. Typhimurium* endotoxin (LPS) through the expression of IL-1 $\beta$ , IL-6, IL-8, TLR15, CCL4 and NOD-like receptor (NLRC5) (Ciraci et al., 2010). Exposure of primary peripheral blood mononuclear cells (PBMC) to *Salmonella* (*S. Typhimurium* or *S. Enteritidis*) or TLR agonists (such as CpG-DNA or flagellin) elicited the expression of TLRs and iNOS and increased NO production (Okamura et al., 2005, He et al., 2006). Using microarray and qRT-PCR analysis, HD11 infected with *S. Enteritidis* expressed higher levels of pro-inflammatory CC and CXC chemokines (Zhang et al., 2008).

#### ***1.9.2.1.3 Heterophils***

It has been shown that heterophils play an important role in the initial immune response against *Salmonella* in poultry. In chickens, heterophils appear to accumulate in the caecal lamina propria as early as during the first 24 hrs following infection with *S. Enteritidis* (Van Immerseel et al., 2002a, Van

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Immerseel et al., 2002b). Chickens with heterophil depletion (Granulocytopenia) are also more susceptible to *S. Enteritidis* infection (Kogut et al., 1993, Kogut et al., 1994). Intraperitoneal administration of *S. Enteritidis*-immune lymphokines protected 18-week-old chickens from organ invasion by *S. Enteritidis* (Tellez et al., 1993). The contribution of heterophils in resistance to *Salmonella* infections has also been shown, since heterophils from resistant chicken lines express high levels of pro-inflammatory cytokines, including IL-6, IL-8 and IL-18, while have low expression of TGF- $\beta$ 4 (Ferro et al., 2004, Swaggerty et al., 2004, Swaggerty et al., 2005, Swaggerty et al., 2008). The role of IFN- $\gamma$  and IL-2 in *Salmonella* infection has also been shown as priming of chicken heterophils with recombinant chicken IFN- $\gamma$  (rChIFN- $\gamma$ ) or rChIL-2 stimulates the expression of a Th1 cytokine response (IL-18 and IFN- $\gamma$ ), in addition to the pro-inflammatory cytokines and chemokines IL-1 $\beta$ , IL-6 and IL-8, in response the exposure to *S. Enteritidis* (Kogut et al., 2003, Kogut et al., 2005b). Also, the expression of TLRs in chicken heterophils has been studied (Kogut et al., 2005a). In this study, TLR agonists, including flagellin and *S. Enteritidis*-LPS, stimulated oxidative burst and degranulation activities in chicken heterophils, with the increased expression of IL-1 $\beta$ , IL-6 and IL-8.

#### **1.9.2.2 Animal studies**

Immune responses to *Salmonella* infections in poultry depend on serotype- and host-specific factors, including the age of infection. Experimental infection of chickens with *S. Typhimurium* have shown that birds infected at older ages (3 or 6 weeks of age) were able to clear the secondary infection better than those infected at younger ages (1 week of age), with the contribution of both cellular and humoral responses (Beal et al., 2004b). It has been also shown that

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differences in breed genetics are associated with differences in the cytokine responses in the caecum (CCLi2, IL-12 $\alpha$ , IL-12 $\beta$  and IL-18) and spleen (CXCLi2, CCLi2, IL-12 $\alpha$  and IL-10) of young chickens in response to *S. Enteritidis* infection (Cheeseman et al., 2007). Evidence of the contribution of the breed genetics to resistance of *Salmonella* infection comes from studies using *S. Enteritidis* susceptible and resistant chicken lines. These studies have shown that resistant lines exhibit less intestinal colonization and increases in the basal intestinal levels of defensins, in the gene expression of pro-inflammatory chemokine IL-8, iNOS, TLR4 and Th1 cytokines IL-18 and IFN- $\gamma$ , compared to lower levels of IFN- $\gamma$  in susceptible birds (Sadeyen et al., 2004, Sadeyen et al., 2006). These differences in immune responses and resistance to *Salmonella* infection appear to correlate with differences in the functionality of the innate effector cells of the avian immune system, heterophils and macrophages, between *Salmonella* susceptible and resistant chicken lines. Studies have shown that macrophages from chicken lines resistant to *Salmonella* infection exhibit higher levels of pro-inflammatory cytokines, including Th1 cytokine IL-18 (Wigley et al., 2006). Indeed, genetic resistance to systemic salmonellosis in chickens is affected by several genetic loci, including major histocompatibility complex (MHC), Toll-like receptors (TLRs), Nramp-1 and a novel genetic trait *SALI* (Mariani et al., 2001, Wigley et al., 2002a, Wigley, 2004). These combined data also show the importance of cellular responses (Th1) in the protection and resistance to *Salmonella* infection, and since resistance to *S. Enteritidis* infection is associated with profound IFN- $\gamma$  cytokine response.

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*Salmonella* infection in chickens stimulates the expression of a wide range of immune responses, depending primarily on the infecting serovar. In chicken experiments, *S. Enteritidis* infection of young chickens up-regulates the production of pro-inflammatory chemokines, CXCLi1 and CXCLi2, and increases macrophage cell populations in the caeca of infected birds (Cheeseman et al., 2008). Again, the up-regulation of a Th1 cytokine response (IFN- $\gamma$  and IL-18) has been reported in the spleen of *S. Enteritidis*-infected chickens, although up-regulation of Th2 cytokine response (IL-4) was correlated to *S. Pullorum* infection (Chappell et al., 2009). In this study, CXCLi1 and CXCLi2 responses were down-regulated in the ileum of newly hatched chicks in response to *S. Pullorum* infection. Infection of newly hatched chicks with *S. Typhimurium* induces significant levels of gut responses, typified by the expression of pro-inflammatory mediators; CXCLi1, CXCLi2, MIP-1 $\beta$  and IL-1 $\beta$ , and a heterophil influx (Withanage et al., 2004). It has been also suggested that cellular, particularly Th1, responses play an important role in the immune clearance of *S. Typhimurium* infection from the intestine (Withanage et al., 2005b). (Fasina et al., 2008) have also shown that *S. Typhimurium* infection in young chicks elicited intestinal immune responses, including the up-regulation of IL-1 $\beta$ , IL-6 and IFN- $\gamma$  and down-regulation of IL-10 in the gut of infected birds. Differential expression of the gut immune responses has been also shown in response to infection with the serotypes of the broad host range (*S. Typhimurium*, *S. Enteritidis*, *S. Hadar* and *S. Infantis*) (Berndt et al., 2007), with *S. Enteritidis* being the highly invasive serotype which stimulated the higher expression of immune mediators in the chicken caeca.

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The above mentioned data may support the role of cell-mediated, particularly Th1, immune responses, including the role of IFN- $\gamma$ , in clearance of *Salmonella* infections in poultry. Studies carried out by (Beal et al., 2004a, Beal et al., 2005) have shown that both humoral and cellular immune responses are important in clearance of and resistance to *Salmonella* infection. Infection of chickens with *S. Typhimurium* elicits antigen-specific immune responses typified by the expression of IFN- $\gamma$ , IL-1 $\beta$  and TGF- $\beta$ 4 from the spleen and elevation in the serum IgA, IgM and IgY. Intraperitoneal administration of recombinant IFN- $\gamma$  has been shown to decrease organ invasion after oral infection with *S. Enteritidis* in chicks (Farnell et al., 2001). A significant role of CD8<sup>+</sup> T-cells in early responses of young chicks to *S. Typhimurium* infection has been shown (Berndt and Methner, 2001), although CD4<sup>+</sup> T-cells would seem to play an important role in response to *S. Enteritidis* infection in older chickens (Holt et al., 2010). Evidence for the importance in cellular immunity in *Salmonella* infection also comes from studies with *S. Enteritidis* or *S. Typhimurium* infection in bursectomized chickens, in which humoral immune responses (antibodies) would be affected (Desmidt et al., 1998, Beal et al., 2006). While surgical bursectomy, which leads to a specific depletion of B lymphocytes, has no effect on the clearance of *S. Typhimurium* from the intestine (Beal et al., 2006), chemical bursectomy, in which T cells also appear to be affected, decreases the ability of chickens to clear the intestinal infection, although systemic clearance was not affected, pointing out a role of cellular immune mechanisms in systemic *Salmonella* infection in chickens (Desmidt et al., 1998).

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**1.10 Reduction of the caecal carriage**

Newly hatched chicks are very susceptible to *Salmonella* infection, because of the immaturity of the gut, in terms of the resident flora and immune responses (Friedman et al., 2003). The oral administration of normal gut flora preparations to very young chicks, as early as immediately after hatching, results in protection against challenge with *Salmonella*, by so called competitive exclusion or colonization inhibition (Nurmi and Rantala, 1973). The prominent effect of competitive exclusion is to prevent pathogenic bacteria, such as *Salmonella*, from multiplying in the caeca and, therefore, they are eliminated from the gut (Impey and Mead, 1989). Orally-administered bacterial microflora also compete for the intestinal receptor sites and nutrients and colonize the gut, so they act as effective physical barrier against *Salmonella* colonization (Soerjadi et al., 1981, Soerjadi et al., 1982). Furthermore, there is evidence that competitive exclusion cultures also modify the composition of gut microflora, and enhance the production of volatile fatty acids, such as butyrates, which have inhibitory effects on *Salmonella* (Barnes et al., 1979, Nisbet et al., 1993, Corrier et al., 1995). Due to their colonization-inhibiting activity, this term has been extended to include the oral administration of live *Salmonella* organisms. It has shown that oral administration of live *S. Montevideo* to newly hatched chicks results in total protection against challenge with *S. Typhimurium* (Barrow and Tucker, 1986). Both *S. Infantis* and *S. Hadar* also express inhibiting activities to the further challenge with *Salmonella* (Berchieri and Barrow, 1990, Nogrady et al., 2003). It has been found that *S. Infantis* mediates a broad spectrum of colonization inhibition against other *Salmonellae* in young chicks (Berchieri and Barrow,

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1990). Recent evidence has shown that while oral administration of *S. Enteritidis* to newly hatched chicks mediates a strong inhibition effect against the 24h later challenge with both monologous and heterologous *Salmonella* strains, *S. Infantis* induces partial protection against the heterologous *Salmonella* (Methner et al., 2010). Studies have also shown that organic acids, such as butyrate, possess an antibacterial activity and reduce the caecal colonization of *S. Enteritidis* in chickens (Van Immerseel et al., 2004c, Van Immerseel et al., 2005a, Van Immerseel et al., 2006) and *S. Enteritidis* invasion in chicken intestinal epithelial cells (Van Immerseel et al., 2003, Van Immerseel et al., 2004a) through down-regulation of SPI-1 genes, including *invB*, *invE*, *invF*, *invI*, *invJ*, *sipA*, *sipD*, *spaP* and *hilD* (Gantois et al., 2006).

### **1.11 Vaccination**

Vaccination, including the use of live and inactivated vaccines, is the best prophylactic method and sustainable approach to control *Salmonella* infection in poultry. Live vaccines stimulate both arms of adaptive responses, cellular and humoral immune responses, and thought to have an advantage over the killed vaccines, which trigger mainly humoral (antibody) response (Collins, 1974, Barrow, 2007). Vaccination against poultry-specific *Salmonella* serotypes, such as *S. Gallinarum*, has contributed, to high extent, to the success in controlling systemic infections in poultry for many years, though evidence of the re-emergence of fowl typhoid is highly anticipated as the disease has been recorded recently in free range and backyard chickens and commercial layers (Cobb et al., 2005, Parmar and Davies, 2007). On the other hand, the efficacy of vaccination against the poultry non-specific *Salmonella* serovars, mainly *S. Typhimurium* and *S. Enteritidis*, is variable and often not

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satisfactory. This is largely as a result of the way by which these vaccines have been produced. Most of currently available vaccines have been produced not on the basis of understanding of avian immunology or host-pathogen interactions. Live *S. Enteritidis* and *S. Typhimurium* vaccines, which are available commercially for use in poultry in Europe, are either auxotrophic double-marker mutants derived through chemical mutagenesis or have been developed on the basis of metabolic drift mutations (EFSA, 2004b). Another live vaccine, which has been developed initially for use against *S. Gallinarum*, is the rough strain *S. Gallinarum* 9R, which has been found to express cross-protection to *S. Enteritidis* (Barrow et al., 1991). Many other attenuated live *Salmonella* vaccines have been developed by genetic mutations, especially for those required for metabolic functions and survival of *Salmonella* in the host tissues, including housekeeping genes [for example, (Cooper et al., 1994, Springer et al., 2000, Methner et al., 2001)]. These mutated genes include (for example) *galE* (synthesis of bacterial LPS), *ompR* (synthesis of outer membrane proteins) and *aroA* (amino acid synthesis) and other genes coded for other metabolic and virulence functions (reviewed in Zhang-Barber et al., 1999, Mastroeni et al., 2001). Nonetheless, it has been shown that some live attenuated *Salmonella* vaccines may express weak colonization inhibition to challenge with *Salmonella* when administered orally (Methner et al., 1997). This points out the need for the development of better live *Salmonella* vaccines that can induce gut immunity without affecting their intestinal colonization and, therefore, colonization inhibition activity, as also suggested by (Van Immerseel et al., 2005b, Barrow, 2007). New evidence shows that a *phoPfliC* mutant of *S. Enteritidis* demonstrates an effective colonization inhibition

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characters to the wild type *S. Enteritidis* and could be a good candidate as a live *Salmonella* vaccine for potential use in poultry which allows the differentiation between the vaccinated and infected birds, through the recognition of *fliC* deletion (Methner et al., 2011).

### **1.12 Aims and objectives of the project:**

Infected poultry flocks remain an important reservoir of non-typhoidal *Salmonellae*, which represent an important public health issue, while fowl typhoid and pullorum disease are important diseases of poultry. The intensive use of antibiotics has led to the emergence of antibiotic-resistant bacteria. Thus, recent regulation prohibiting the use of antibiotic or growth promoters necessitates the search for alternative ways of control in poultry. New legislation, welfare consideration and improvement in public awareness create continuous pressure on poultry producers, calling for the development of alternative methods to contain the problem. This includes the development of improved vaccines and breeding for disease resistance.

The development of novel immune-based control strategies against enteric pathogens requires a detailed understanding of the basic immunology of host-pathogen interactions. This should take in account the differences in the disease biology between the different serovars, including systemic and paratyphoid *Salmonella* serotypes. In this context, there is a growing thought that oral vaccine technology will require a better understanding of GALT, in terms of structure and function (Lo, 2004). Evidence has also indicated that the initial interaction of certain pathogens with the innate effector cells can modulate the further adaptive immune responses. As the blind caeca are considered the main

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site of *Salmonella* colonization, it is suggested that caecal tonsils represent the key regulator of immune responses against *Salmonella*. However, the role of the caecal tonsils during the course of *Salmonella* infection is poorly understood and the information available about the early cellular responses of chickens to *Salmonella* is scarce.

The principle aim of this project was to study and compare the immunobiology of *Salmonella* infections at the cellular or molecular level, both *in vivo* and *in vitro*, and to elucidate the type of immune response derived (either Th1 or Th2 immune mechanism) in response to *Salmonella* infection in poultry. This has been approached by carrying out infections of tissue culture models and chickens with *Salmonella* serotypes Gallinarum, Pullorum, Typhimurium, Enteritidis, Hadar and Infantis and studying the interaction of the cell cultures (epithelial cells and macrophages) and caecal tonsils with these bacteria.

The objectives of this study were to:

- 1- Study the interaction of *Salmonella* with chicken epithelial cells and macrophages and to examine the gene expression of chicken blood lymphocytes infected with typhoidal and non-typhoidal *Salmonella* serotypes (chapter 3).
- 2- Study the interaction of *Salmonella* mutants with avian cells, and to clarify the role of specific *Salmonella* virulence determinant markers in the developed immune mechanisms (chapter 4).

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- 3- Elucidate the immune responses of caecal tonsils to infection in newly hatched chicks and older chickens and to examine and record the cellular changes in the caecal tonsils in response to infection (chapter 5).

The objectives will be discussed in further detail in the beginning of each chapter in the results section.

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## 2 General Materials and Methods

### 2.1 Bacteriology

#### 2.1.1 Media

Nutrient agar (Oxoid, UK) was used for routine cultivation of *Salmonella* and determination of intracellular bacterial counts in infected tissue culture cells. Nutrient broth (Oxoid, UK) was prepared and used for growing *Salmonella* prior to experimental infection of cultured cells as well as experimental chickens. MacConkey agar (Oxoid, UK) was used as a selective medium for isolation of *Salmonella* serotypes. A highly selective medium, Brilliant Green agar (Difco, UK), was also used for selective isolation and counting of *Salmonella* in the caecal contents of infected birds. All media were prepared according to the manufacture's instructions and sterilized by autoclaving at 121°C for 15 min.

Selenite broth (Oxoid, UK) was used as an enrichment medium for isolation of *Salmonella*. The medium was prepared according the manufacture's instructions and sterilized by the use of a boiling water bath for 10 min. Soft agar was used for motility testing of *Salmonella* stains. The medium was prepared by adding 1g of nutrient agar base and 3.6g of heart infusion agar base (Oxoid, UK) to 400 ml distilled water. The medium was then mixed and sterilized by autoclaving at 121°C for 15 min.

#### 2.1.2 Antibiotics

When required appropriate antibiotics (Sigma-Aldrich, UK) were added to the bacteriological media. Antibiotics were solubilised in sterile distilled water,

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filter sterilized and then aliquoted (10 ml) and stored at -20°C. The antibiotic used and their concentrations are shown in Table 2.1.

**Table 2.1:** Antibiotics used throughout this study

The antibiotic	Stock concentration	Final concentration
Sodium nalidixate	10 mg/ml	20 µg/ml
Novobiocin	1 mg/ml	1 µg/ml
Vancomycin	100 µg/ml	100 µg/ml

### 2.1.3 Bacterial strains

The infection studies were carried out using spontaneous nalidixic acid-resistant (Nal<sup>r</sup>) as well as mutant strains of well-characterised *Salmonella* serovars. The *Salmonella* strains used in this study are shown in Table 2.2. To study the immune responses of avian cells to *Salmonella*, parent strains of *S. Typhimurium* 4/74, *S. Enteritidis* P125109, *S. Pullorum* 449/87, *S. Gallinarum* 287/91, *S. Hadar* 18 and *S. Infantis* 1326.28, in addition to *Escherichia coli* K-12 laboratory strain (Smith, 1978, Kaiser et al., 2000) were used. For *in vivo* experiments, *S. Enteritidis* P125109, *S. Infantis* 1326.28, *S. Pullorum* 449/87 and *S. Gallinarum* 287/91 were used for infection of experimental chickens. In addition to the wild type *Salmonella* serotypes, mutant strains of *S. Enteritidis* 125109, *S. Pullorum* 449/87 and *S. Gallinarum* 287/91 serotypes were used to elucidate the behaviour of avian cells in response to infection with *Salmonella* that defective in TTSS-1, TTSS-2 or flagellar machineries. Infection of chicken

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macrophages and epithelial cells was conducted using *S. Enteritidis* strains *invA*, *ssaR* and *fliJ*, *S. Pullorum* strains *sipB*, *spaS* and *ssaU* and *S. Gallinarum* Flg<sup>+</sup>.

In addition to the wild type *Salmonella* serotypes, mutant strains of *S. Enteritidis* 125109, *S. Pullorum* 449/87 and *S. Gallinarum* 287/91 serotypes were used in this experiment. Mutant strains were produced and kindly provided by Oliveira O. Neto (Paul Barrow's group, University of Nottingham). *S. Enteritidis* strains *invA* and *fliJ*, *S. Pullorum* strains *sipB*, *spaS* and *ssaU* and *S. Gallinarum* Flg<sup>+</sup> (*flgL*) were grown on nutrient agar containing 15 µg/ml chloramphenicol while *S. Enteritidis* *ssaR* strain was grown on nutrient agar containing 20 µg/ml kanamycin. *S. Gallinarum* Flg<sup>+</sup> was motile on soft nutrient agar while *S. Enteritidis* *fliJ* was non motile. The presence or absence of flagella was confirmed in Flg<sup>+</sup> and *fliJ* strains, respectively, by electron microscopy. Prior to infection, bacteria were grown to log phase in nutrient broth (Oxoid ltd, UK) at 37°C in an orbital shaking incubator at 150 rpm/min.

**Table 2.2:** Strains of *S. enterica* used in this study

<i>Salmonella</i> strain	Relevant data	Source or reference
<i>S. Typhimurium</i> 4/74	Wt, nal <sup>r</sup> , non-host-specific SL1344 strain, first isolated from cattle in the UK in 1960s Colonizing and invasive to chicken gut	(Jones et al., 1988, Chadfield et al., 2003, Foster et al., 2006)
<i>S. Enteritidis</i> P125109	Wt, nal <sup>r</sup> , non-host-specific, PT4 Isolated from a poultry-associated food-poisoning outbreak in the UK Virulent in newly hatched chicks, invasive and causing egg contamination in laying hens	<a href="http://www.sanger.ac.uk/Projects/Salmonella">www.sanger.ac.uk/Projects/Salmonella</a> ; (Barrow, 1991, Barrow and Lovell, 1991, Thomson et al., 2008)
<i>S. Hadar</i> 18	Wt, nal <sup>r</sup> , non-host-specific From Prof. B. Nagy (Veterinary Medical Institute of the Hungarian Academy of Sciences, Budapest) in the 1980s Colonizing and invasive to chicken gut	(Berndt et al., 2007)
<i>S. Infantis</i> 1326.28	Wt, nal <sup>r</sup> , non-host-specific Isolated from health broiler poultry in the UK in 1970s Colonizing but weak invasive to chicken gut	(Barrow et al., 1988, Berndt et al., 2007, Methner et al., 2010)
<i>S. Pullorum</i> 449/87	Wt, nal <sup>r</sup> , poultry-specific Isolated from free range poultry in the UK in late 1980s Pullorum disease	(Berchieri et al., 2001, Suar et al., 2006)
<i>S. Gallinarum</i> 287/91	Wt, nal <sup>r</sup> , poultry-specific Isolated by Prof. A. Berchieri (University of Sao Paulo) from diseased egg-laying hens in Brazil Fowl typhoid	<a href="http://www.sanger.ac.uk/Projects/Salmonella">www.sanger.ac.uk/Projects/Salmonella</a> ; (Jones et al., 2001, Thomson et al., 2008)
<i>S. Enteritidis invA</i>	SPI-1, cm <sup>r</sup>	Barrow, P. and Neto, O.C. (unpublished )
<i>S. Enteritidis ssaR</i>	SPI-2, kn <sup>r</sup>	Barrow, P. and Neto, O.C. (unpublished )
<i>S. Enteritidis fliJ</i>	Non-flagellated, non-motile, cm <sup>r</sup>	Barrow, P. and Neto, O.C. (unpublished )
<i>S. Pullorum sipB</i>	SPI-1, cm <sup>r</sup>	Barrow, P. and Jones, M. (unpublished)
<i>S. Pullorum spaS</i>	SPI-1, cm <sup>r</sup>	(Wigley et al., 2002b)
<i>S. Pullorum ssaU</i>	SPI-2, cm <sup>r</sup>	(Wigley et al., 2002b)
<i>S. Gallinarum</i> Flg <sup>+</sup>	Flagellated, motile, cm <sup>r</sup>	Barrow, P. and Neto, O.C. (unpublished )

Wt: wild type strain; nal<sup>r</sup> : nalidixic acid resistant; cm<sup>r</sup>: chloramphenicol resistant; kn<sup>r</sup>: Kanamycin resistant.

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#### 2.1.4 Nalidixic acid mutation

Nalidixic acid resistance (Nal<sup>r</sup>) was induced in all serotypes for use in the experimental infections both *in vitro* and *in vivo*. This was originally generated for infection of chickens (*in vivo*) to facilitate enumeration of targeted serotypes to avoid contamination from the gastrointestinal flora and commensal bacteria. Nal<sup>r</sup> *Salmonella* strains have been extensively used in experimental infection of chickens (Beal et al., 2005, Withanage et al., 2005b, Sadeyen et al., 2006). To produce there mutants, all strains were incubated overnight on nutrient agar plates containing sodium nalidixate (40µg/ml) (Sigma-Aldrich, UK) at 37°C for the growth of single colonies. 10 plates / serotype were used with different sets of bacterial culture aliquots added (0.4, 0.2 and 0.1 ml /plate). Single colonies were then streaked to another nalidixate agar plate for purification. A single colony was then picked up for plating on MacConkey agar and plain nutrient agar plate with addition of nalidixic acid disk (NA 30, Oxoid, UK). These colonies were further tested with specific antisera, acriflavine and phosphate buffered saline (PBS) to check for smoothness and then smooth colonies were further kept as glycerol stocks at -80°C.

#### 2.1.5 Growth patterns of different serotypes

To determine the growth characteristics for all serotypes and the proper doses for *in vitro* infections, growth curves for different *Salmonella* serotypes were performed using both the dilution method (viable colony count, VCC) and spectrophotometry (total bacterial count) (Miles et al., 1938). One ml of a over night nutrient broth culture was transferred into 100 ml of nutrient broth and kept in shaking incubator (150 rpm/min) at 37°C with regular monitoring

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of the growth rate at different time points, 0, 1, 2, 3, 4, 5 h and over night. Growth curves were repeated three times. To determine the bacterial growth, the optical density (OD) for each bacterial culture was determined at each time point using spectrophotometer cuvettes at 600 nm wave length. To determine the viable count, 20  $\mu$ l of bacterial culture, at each time point, was transferred to 180  $\mu$ l of sterile PBS and then mixed gently before preparing a of 10 fold serial dilution. An aliquot of 100  $\mu$ l from each dilution was then transferred to the surface of NA plate and left to dry before being incubated at 37°C o/n. Colonies were counted and the viable count was converted to the log<sub>10</sub> format and expressed in a chart form. Growth rate of Nal<sup>r</sup> strains at their exponential growth phase was also determined using more close time points, 0, 1, 1.20, 1.40, 2, 2.20, 2.40, and 3 h. A calibration graph between the OD and the log<sub>10</sub> counts was designed for each strain for the determination of infection doses which were subsequently confirmed by counting on nutrient agar plates. Growth curves for all bacterial strains used in this study are shown in the appendix section.

## **2.1.6 Confirmation of mutations by polymerase chain reaction (PCR)**

### ***2.1.6.1 Extraction of bacterial DNA***

1.5 ml of the bacterial suspension was transferred in a 1.5 ml centrifuge tube and centrifuged at 9000 rpm for 3 min. The supernatant was discarded and the cell pellet was re-suspended in 200  $\mu$ l of RNase-free water. The bacterial suspension was then heated at 100°C for 20 min followed by freezing at -20°C for about 30 min and then thawing. The bacterial suspension was then centrifuged and 1  $\mu$ l of the suspension (contains the bacterial DNA) was transferred into a PCR tube.

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**2.1.6.2 Preparation of PCR Master Mix**

Each PCR reaction (25 µl) contains 16 µl RNase free water, 2.5 µl buffer, 2 µl Magnesium chloride, 1 µl dNTP, 1 µl Forward primer, 1 µl Reverse primer, 0.5 µl Tag polymerase enzyme and 1 µl Template (DNA). PCR was conducted using the XP Thermal Cycler (Bioer Technology, China) with the following cycle profile: an initial DNA denaturation step at 94°C for 3 minutes followed by 35 cycles of 94°C for 20 seconds, 60°C for 30 seconds and 72°C for 90 seconds and then a final extension step at 72°C for 5 minutes. PCR primer sequences are presented in Table 2.3.

**Table 2.3:** Primers used for PCR experiment

Primer	Sequence
<i>invA</i> F	5-AGCCACATGAAGTGGATTCGCTG-3
<i>invA</i> R	5-GCCGCGGGCAAAATGGCATC-3
<i>ssaR</i> F	5-ACGGGGGACGTTTTGCCTGT-3
<i>ssaR</i> R	5-GCCACAGCCAATGCAATAAGCC-3
<i>fliJ</i> F	5-TTATTGTGCGTCGTCTGGC-3
<i>fliJ</i> R	5-TGGCGTTAGCGTAGACAGTAGAT-3
<i>flgL</i> F	5-AGTACGCTGGATTCACTGGG-3
<i>flgL</i> R	5-GACGATCATAATCACGCCAG-3
F, forward; R, reverse	

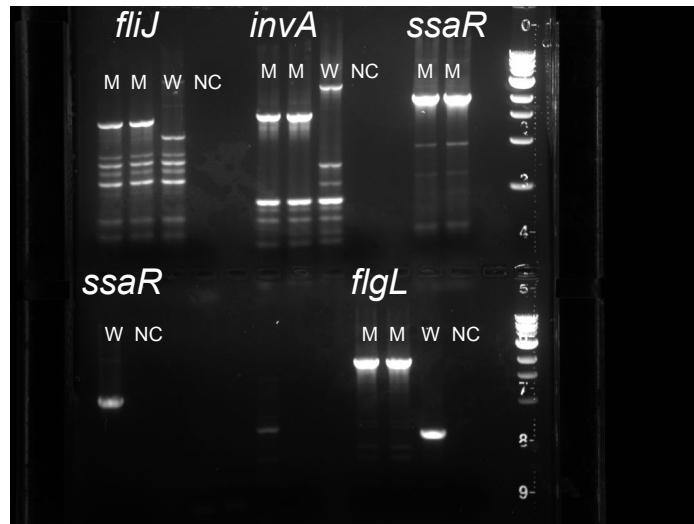
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**2.1.6.3 Agarose gel electrophoresis and detection of PCR products**

One gram of agarose was added to 80 ml of the working solution of TAE buffer followed by microwaving for 2 min with shaking. After a few minutes of leaving the preparation at room temperature, 4  $\mu$ l of ethidium bromide was added, mixed, followed by pouring into the PCR tank with a comb and allowed to set for the formation of the gel.

Five  $\mu$ l of the loading buffer was added to 25  $\mu$ l of PCR mix, mixed well and then 11  $\mu$ l was taken and loaded in the gel. 6  $\mu$ l of the marker was also loaded in the first lane followed by the run at 110 volts. No template controls were used. PCR reaction products were visualized by UV transillumination of the ethidium bromide-stained gel and shown in Figure 2.1.





**Figure 2.1:** PCR of *S. Enteritidis* and *S. Gallinarum* used in this experiment.

Ethidium bromide-stained PCR products of mutant (M) and wild (W) strains after gel electrophoresis are shown for *fliJ*, *invA* and *ssaR* (*S. Enteritidis*) and *flgL* (*S. Gallinarum*). Note the presence of specific bands (products) by the mutant strains while the wild type strains show no amplification. The image also shows a DNA ladder (marker) on the right side of the image. NC, non template control.

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## 2.2 *Salmonella* infection of avian cells (*in vitro*)

### 2.2.1 Tissue culture cell lines

#### 2.2.1.1 *Chicken macrophages (HD11)*

For culture and harvest of HD11 cells, the old medium was removed and flasks were washed out with 10ml PBS to remove any unattached cells. About 1-3ml of Trypsin-PBS (10%) was added and flasks were then incubated at 37°C up to 5 min until the cells were released from the flask. An equal volume of the growth media was added and cells were counted to determine the proper seeding rate. Cells were re-suspended in the proper volume, distributed into flasks and then incubated at 37°C.

For storage (freezing), HD11 cells were harvested from maintenance flask as mentioned above. The cell count was identified for freezing. Cells were then centrifuged at 2500 rpm for 10 min and the cell pellet was re-suspended in ice-cold cryopreservation media (90% serum with 10% dimethyl sulfoxide (Fisher Scientific, UK)). One ml containing ca 1x10<sup>6</sup> cells / ml was transferred into each cryovial and vials were labelled with an identification code/number, cell type, date and passage number and then placed in Nalgene cryopreserver (contains the necessary quantity of propanol) and then placed in -80 over night before it was transferred to liquid nitrogen. To revive cells, vials were thawed using a water bath at 37°C. The content was then transferred to a flask containing the culture media. The flask was then incubated to allow growing HD11 cells.

For invasion assay, chicken macrophage-like cells (HD11) were cultured as previously described (Kaiser et al., 2000). Briefly, cells were seeded at 3-4 x

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$10^5$  cells/ml in 24-well plates (1 ml/well) and grown at 37°C in 5% CO<sub>2</sub> for 48 hrs in RPMI 1640 medium containing 20 mM L-glutamine, 2.5% foetal bovine serum (FBS), 2.5% chicken serum, 10% tryptose phosphate broth (TPB) and 100 U / ml penicillin/streptomycin (P/S).

### ***2.2.1.2 Chicken kidney cells (CKC)***

Primary CKC were prepared from the kidneys of 1-3 week old Ross 308 broiler chicks supplied by PD Hook Hatcheries (Oxford, UK) using the protocol essentially described by (Barrow and Lovell, 1989). Cells were seeded in 24-well plates at  $1 \times 10^6$  cells / ml (1 ml/well) in complete Dulbecco's modified Eagle's medium (DMEM) provided with 12.5% FBS, 10% TPB, 25 U / ml nystatin and P/S and incubated for 72 hrs at 37 °C in 5% CO<sub>2</sub> (Figure 2.3).

The back and under the wings of the bird(s) were sprayed with IMS to dampen the feathers and to clean the bird(s). Using a sturdy pair of scissors and with taking care to avoid piercing the gut, the back section of the birds(s) was removed by cutting through the spine halfway along, and through each leg. Using clean sterile instruments, the kidneys were removed and placed into a beaker containing PBS. The kidneys were agitated hard and the PBS was discarded. This step was repeated until the PBS ran clear as this removes a lot of blood. The contents of the beaker were transferred into a Petri-dish and then, using two scalpel blades, the kidneys were shredded and chopped to remove blood clots, connective tissue and kidney core as it is important to remove as much blood as possible. The chopped tissue was then moved into a medical flat bottle and washed with PBS until the supernatant runs clear, allowing the contents to settle for 1 minute in between washes. All supernatants were

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discarded to waste. 50-80 ml of Trypsin/PBS solution was added to the tissue and agitated moderately hard for 2 minutes. Tissues were allowed to settle for 1 min. the supernatant was discarded. Another volume of Trypsin/PBS solution was added and agitated for 4 minutes before allowing the contents to settle for 1 minute. The supernatant was then placed into conical flask containing 50-100 ml of FBS. The latter step was repeated, collecting the supernatant in the same flask, until no more tissue remains. The supernatant was filtered through a funnel and metal gauze (Potter and Soar Ltd, UK) into a clean flask then decanted into 50 ml tubes and centrifuged at about 1500 rpm for approx. 10 minutes. The supernatant was discarded and the pellet cells were re-suspended in growth medium and then triturated not fewer than five times and then filtered through a 70- $\mu$ m cell strainer (Fisher Scientific, UK). The volume was measured and recorded. The cells were counted and then suspended at the appropriate seeding rate and distributed as required then incubated.

At least two hours before the invasion assays of cells, media were replaced with either RPMI 1640 (HD11) or DMEM (CKC) without antibiotics.

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### 2.2.2 Invasion assay

For *in vitro* infection experiments, bacteria were grown to their exponential growth phase by inoculating 1 ml of o/n bacterial culture into 100 ml of NB and kept in shaking incubator (150 rpm/min) at 37°C for about approx. 2 hours. Bacteria were pelleted prior to suspension in PBS according to the required challenge dose which was calculated by measuring the optical density of bacterial cultures at 600 nm and comparing the values with log counts. Infections were carried out using a multiplicity of infection (MOI) of 10 (10 bacteria to 1 cell) (Kaiser et al., 2000) and tissue culture cells were then incubated for 48 hr at 37°C. Intracellular bacterial counts and nitric oxide production were determined at different time points 2, 6, 24, and 48 hr post infection using the gentamicin protection assay and Greiss assay, respectively (see below). Intracellular counts were detected after lysing cells with 1% Triton X-100 solution (Fisher Scientific, UK). Cell supernatants were kept at -20°C for the estimation of nitrite.

The invasion and persistence of *Salmonella* in epithelial cells and macrophages was determined by using the gentamicin protection assay as previously described by (Jones et al., 2001, Smith et al., 2005). Briefly, HD11 cells or CKC grown in 24 well plates were infected with different *Salmonella* serotypes (100 µl per well). After 1 hr of incubating the bacteria with the cells, the media was changed with RPMI or DMEM containing gentamicin sulphate (100µg / ml) (Sigma-Aldrich, UK) and further incubated for another 1 hr to kill the extracellular bacteria. The cells were then washed three times with antibiotic free medium. The count of bacterial invasion and/or uptake was made by adding of 100 µl of 1% Triton X-100in PBS (for lysing cells) and then

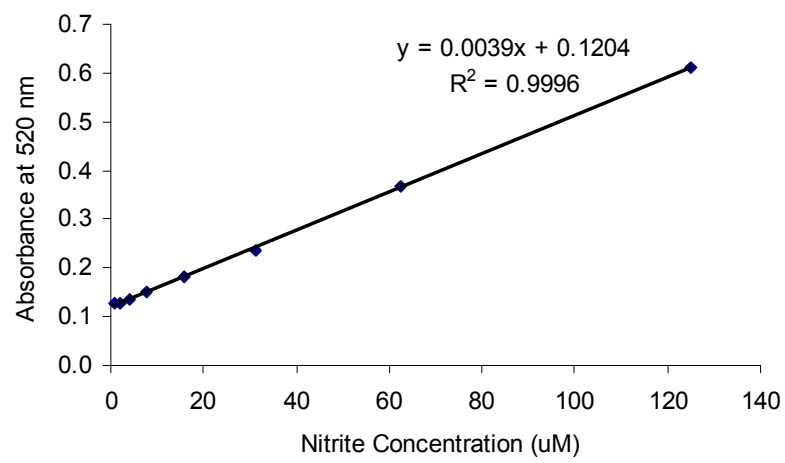
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plating on NA plates. To determine bacterial survival inside infected cells, cells were kept in media containing gentamicin sulphate (20 µg / ml) to inhibit the extracellular bacterial multiplication. At 6, 24 and 48 hr post-infection, the cells monolayer was washed three times in pre-warmed PBS before lysing cells with 100 µl of 1% Triton X-100 solution. Viable counts were determined and are shown as CFU/ml.

### **2.2.3 Griess assay**

Nitrite, a metabolite of nitric oxide (NO), produced from infected macrophages was measured by testing the tissue culture supernatants using the Griess assay (Green et al., 1982, Ding et al., 1988). Griess reagent was prepared by dissolving 1% sulphanilamide and 0.1% naphthylethylenediamine dihydrochloride separately in 2.5% phosphoric acid (Sigma-Aldrich, UK). Briefly, an aliquot of 50 µl of the culture fluid from each sample was collected and transferred to a 96- well flat-bottom microtitre plate before 50 µl of Griess reagent solution was added. After 10 min of incubation at room temperature, nitrite concentration was detected by measuring the absorbance at 520 nm using a Fluostar Optima micotitre plate reader (BMG Labtech Ltd, UK). Sodium nitrite (Sigma-Aldrich, UK) double fold serial dilutions (125 to 0.97 µM final concentrations) were used as a standard to determine NO concentrations in the cell-free medium (Figure 2.2). Three wells were used for each sample in each time point to get an accurate reading for the reaction. A LPS-stimulated cell culture was used as a positive control.



**Figure 2.2:** Sodium nitrite standard curve.

An example of sodium nitrite standard curve for determination of nitrite concentration in HD11 cells supernatants.

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#### 2.2.4 Oxidative burst assay

ROS produced from infected HD11 cells as a result of phagocytosis was measured by oxidation of 2',7' dichlorofluorescein-diacetate (DCFH-DA) (Sigma-Aldrich, UK) to fluorescent DCF as essentially described (He et al., 2009). Briefly, 1 ml of HD11 cells containing  $1 \times 10^6$  cells / ml RPMI was infected with different *Salmonella* serovars in sterile centrifuge tubes containing 10 µg/ml of DCFH-DA and incubated at 37°C in 5% CO<sub>2</sub>. After 1 h of incubation, 150 µl of cell culture aliquots were then transferred to a black 96-well plate and the relative fluorescent units (RFU) were measured at 485/520 nm using Fluostar Optima micotitre plate reader (BMG Labtech Ltd, UK). Phorbol 12-myristate 13-acetate (PMA)-stimulated cell cultures were used as positive controls and uninfected cells as negative controls.

#### 2.2.5 Formalized *Salmonella*

To study the immune dynamic response of both HD11 and CKC to killed or inactivated *Salmonella*, killed formalized bacteria were used. Serial dilutions (0.5-3%) were made to determine the proper concentration of formaldehyde (Sigma-Aldrich, UK) which is sufficient to kill 100% of bacterial cells. Infection doses were determined as described earlier and 10 µl of formalin was added to each 1 ml of bacteria suspended in PBS, mixed and kept o/n at 4°C, this gives the concentration of 1% formaldehyde. On the next day, bacterial suspensions were centrifuged at 5500 rpm for 12 min and supernatants were removed. The cell pellets were re-suspended again in warm PBS. Two inoculums (100 µl each) were taken from each strain, one for infecting the tissue culture cells and the other for plating on nutrient agar to ensure the absence of any viable bacteria. Invasion assay was carried out and further

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procedures were conducted as described above for RNA extraction, cDNA synthesis and qRT-PCR for CXCLi2 and IL-6 genes.

### **2.2.6 Isolation of blood lymphocytes**

Blood was collected from chickens at 20 and 21 days of age using sterile needles (G23). Each bird was euthanized (by cervical dislocation) and blood was collected immediately into 15 ml tubes containing 5 µl of 10 mg/ml heparin (Sigma-Aldrich, UK). Blood was diluted 1:1 using sterile PBS and then layered over histopaque-1077 (Sigma-Aldrich, UK), the proportion of histopaque : blood being 2:3. Diluted blood was poured onto the histopaque very carefully and then centrifuged at 3000 rpm for 20 min. After removing the upper layer without disturbing the lymphocyte layer, the ring was harvested using sterile Pasteur pipette, washed twice with RBCs lysis buffer and then with a sterile PBS. Cells were counted using the trypan blue exclusion and counts were recorded. Cells were then re-suspended in RPMI containing gentamicin (20 µg / ml) at the concentration of  $1 \times 10^5$  cells/ml.

### **2.2.7 Macrophage-lymphocyte co-culture and *Salmonella* infection**

HD11 was seeded at  $2 \times 10^4$  cells/ml in 24-well tissue culture plates one day prior to the collection of blood. On the day of co-culture, HD11 was infected with either *S. Enteritidis* or *S. Pullorum* at MOI of 5 (i.e.  $1 \times 10^5$  CFU/well) or stimulated with LPS, incubated for 1 h and then gentamicin assay was conducted as described in chapter 2. 2 h post-infection, media was removed and lymphocytes  $1 \times 10^5$  cells/ml in RPMI containing gentamicin (20 µg / ml) were added and incubated at 37°C for 7 days (to allow differentiation of lymphocytes). At the same time point, HD11 cells were lysed using 1% Triton

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X-100 and intracellular bacterial counts were determined as described earlier in chapter 2.

## **2.3 *Salmonella* infection of poultry**

### **2.3.1 Chickens and experimental design**

For this study, a total of seventy five one-day-old Ross 308 commercial broiler chicks were obtained from PD Hook Hatcheries (Oxfordshire, UK). The birds were divided into groups (the number varied according to the relevant experiment) and were given *ad libitum* access to antibiotic-free feed and water. In the first experiment, a total of twenty five one-day-old chicks were divided into five groups, five birds each. The first four groups were infected orally with *Salmonella* serotypes (one serotype per group). Each bird was inoculated orally with 0.1 ml of bacterial suspension containing *ca.*  $1 \times 10^8$  CFU of the corresponding *Salmonella* serovar. The control group was mock infected with 0.1 ml of sterile nutrient broth. In the second experiment, a total of fifty one-day-old chicks were divided into five groups of 10 birds each, and were given access to antibiotic-free feed and water *ad libitum*, until they reached three weeks of age. To confirm that birds were free from contaminating *Salmonella* prior to infection, random litter samples were taken from different groups and tested for the presence of *Salmonella*. Samples were incubated in Selenite broth (Sigma-Aldrich, UK) at 37°C for 24h and then plated on Brilliant Green agar (Oxoid Ltd, UK) at 37°C for 24h before testing suspect colonies for slide agglutination with *Salmonella*-specific polyclonal antibodies. The first four groups of three-week-old chickens were infected orally with *Salmonella* serotypes (one serotype per group). Each bird was inoculated orally with 0.3 ml of the bacterial culture containing *ca.*  $3 \times 10^8$  CFU of the *Salmonella* serovar.

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The control group was mock-infected with 0.3 ml of sterile nutrient broth. At four days post-infection, birds were euthanized and tissue samples were taken post-mortem for further investigations. This work was carried out under Home Office license (project license PPL 40/3048) and had local ethical approval.

### **2.3.2 Vancomycin susceptibility test**

A loopful from an overnight culture of each *Salmonella* serotype tested in this experiment was collected and streaked onto the whole surface of a nutrient agar plate. A drop of 20µl of 100 µg/ml vancomycin solution was added onto a blank paper disc which was further placed onto the lawn *Salmonella* culture. Plates were incubated overnight at 37°C. The presence of an inhibitory zone around the disc was read as a positive result for resistance. All tested *Salmonella* serotypes were observed to be resistant to vancomycin by sensitivity testing.

### **2.3.3 Vancomycin administration**

To enhance colonisation by the different serovars, especially *S. Gallinarum* and *S. Pullorum* such that differences in colonisation would not become a major factor in any quantitative immunological responses observed, the glycopeptide antibiotic vancomycin (Sigma-Aldrich, UK), related to avoparcin (Barrow, 1989), was administered one day prior to infection of three-week-old chickens. The antibiotic was added in drinking water at the concentration of 100 µg/ml and each bird was inoculated orally on one occasion with 0.5 ml of vancomycin solution 40 mg/ml.

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### 2.3.4 Sampling

At the day of sampling, birds were euthanized and caecal contents were collected in a sterile cold 15 ml centrifuge tubes before being transferred to the microbiology lab for further bacteriological examination. Tissue samples of caecal tonsils and spleens were placed directly in RNA-later (Sigma-Aldrich, UK) at 4°C for 24 hours and then stored at -80°C prior to RNA extraction. Caecal tonsils were also collected and placed in 10% NBF for histological examination or snap frozen for immunohistochemical staining procedures or transferred into a sterile 15 ml centrifuge tubes containing 5 ml of sterile PBS for flow cytometry.

### 2.3.5 Bacteriology

Previously weighed 15 ml sterile tubes were used to determine the weight (in grams) of the caecal contents from each individual bird. Caecal contents were then decimal-diluted using phosphate buffered saline (PBS) while spleen samples were homogenized using Griffiths tubes in a 1 ml sterile PBS and then plated on Brilliant Green agar containing sodium nalidixate (20 µg/ml) and novobiocin (1 µg/ml) at 37°C for 18-24h. Caecal counts of different *Salmonella* strains were easily enumerated and then expressed as Log<sub>10</sub> CFU/g.

### 2.3.6 Histopathology

Caecal tonsils were processed using a routine histological procedure. Briefly, samples were fixed in 10% neutral buffered formalin (NBF) for 24h, dehydrated and embedded in paraffin wax and then cut as 6-µm sections. Sections were stained with haematoxylin and eosin (H&E) using standard procedures and observed with the light microscope (Leica Microsystems, UK)

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using Image-Pro Plus software (Media Cybernetics, UK). This work was performed according to the following protocols:

#### ***2.3.6.1 Tissue fixation***

The tissue was removed from the bird and placed into NBF solution at room temperature. After 24 h, NBF was removed (to avoid over-fixation) and PBS rinse solution (0.5 mM phosphate, 7.5 mM sodium chloride, pH 7.4) was added. The tissue was kept in PBS rinse solution for 24 h. PBS rinse solution was then removed and the tissue was transferred into 70% ethanol until the tissue was processed/embedded.

#### ***2.3.6.2 Paraffin embedding***

Fixed tissues were placed in the embedding cassettes and then placed in the tissue processor (Leica Microsystems Ltd, UK) to be processed for paraffin embedding programme (10 hours 30 min) as follow:

1. Ethanol 70% for 1 h
2. Ethanol 80% for 1 h
3. Ethanol 95% for 1 h
4. Ethanol 100%, four changes, 30 min each
5. HistoClear, three changes, 1 h each
6. Paraffin, two changes, the first for 1 h and the second for 1.30 h.

Tissues were then embedded into paraffin blocks.

#### ***2.3.6.3 Sectioning***

Paraffin blocks were trimmed and then cut as 6- $\mu$ m sections using a microtome onto a small glass moistened with 30% ethanol. The paraffin sections were placed in a water bath at 40°C and then mounted onto polysene coated glass

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slides and were allowed to dry at 40°C for around 20 min. Sections were then placed in an oven at 52°C overnight.

#### **2.3.6.4 *H&E staining***

Sections were deparaffinised in Histoclear for two times 5 min each. Sections were then placed in 100% ethanol for 2 min followed by 95% ethanol for another 2 min and then 70% ethanol for 2 min. Slides were rinsed in running water, stained with haematoxylin for up to 3 min and then washed using tap water. Slides were dipped in 1% IMS, washed in tap water, dipped in ammoniated water and then the staining was checked under the microscope. Sections were washed with water. Sections were stained with eosin for 5 min and then washed with water until water runs clear. Slides were then placed in 70% ethanol for 2 min followed by 95% ethanol for another 2 min and then 100% ethanol for 2 min. Slides were placed in a histoclear jar and kept for 5 min and then in xylene for another 5 min. Slides were then mounted using DPX mountant (Fisher Scientific, UK) and kept overnight to dry. Slides were then examined under the microscope.

#### **2.3.7 Immunohistochemistry**

Frozen sections were prepared from caecal tonsils as previously described (Cheeseman et al., 2008) and sections were stained using the Vectastain ABC kit (Vector Laboratories Ltd, UK), following the manufacturer's instructions. Briefly, caecal tonsils were placed in OCT medium in cryomolds and snap frozen in liquid nitrogen and then kept at -80°C prior to cutting for immunohistochemical staining. Sections of 6-µm thickness were cut using cryostat, mounted on polysene coated slides and fixed with ice cold acetone.

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After incubating slides with diluted normal serum for 30 min, slides were incubated with primary mouse anti-chicken monoclonal antibodies (Cambridge Bioscience, UK) against chicken monocytes/macrophages (KUL01, 1:200) or B-lymphocytes (AV20, 1:300) for 30 min in room temperature. Slides were washed twice with PBS-Tween for 5 min and then incubated with diluted biotinylated secondary antibody solution for 30 min. slides were washed twice and then incubated with 3-4 drops of ABC-Peroxidase Solution (Vectastain ABC reagent) for 30 min. Slides were washed twice, incubated with 0.05% 3, 3' diaminobenzidine (DAB) (Sigma-Aldrich, UK) in 0.01 M PBS (pH 7.2) containing 0.015% hydrogen peroxide for 1-3 min, dehydrated and counterstained with haematoxylin. Two sections were stained per bird and two separate random images were taken per section (Leica Microsystems, UK), as previously described by (Cheeseman et al., 2008). Data were expressed as an average of four measurements in the form of a percentage of brown stained area (positive) to blue stained area (negative) using Image-Pro Plus software (Media Cybernetics, UK). For each slide, a tissue section from the caecal tonsil was used as negative control (no primary antibody was added).

### **2.3.8 Isolation of lymphocytes for flow cytometry**

Isolation of lymphocytes from the caecal tonsils was performed according to the modified protocol of (Fan et al., 2009). Briefly, caecal tonsils were placed in Petri dishes containing 5 ml of cold, Hank's balanced salt solution (HBSS). Each caecal tonsil was gently glass-ground using the Griffith's tubes, and the cell suspension was passed through a 70 µm white cell strainer into a beaker before it has been transferred to a 15-ml centrifuge tube for centrifugation at 400 g for 10 min at room temperature. The pellet was then disrupted and re-

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suspended in 8 ml of HBSS, layered over 5 ml of 67% Percoll and centrifuged at 600 g for 20 min at room temperature. The cells at the interface were harvested with a Pasteur pipette and washed twice with HBSS. Cells were then kept in 24 well plates for 2 hrs to allow macrophages to settle down and then the supernatants (containing lymphocytes) were collected and incubated with serum for 30 min to block any non specific reaction. Viability was assessed by trypan blue exclusion and the total caecal tonsil cell yield was determined with a haemocytometer. Cells were then fixed using 70% ice-cold methanol and kept in -20°C until the day of processing.

Cells counts were adjusted to  $10^5$ / ml using HBSS and 1 ml of the cell suspension was placed into 1.5 ml microcentrifuge tubes for cell staining. To detect the phenotypes of lymphocytes isolated from caecal tonsils,  $10^5$  cells were incubated with mouse anti-chicken CD3, CD4 and CD8 $\alpha$  (Cambridge Bioscience, UK) for 30 min on ice in the dark. All monoclonal antibodies were either conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or PE-Cy5. Then the incubated cells were washed twice with cold HBSS and then the antigen expression was measured by flow cytometry using BD FACSCanto II flow cytometer (BD Biosciences, UK).

## **2.4 Quantification of mRNA gene transcripts**

### **2.4.1 RNA extraction and cDNA synthesis**

To study cytokine gene expression after *Salmonella* infection, total RNA was extracted from infected cells or tissues using the Nucleospin RNA II kit (Macherey-Nagel; Fisher Scientific, UK) following the manufacturer's instructions. Caecal tonsils and spleens were homogenized in a mortar and pestle using liquid nitrogen into a powder form. Homogenized tissue samples

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or tissue culture cells were then lysed in 350 µl lysis buffer (RA1, provided by the kit) containing 10% of  $\beta$ -mercaptoethanol, snap frozen in liquid nitrogen and then moved to -80°C prior to the extraction of total RNA. For the extraction of RNA, the lysate was filtered through a filter column by centrifugation at 11.000 g for 1 min followed by the addition of 350 µl of ethanol 70% to homogenize the lysate. The latter mixture was then passed through the RNA column to bind RNA then 350 µl of membrane desalting buffer was added. DNase treatment was conducted to eliminate contaminating DNA. The RNA column was washed using RA2 and RA3 wash buffers (provided by the kit) and the purified RNA was eluted in 60 µl of RNase-free water and then tested for quality and quantity using spectral analysis by NanoDrop spectrophotometer ND-1000 (Labtech International Ltd, UK) and then kept at -80°C until use. For cDNA synthesis, RNA samples (1 µg per sample) were reverse-transcribed using the Transcriptor First Strand cDNA Synthesis kit (Roche Applied Science, UK) following the manufacturer's guidelines. Briefly, the template-primer mixture for each 20 µl PCR reaction was prepared by adding 1 µg of total RNA in a sterile, nuclease-free PCR tube followed by the addition of 4 µl of reverse transcriptase reaction buffer and 0.5 µl of protector RNase inhibitor. 2 µl of deoxynucleotide mix was added followed by 2 µl of random hexamer primer. Finally, 0.5 µl of reverse transcriptase was added and the reagents were mixed carefully. Amplification and cDNA synthesis were done using Techne TC-512 thermal cycler (Bibby Scientific Ltd, UK) with following cycle profile: 10 min at 25°C, 30 min at 55°C followed by 5 min at 85°C. The resulting cDNAs were randomly tested

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using the NanoDrop spectrophotometer ND-1000 and then stored at -20°C prior to the performance of quantitative real-time RT-PCR.

#### **2.4.2 Quantitative real-time RT-PCR (qRT-PCR).**

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) is considered a highly sensitive and reliable mean to measure and quantify the avian cytokines and chemokines, as illustrated by a relatively recently published literature (Kaiser et al., 2000, Kaiser et al., 2003, Withanage et al., 2004, Smith et al., 2005, Swaggerty et al., 2008). QRT-PCR is used to amplify and quantify a targeted DNA based on sequence-specific hybridization using a specific probe and pair of flanking primers. In the present study, gene expression of cytokines and chemokines was measured by quantitative real-time PCR using the Light Cycler 480 System (Roche Applied Science, UK). Primer and probe sequences are shown in Table 2.4. Genes were selected to test the stimulation both arms of immune responses (innate and adaptive) that could be stimulated following infection with different serotypes of *Salmonella* (Table 2.5). Gene expression of TLR4 and TLR5 was also studied *in vitro*. Primers and probes for the house keeping gene (28S), inducible nitric oxide synthase (iNOS), CXCLi1, CXCLi2, IL-6, IFN- $\gamma$ , IL-4, IL-13 and IL-10 genes have been described previously (Kaiser et al., 2000, Withanage et al., 2004, Avery et al., 2004). Primers and probes sequences for lipopolysaccharide-induced tumour necrosis alpha factor (LITAF), IL-12 $\beta$ , IL-18, MIP-1 $\beta$  (CCLi2), TLR4 and TLR5 were designed using ENSEMBL database (<http://www.ensembl.org>) and Roche probe design centre (<https://www.roche-applied-science.com>). Probes were labelled with the fluorescent reporter dye 5-carboxyfluorescein (FAM) at the 5' end and the

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quencher N,N,N,N'-tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. QRT-PCR was performed using the Light Cyclers 480 Probes Master kit (Roche Applied Science, UK) with the following cycle profile: one cycle at 95°C for 10 min, 45 cycles at 95°C for 10 s, 60°C for 30 s and 72°C for 1 s and one cycle at 40°C for 30 s. For qRT-PCR, 96-well PCR plates were used with 20 µl added per reaction (well). For the test samples, each RT-PCR mixture consisted of 1x light cycler probe master, 300 nM forward primer, 300 nM reverse primer, 100 nM probe, 2 µl (50 ng) template (DNA), made up to 20 µl with RNase free water. Each qRT-PCR experiment contained three no-template controls, test samples, a calibrator from uninfected cells and a standard log<sub>10</sub> dilution series. Each PCR reaction was performed in triplicate. In this study, RNA from lipopolysaccharide-stimulated HD11 cells or COS-7 cells was used as standard control for generation of standard curves (Figure 2.3). Log<sub>10</sub> serial dilutions were made and PCR efficiencies were calculated using the LC480 software (Table 2.6). Ideally the PCR efficiency should be 100%, which means that the amount of the product doubles each cycle ( $E=2$ ). Normalized values were determined using the advanced relative quantification method (Pfaffl et al., 2002) using LC480 analysis software.

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**Table 2.4:** Primer and probe sequences for qRT-PCR

Target RNA	Probe / primer*	Sequence	Accession number
28S	P	5'-(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)-3'	X59733
	F	5'-GGCGAAGCCAGAGGAAACT-3'	
	R	5'-GACGACCGATTGTCACGTC-3'	
IL-6	P	5'-(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)-3'	AJ250838
	F	5'-GCTCGCCGGCTTCGA-3'	
	R	5'-GGTAGGTCTGAAAGGCGAACAG-3'	
CXCLi1	P	5'-(FAM)-CCACATTCTTGAGTGAGGTCCGCT-(TAMRA)-3'	AF277660
	F	5'-CCAGTGCATAGAGACTCATTCCAAA-3'	
	R	5'-TGCCATCTTTCAGAGTAGCTATGACT-3'	
CXCLi2	P	5'-(FAM)-TCTTTACCAGCGTCTACCTTGCGACA-(TAMRA)-3'	AJ009800
	F	5'-GCCCTCCTCCTGGTTTCAG-3'	
	R	5'-TGGCACCGCAGCTCATT-3'	
iNOS	P	5'-(FAM)-TCCACAGACATACAGATGCCCTTCCTCTTT-(TAMRA)-3'	U46504
	F	5'-TTGGAACCAAAGTGTGTAATATCTTG-3'	
	R	5'-CCCTGGCCATGCGTACAT-3'	
IFN- $\gamma$	P	5'-(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)-3'	Y07922
	F	5'-GTGAAGAAGGTGAAAGATATCATGGA-3'	
	R	5'-GCTTTGCGCTGGATTCTCA-3'	
IL-4	P	5'-(FAM)-AGCAGCACCTCCCTCAAGGCACC-(TAMRA)-3'	AJ621735
	F	5'-AACATGCGTCAGCTCCTGAAT-3'	
	R	5'-TCTGCTAGGAACCTCTCCATTGAA-3'	
IL-13	P	5'-(FAM)-CATTGCAAGGGACCTGCACTCCTCTG-(TAMRA)-3'	AJ621735
	F	5'-CACCCAGGGCATCCAGAA-3'	
	R	5'-TCCGATCCTTGAAAGCCACTT-3'	
IL-10	P	5'-(FAM)-CGACGATGCGGCGCTGTCA-(TAMRA)-3'	AJ621614
	F	5'-CATGCTGCTGGGCCTGAA-3'	
	R	5'-CGTCTCCTTGATCTGCTTGATG-3'	
LITAF	P	5'-(FAM)-TGGTGGCC-(TAMRA)-3'	NM_204267
	F	5'-GAGCGTTGACTTGGCTGTC-3'	
	R	5'-AAGCAACAACCAGCTATGCAC-3'	
IL-18	P	5'-(FAM)-GGAAGGAG-(TAMRA)-3'	AJ276026
	F	5'-AGAGCATGGGAAAATGGTTG-3'	
	R	5'-CCAGGAATGTCTTTGGGAAC-3'	
MIP-1 $\beta$ (CCLi2)	P	5'-(FAM)-TCCTGCTG-(TAMRA)-3'	AJ243034
	F	5'-TGCCATCTGCTACCAGACCT-3'	
	R	5'-GCCGGGAGATGTAGGTGA-3'	
IL-12 $\beta$	P	5'-(FAM)-TGATGAGC-(TAMRA)-3'	AJ564201
	F	5'-AGCACAAAGAAATACAAAAGCAAG-3'	
	R	5'-GTCTTTTGGCCCAGTCTTTG-3'	
TLR4	P	5'-(FAM)-CCTGGAGG-(TAMRA)-3'	NM_001030693
	F	5'-ACTCTTGGGGTGCTGCTG-3'	
	R	5'-GTGCATCTGAAAGCTGTGCT-3'	
TLR5	P	5'-(FAM)-CATCACCA-(TAMRA)-3'	NM_001024586
	F	5'-GGGCATTGTTTTGTCTGGT-3'	
	R	5'-GGTGGATGGCTTCCTATCAA-3'	

\*P, probe; F, forward primer; R, reverse primer

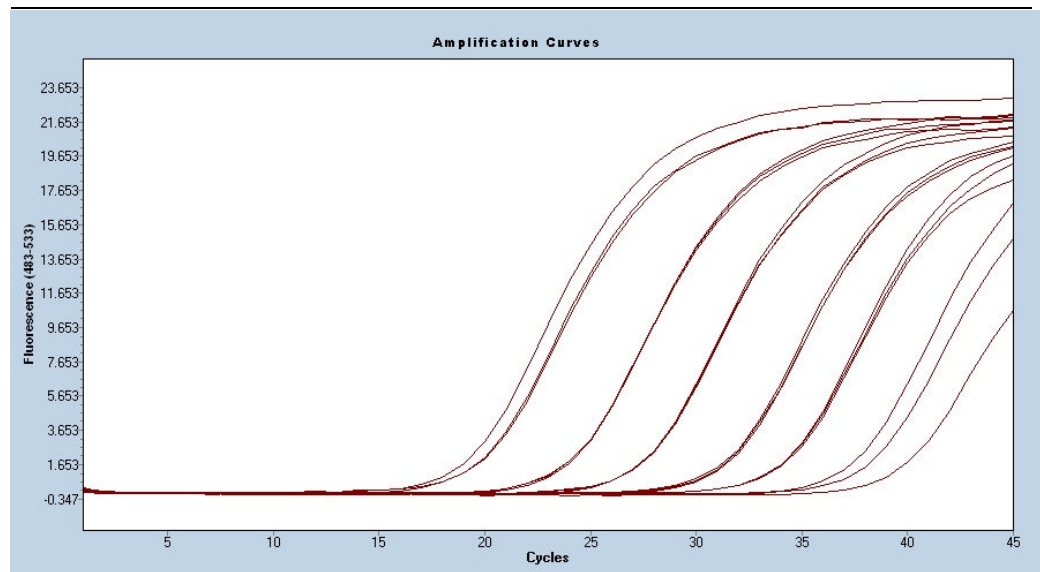
**Table 2.5:** A list of the avian immune mediators tested and their function.

Immune mediator	Function	Reference
CXCLi1	Chemoattraction of cells, particularly heterophils Inflammation	(Withanage et al., 2004, Kaiser and Staheli, 2008)
CXCLi2	Chemoattraction of cells, particularly monocytes and heterophils Inflammation	(Withanage et al., 2004, Kaiser and Staheli, 2008)
CCLi2	Chemoattractant for splenic B cells	(Rossi et al., 1999, Withanage et al., 2005b)
IL-6	Pro-inflammatory and acute phase responses Immune regulation and haematopoiesis	(Kaiser et al., 2000, Schneider et al., 2001)
iNOS	NO production and Inflammation Bacterial clearance	(He and Kogut, 2003, Berndt et al., 2007)
IFN- $\gamma$	Potent macrophage activating factor Increase the expression of MHC class II Class switching of immunoglobulins Th1 (cellular) responses	(Wigley and Kaiser, 2003, Kaiser and Staheli, 2008)
IL-4	Stimulate antibody production Th2 (humoral) responses	(Degen et al., 2005, Kaiser and Staheli, 2008, Chappell et al., 2009)
IL-13	Stimulate antibody production Th2 (humoral) responses	(Degen et al., 2005, Kaiser and Staheli, 2008)
IL-10	Immune regulation Anti-inflammatory	(Rothwell et al., 2004, Couper et al., 2008)
LITAF	TNF- $\alpha$ expression Bacterial clearance and inflammation	(Myokai et al., 1999, Hong et al., 2006b)
IL-12 $\beta$	Macrophage activation, T-cell growth Th1 responses	(Wigley and Kaiser, 2003)
IL-18	Stimulate production of IFN- $\gamma$ Th1 responses	(Wigley and Kaiser, 2003)

**Table 2.6:** Data from qRT-PCR standard curves

Target gene	Log dilutions	PCR efficiencies (E)*						
28S	10 <sup>-1</sup> -10 <sup>-6</sup>	2.17 <sup>a</sup>	1.99 <sup>b</sup>	2.02 <sup>c</sup>	2.00 <sup>d</sup>	1.97 <sup>e</sup>	1.75 <sup>f</sup>	1.79 <sup>g</sup>
IL-6	10 <sup>-1</sup> -10 <sup>-5</sup>	2.18	2.10	2.21	2.21	1.96	1.92	2.02
CXCLi1	10 <sup>-1</sup> -10 <sup>-5</sup>	1.96	2.02	2.33	1.89	2.10	1.90	2.00
CXCLi2	10 <sup>-1</sup> -10 <sup>-5</sup>	2.17	2.10	2.18	2.19	2.09	1.82	1.92
iNOS	10 <sup>-1</sup> -10 <sup>-5</sup>	1.96	2.00	2.13	1.84	1.97	1.74	1.76
IFN- $\gamma$	10 <sup>-1</sup> -10 <sup>-6</sup>	1.70	2.15			1.94	1.88	1.85
IL-4	10 <sup>-1</sup> -10 <sup>-6</sup>	1.77	2.12			1.92	1.83	1.75
IL-13	10 <sup>-1</sup> -10 <sup>-6</sup>	1.94	2.13			2.08	2.05	1.86
IL-10	10 <sup>-1</sup> -10 <sup>-5</sup>	2.18	1.96			2.50	2.10	2.06
LITAF	10 <sup>-1</sup> -10 <sup>-5</sup>	2.11	2.01			1.91	1.95	2.01
IL-18	10 <sup>-1</sup> -10 <sup>-5</sup>	1.93						
CCLi2	10 <sup>-1</sup> -10 <sup>-5</sup>	2.04						
IL-12 $\beta$	10 <sup>-1</sup> -10 <sup>-3</sup>	2.00						
TLR4	10 <sup>-1</sup> -10 <sup>-4</sup>	1.90						
TLR5	10 <sup>-1</sup> -10 <sup>-4</sup>	2.35		2.32				

\*QRT-PCR experiments of (a,b) HD11 cells and (c,d) CKC infected with *S. enterica*, (e) caecal tonsils of *Salmonella*-infected one-day-old chicks, (f) caecal tonsils and (g) spleens of *Salmonella*-infected three-week-old chickens.



**Figure 2.3:** Snapshot of qRT-PCR standard curve.

In this case, Th2 cytokine, IL-4, standard curve was generated using LC480 for determination of mRNA gene expression in caecal tonsils of three-week-old chickens infected with *Salmonella* serotypes. Ten-fold serial dilutions  $10^{-1}$  to  $10^{-6}$ , PCR efficiency of 1.83.

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## 2.5 Statistical analysis

For qRT-PCR experiments, data were normalized using an advanced relative quantification method using LC480 analysis software. The ratio of expression of each target gene was based on its relative expression against the level of expression of 28S reference gene (Pfaffl et al., 2002, Haghighi et al., 2008). Normalized values were expressed according to the following formula:

$$\text{Normalized ratio} = E_T^{\text{CpT(C)} - \text{CpT(S)}} \times E_R^{\text{CpR(S)} - \text{CpR(C)}} \quad \text{where:}$$

CpT/CpR : Cycle number at target/reference detection threshold (crossing point)

$E_T/E_R$  : Efficiency of target/reference amplification

T : Target

R : Reference

S : Sample

C : Calibrator

Statistical analysis was carried out using GraphPad Prism software (using the analysis of variance (ANOVA)) and Microsoft Excel (using Student's *t* test). Data were analysed using either two-way ANOVA followed by Bonferroni post-test or one-way ANOVA followed by Tukey's multiple comparison test to detect differences between the treated groups. In all cases, differences were considered significant if  $P < 0.05$ . Specific statistical analyses are described in more details in the experimental chapters where appropriate.



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### **3 Immune dynamics following *Salmonella* infection of cultured avian cells; invasion and persistence, nitric oxide and oxygen production and differential host gene expression**

#### **3.1 Introduction**

HD11 cells, a transformed line of chick macrophage-like cells, together with chicken kidney cells (CKC) models (Kaiser et al., 2000) were chosen to study their response to *Salmonella* infection. These cells are standard models for *in vitro* interactions of this sort. Invasion of CKC by *S. Typhimurium* and *S. Enteritidis* induces a strong pro-inflammatory response indicated by high levels of IL-6 cytokine production (Kaiser et al., 2000). The production of pro-inflammatory cytokines has been associated with infection of HD11 with many bacterial pathogens including *Salmonella* (Zhang et al., 2008), *Campylobacter* (Smith et al., 2005) and *Chlamydomphila* (Beeckman et al., 2010). Also, up-regulation of interferon- $\gamma$  (IFN- $\gamma$ ) has been correlated with baculovirus infection of HD11 (Han et al., 2009).

Studies have shown that *Salmonella* infections induce cellular changes in the tissues of infected birds, including the caeca (Van Immerseel et al., 2002a, Berndt et al., 2007), spleen (Sasai et al., 1997), thymus (Sasai et al., 1997), bursa of Fabricius (Berndt and Methner, 2004) and reproductive organs (Withanage et al., 1998, Withanage et al., 2003). However, the gene expression of chicken lymphocytes in response to *Salmonella* has not been described and it is unclear which mechanism (Th1 or Th2) is developed following infection with *Salmonella*. Therefore, the aim of this experiment was to elucidate and compare the immune responses of avian cells infected with different serovars

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of *Salmonella*. Given that the biology of the various pathotypes of *S. enterica* are so different and include (i) typhoid infection (*S. Gallinarum*), (ii) pullorum disease with persistent carrier infection (*S. Pullorum*), (iii) highly invasive and colonising (*S. Enteritidis*, *S. Typhimurium*) and (iv) poorly invasive and colonising (*S. Infantis*, *S. Hadar*), this experiment was conducted to investigate the host response to individual representative strains from these four pathotypes. The nature of the adaptive immune responses developed in response to infection with a paratyphoid serotype (*S. Enteritidis*) or a systemic serotype (*S. Pullorum*) in primary blood lymphocytes was also examined.

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## 3.2 Results

### 3.2.1 Invasion and intracellular survival of *Salmonella* in avian cells

The intracellular bacterial survival in HD11 cells and CKC was determined using a gentamicin protection assay. The results of invasion and survival of *Salmonella* and *E. coli* K-12 in both cells lines are given in Tables 3.2 and 3.3. Generally *S. Typhimurium*, *S. Enteritidis*, *S. Hadar* and *S. Infantis* invaded and/or were taken up by both types of cells in greater numbers than *S. Pullorum*, *S. Gallinarum* and *E. coli*-K12 ( $P < 0.05$ ). Bacterial counts in CKC remained stable during the 48h period. In contrast, bacterial counts in HD11 began to decline at 6h post-infection with the 48h being significantly lower than 2h ( $P = 0.0009$ ), 6h ( $P = 0.001$ ) or 24h ( $P = 0.02$ ). The number of *S. Typhimurium* recovered from CKC was higher than that of the other serotypes at 2 h post-infection. Compared to CKC, the number of intracellular bacteria detected from HD11 cells was higher during the 48 h infection period. *E. coli* K-12 could not be detected from infected CKC at 24 or 48 h post-infection.

For macrophage-lymphocyte co-culture experiment, the intracellular bacterial were determined in HD11 cells at 2 h post-infection. The intracellular counts of *S. Enteritidis* and *S. Pullorum* were  $\text{Log}_{10} 3.34 \pm 0.04$  and  $\text{Log}_{10} 2.77 \pm 0.06$ , respectively. Consistent with the earlier findings, the number of *S. Enteritidis* internalized by HD11 cells was statistically significantly higher than that of *S. Pullorum* ( $P < 0.05$ ).

**Table 3.1:** Invasion of HD11 with *Salmonella* serotypes and *E. coli*

Serotype	Log <sub>10</sub> inoculum counts	Intracellular bacterial counts (log <sub>10</sub> CFU/ml)*			
		2 h	6 h	24 h	48 h
<i>S. Typhimurium</i>	6.955±0.11	6.390±0.03	6.321±0.07	5.358±0.07	4.305±0.03
<i>S. Enteritidis</i>	6.867±0.08	6.389±0.05	6.176±0.08	5.204±0.18	3.963±0.39
<i>S. Hadar</i>	6.938±0.12	6.366±0.01	5.958±0.04	5.477±0.13	4.558±0.24
<i>S. Infantis</i>	7.031±0.12	6.494±0.05	6.067±0.09	5.323±0.02	4.264±0.10
<i>S. Pullorum</i>	6.856±0.11	4.811±0.34	4.577±0.29	4.039±0.27	3.004±0.21
<i>S. Gallinarum</i>	6.907±0.03	4.922±0.34	4.889±0.32	4.558±0.32	2.978±0.51
<i>E. coli</i> K-12	6.945±0.12	4.363±0.13	4.169±0.16	3.332±0.14	2.626±0.28

\*Values are expressed as means ± standard errors of three independent experiments.

At all time points, *S. Typhimurium*, *S. Enteritidis*, *S. Hadar*, and *S. Infantis* were more internalized by HD11 cells than *S. Pullorum*, *S. Gallinarum* or *E. coli*-K12 (P<0.05).

**Table 3.2:** Invasion of CKC with *Salmonella* serotypes and *E. coli*

Serotype	Log <sub>10</sub> inoculum counts	Intracellular bacterial counts (log <sub>10</sub> CFU/ml)*			
		2 h	6 h	24 h	48 h
<i>S. Typhimurium</i>	7.090±0.06	5.517±0.45	5.938±0.13	6.756±0.32	5.368±0.13
<i>S. Enteritidis</i>	7.188±0.03	4.898±0.04	5.299±0.11	6.158±0.09	5.292±0.07
<i>S. Hadar</i>	7.221±0.11	4.618±0.18	5.675±0.09	6.932±0.09	5.754±0.32
<i>S. Infantis</i>	7.211±0.01	4.472±0.16	5.973±0.06	6.574±0.47	5.490±0.29
<i>S. Pullorum</i>	6.956±0.22	3.329±0.26	3.509±0.31	3.777±0.39	2.534±0.27
<i>S. Gallinarum</i>	6.978±0.21	3.122±0.12	4.312±0.35	4.686±0.15	2.859±0.28
<i>E. coli</i> K-12	7.206±0.06	2.201±0.20	2.00±0.00	-	-

\*Values are expressed as means ± standard errors of three independent experiments.

At all time points, *S. Typhimurium*, *S. Enteritidis*, *S. Hadar*, and *S. Infantis* were more internalized by CKC than *S. Pullorum*, *S. Gallinarum* or *E. coli*-K12 (P<0.05).

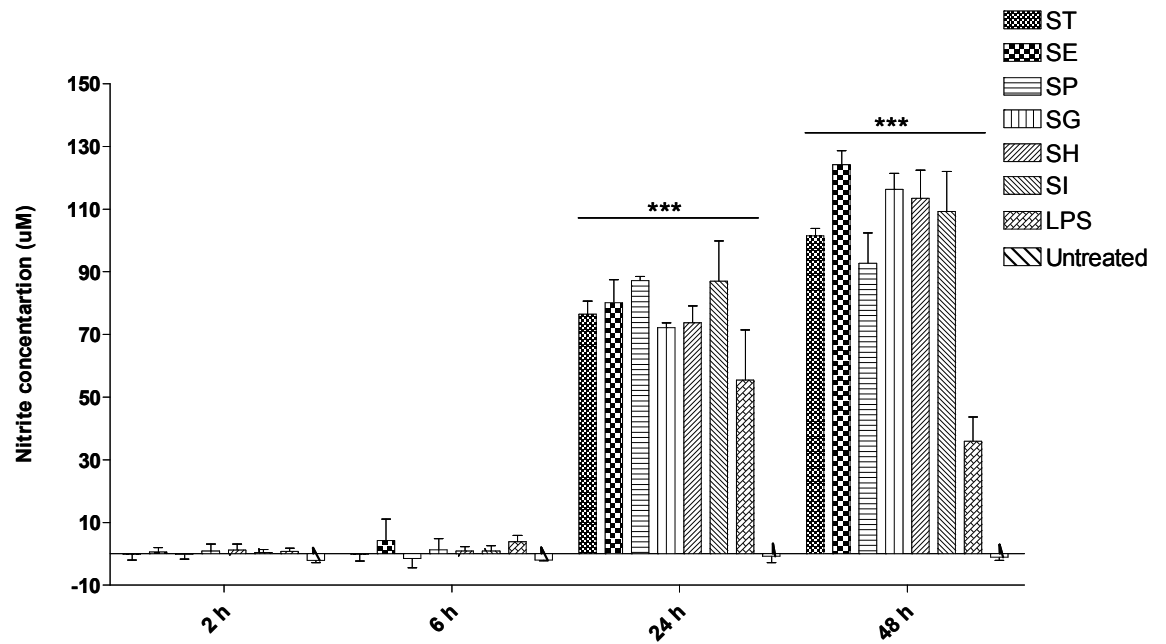
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### **3.2.2 Nitric oxide production by HD11 cells in response to *Salmonella* infection**

Experimental infection of HD11 with different serotypes of *Salmonella* produced significantly higher NO concentrations at 24 and 48 h post-infection. The maximal production was observed at 48 h post-infection ( $P < 0.001$ ) (Figure 3.1). At 48 h post-infection, NO produced from *S. Pullorum*-infected cells were significantly lower than that produced from *S. Enteritidis*, *S. Hadar* and *S. Gallinarum*-infected HD11 cells ( $P < 0.05$ ).

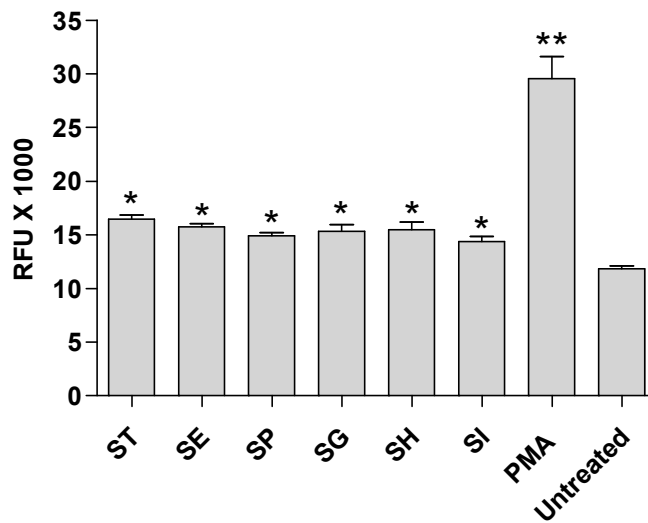
### **3.2.3 Oxygen production following infection of HD11 cells with *Salmonella***

The production of ROS from *Salmonella*-infected HD11 was also assessed. The oxidative burst occurring as a result of phagocytosis was measured as relative fluorescent units (RFU). Results showed a minimal but significant increase in oxidative burst after *Salmonella* exposure ( $P < 0.05$ ) with no significant difference between serovars (Figure 3.2).



**Figure 3.1:** Nitric oxide production by HD11 cells following infection with different *Salmonella* serovars.

ST; *S. Typhimurium*, SE; *S. Enteritidis*, SH; *S. Hadar*, SI; *S. Infantis*, SP; *S. Pullorum*, SG; *S. Gallinarum*, LPS; *E.coli* Lipopolysaccharide O55:B55 5 µg/ml final concentration, and untreated control cells. Values shown are averages and SEM from three independent experiments. Asterisks indicate significance from the uninfected controls (\*\*\*)  $P < 0.001$ .



**Figure 3.2:** Oxidative burst of HD11 cells following infection with different *Salmonella* serovars.

ST; *S. Typhimurium*, SE; *S. Enteritidis*, SP; *S. Pullorum*, SG; *S. Gallinarum*, SH; *S. Hadar*, SI; *S. Infantis*, PMA: final concentration of 10 µg/ml and untreated control cells. Values shown are means and SEM from three independent experiments. Asterisks indicate significance from the non-infected control cells (\* $P < 0.05$ , \*\* $P < 0.01$ ).



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**3.2.4 Quantification of gene expression of immune mediators following*****Salmonella* infection of cultured avian cells**

Gene expression of iNOS, the pro-inflammatory cytokine IL-6 and chemokines CXCLi1 and CXCLi2 were determined 6 h after infection in both cell lines. The mRNA expression profile of LITAF, Th1 signature cytokine IFN- $\gamma$ , Th2 key cytokines IL-4 and IL-13 and IL-10 regulatory cytokine were investigated in HD11 after infection with different *Salmonella* serovars and *E. coli* K-12.

*Salmonella* infections of HD11 resulted in higher levels of iNOS, the pro-inflammatory cytokine IL-6 and chemokines CXCLi1 and CXCLi2 ( $P < 0.05$ ) (Figures 3.3 and 3.4). Interestingly, the IL-6 fold increase from *S. Enteritidis*-infected HD11 was greater than that of *S. Pullorum*-infected cells (up to 219 fold change for *S. Enteritidis* and up to 69 fold change for *S. Pullorum*) ( $P < 0.05$ ). The CXCLi2 expression level was higher in *S. Typhimurium*, *S. Enteritidis* and *S. Hadar*-infected cells when compared with *S. Pullorum*-stimulated HD11 (up to 63 fold change for *S. Typhimurium*, up to 75 fold change for *S. Enteritidis*, up to 70 fold change for *S. Hadar* and up to 28 fold change for *S. Pullorum*) ( $P < 0.05$ ). Also, *S. Infantis*-infected HD11 stimulated the production of lower levels of CXCLi2 in comparison with *S. Enteritidis*- and *S. Hadar*-infected cells ( $P < 0.05$ ). The iNOS mRNA fold change was also higher in *S. Typhimurium* and *S. Enteritidis*-infected cells when compared with *S. Pullorum* and *S. Gallinarum*-infected HD11. The results obtained showed low but significant levels of IL-10 following infection with *Salmonella* with no difference between serovars (Figure 3.5). The fold change in mRNA expression measured for LITAF, IFN- $\gamma$  and IL-4 was not significant in

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*Salmonella*-infected HD11 cells in comparison with uninfected control cells ( $P>0.05$ ). *E.coli*-infected HD11 cells stimulated variable levels of CXCLi1, CXCLi2, IL-6, iNOS and IL-10 expression ( $P<0.05$ ). In CKC, the expression of IL-6, CXCLi2 and iNOS was significantly higher in *S. Typhimurium*, *S. Enteritidis*, *S. Hadar* and *S. Infantis*-infected cells when compared with uninfected controls ( $P<0.05$ ) (Figure 3.6). Generally the mRNA gene expression of IL-6, CXCLi1, CXCLi2 and iNOS was significantly highly up-regulated in *S. Typhimurium* and *S. Enteritidis*-infected CKC when compared with *S. Hadar*- and *S. Infantis*-infected cells (up to 14 fold change for IL-6, up to 10 fold change for CXCLi2, up to 4 fold change for CXCLi1 and up to 31 fold change (*S. Typhimurium*) and 18 fold change (*S. Enteritidis*) for iNOS) ( $P<0.05$ ). Both *S. Pullorum* and *S. Gallinarum*-stimulated CKC did not produce any of the cytokines and chemokines examined ( $P>0.05$ ) while *E.coli* infection stimulated the production of CXCLi1 and CXCLi2 from CKC ( $P<0.05$ ).

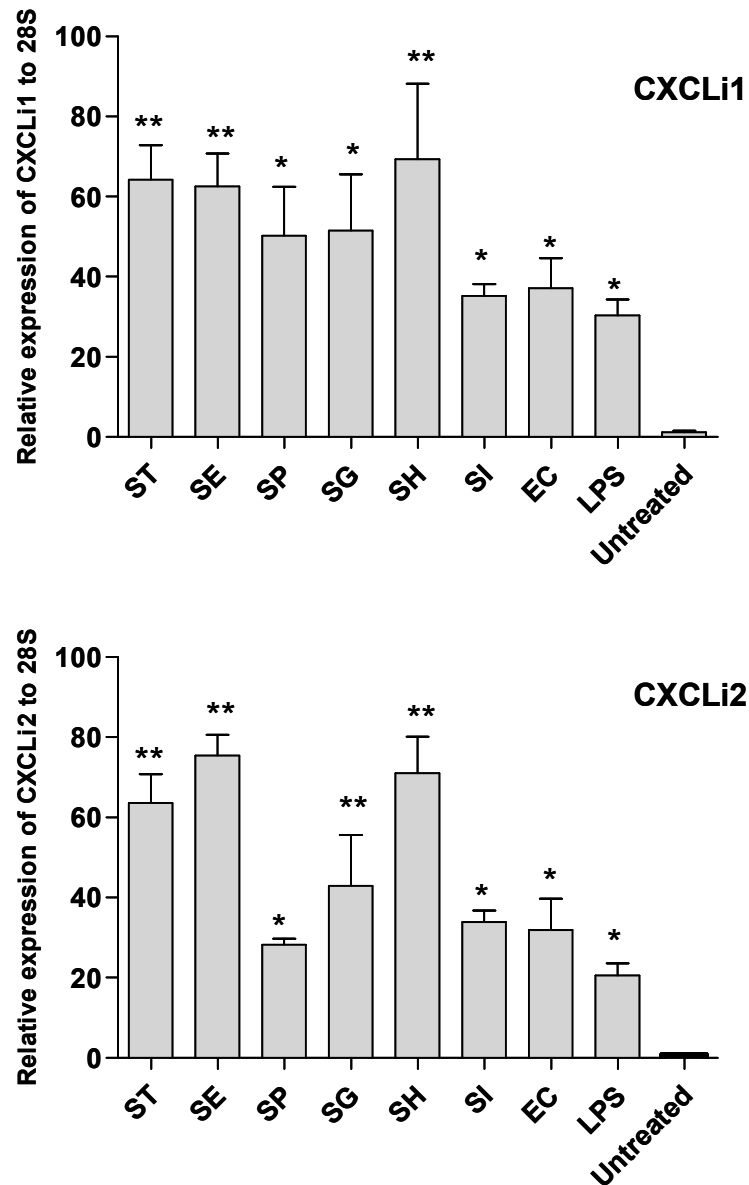
Quantification of the mRNA gene transcripts from chicken lymphocytes revealed expression of IL-18 and IL-4 (Figure 3.7). IL-18 was down-regulated in response to infection with *S. Pullorum*, while not significantly changed as a result of *S. Enteritidis* infection, though numerical evidence of expression was apparent. No significant changes were detected in IL-4 expression between *Salmonella*-infected and untreated cells ( $P>0.05$ ). Th1 cytokine, IFN- $\gamma$ , and Th2 cytokine, IL-13, were not expressed in this experiment.

Compared with the above mentioned data of gene expression, the mRNA gene expression of CXCLi2 and IL-6 decreased dramatically following the

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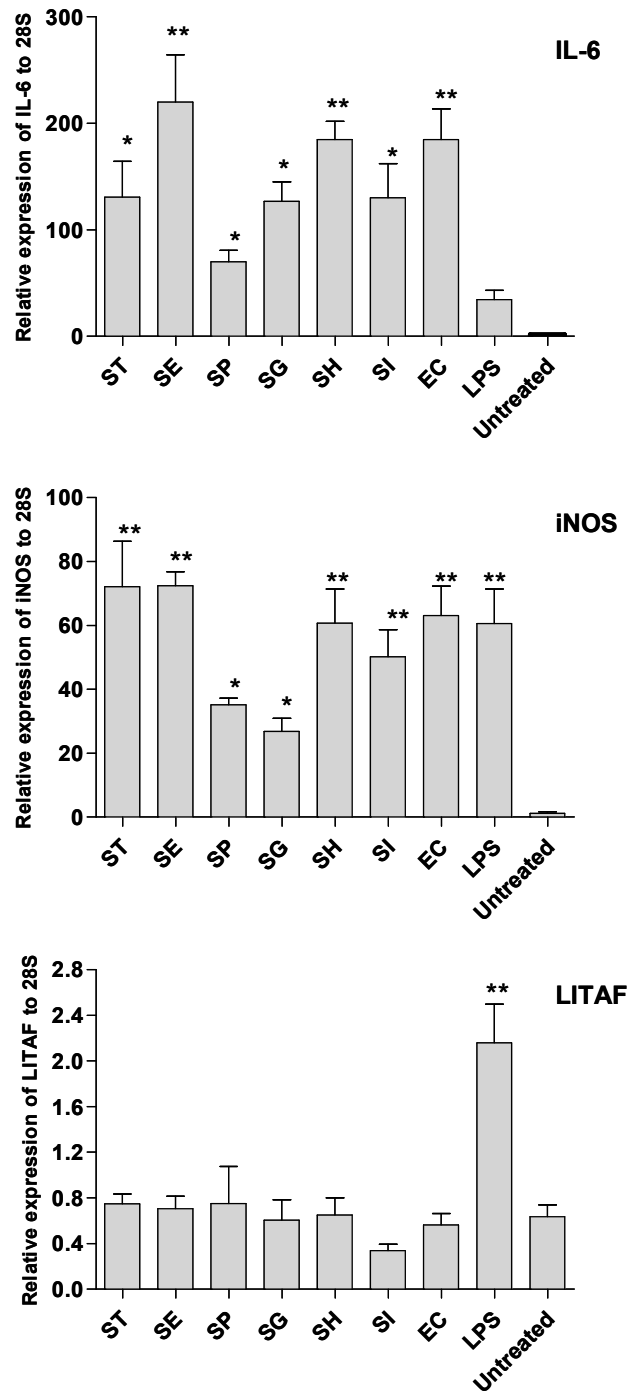
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exposure of HD11 and CKC to formalized *Salmonella* serovars as numerical trends in reduction of gene expression were observed (Figures 3.8 and 3.9). In HD11, IL-6 expression was not changed when compared with the uninfected control cells while CXCLi2 showed a little expression (9.3 to 10.9 fold change) which was greatly lowered when compared to that measured from the infection with the parent (viable) *Salmonella*. In CKC, the gene expression of CXCLi2 and IL-6 was lower than that measured in non infected control cells.



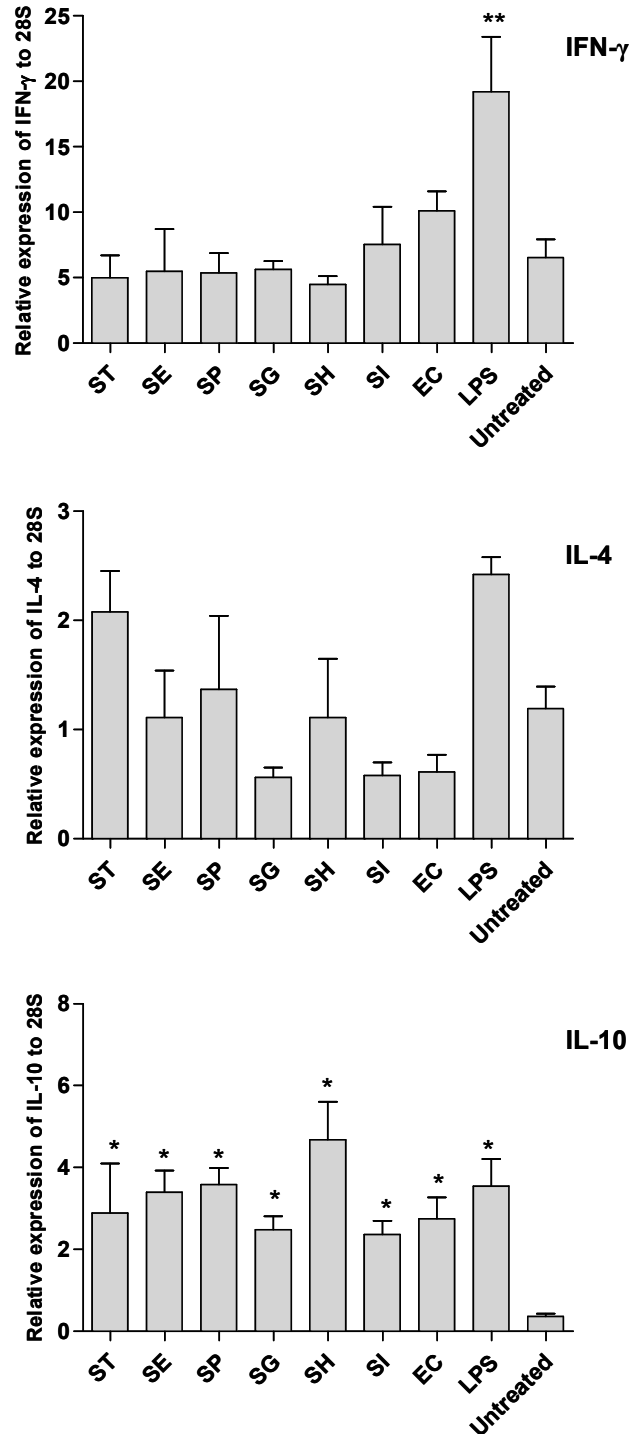
**Figure 3.3:** Gene expression of pro-inflammatory chemokines CXCLi1 and CXCLi2 from HD11 at 6 hrs post-infection.

ST; *S. Typhimurium*, SE; *S. Enteritidis*, SP; *S. Pullorum*, SG; *S. Gallinarum*, SH; *S. Hadar*, SI; *S. Infantis*, EC; *E. coli* K-12, LPS; *E. coli* Lipopolysaccharide O55:B55 5 µg/ml final concentration, and uninfected control cells. Values shown are averages and SEM of three independent experiments. Asterisks indicate significance from the uninfected control cells (\* $P < 0.05$ , \*\* $P < 0.01$ ).



**Figure 3.4:** Gene expression of inflammatory mediators IL-6, iNOS and LITAF from HD11 at 6 hrs post-infection with *Salmonella*.

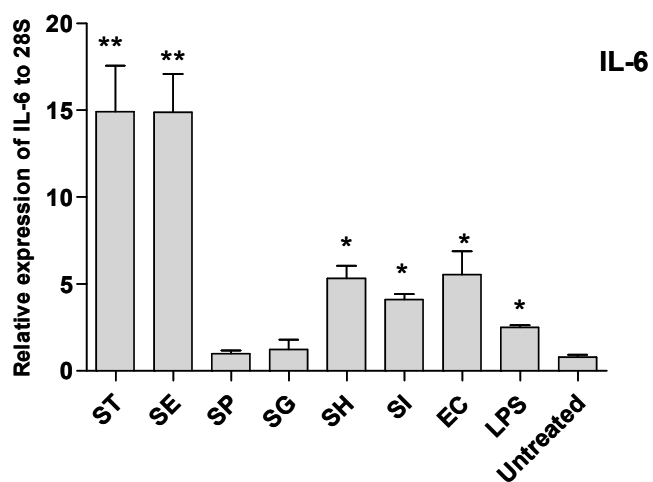
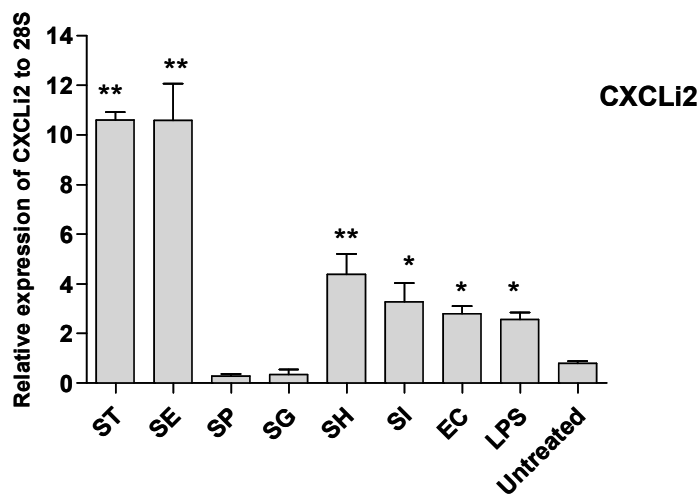
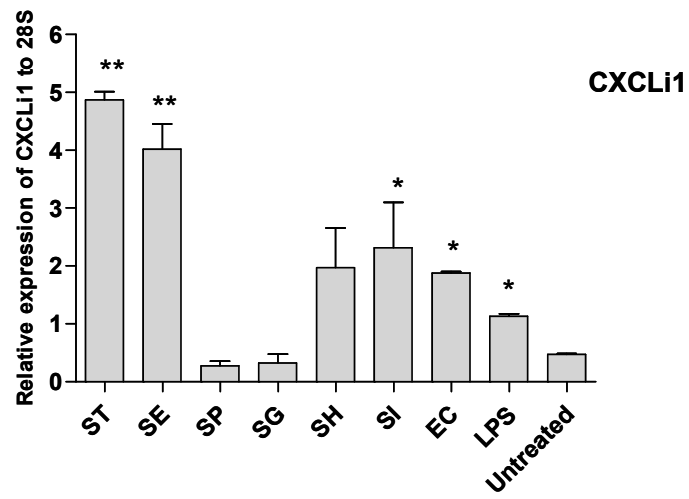
ST; *S. Typhimurium*, SE; *S. Enteritidis*, SP; *S. Pullorum*, SG; *S. Gallinarum*, SH; *S. Hadar*, SI; *S. Infantis*, EC; *E. coli* K-12, LPS; *E. coli* Lipopolysaccharide O55:B55 5 µg/ml final concentration, and uninfected control cells. Values shown are averages and SEM of three independent experiments. Asterisks indicate significance from the uninfected control cells (\*P<0.05, \*\*P<0.01).



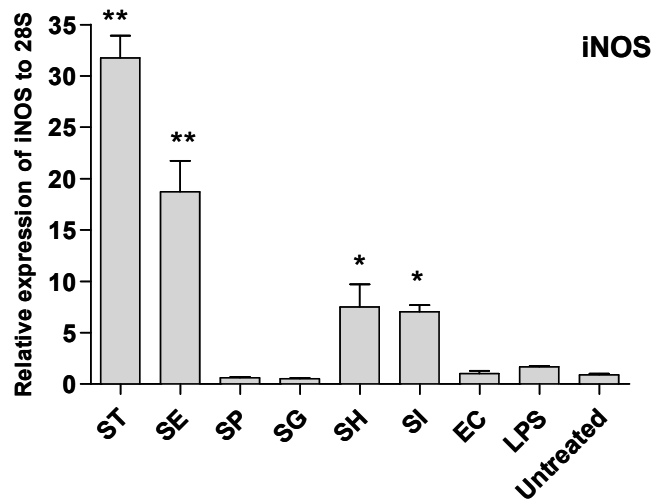
**Figure 3.5:** Gene expression of IFN- $\gamma$ , IL-4 and IL-10 from HD11 at 6 hrs post-infection with different serotypes of *Salmonella*.

ST; *S. Typhimurium*, SE; *S. Enteritidis*, SP; *S. Pullorum*, SG; *S. Gallinarum*, SH; *S. Hadar*, SI; *S. Infantis*, EC; *E. coli* K-12, LPS; *E. coli* Lipopolysaccharide O55:B55 5  $\mu$ g/ml final concentration, and untreated control cells. Values shown are means and SEM of three independent experiments. Asterisks indicate significance from the uninfected control cells (\* $P < 0.05$ , \*\* $P < 0.01$ ).

A



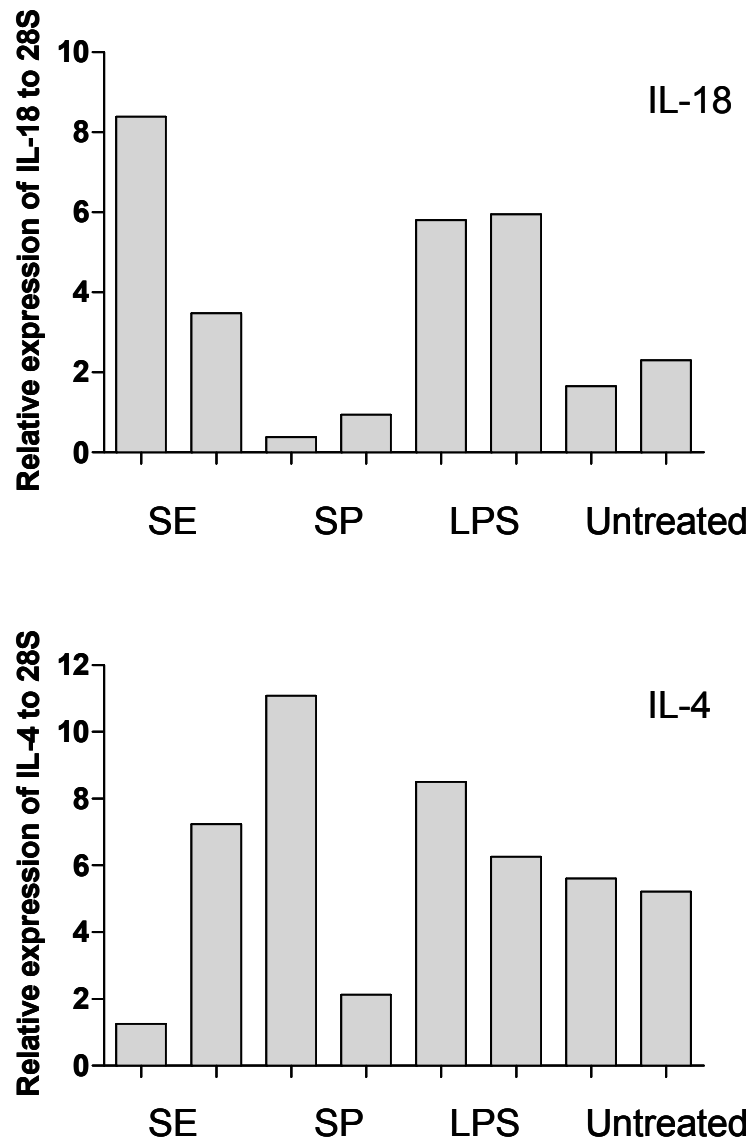
B



**Figure 3.6:** Quantification of cytokine and chemokine (A) and iNOS (B) mRNA gene expression from CKC at 6 hrs post-infection with *Salmonella* serovars.

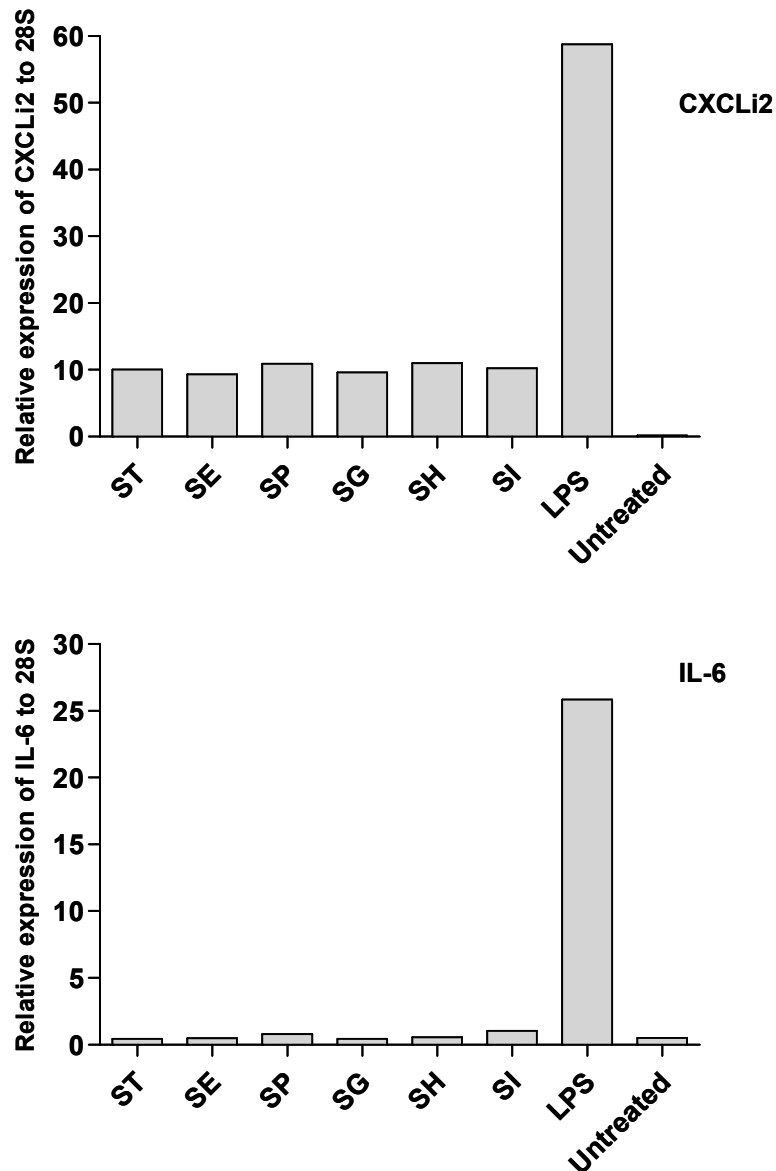
ST; *S. Typhimurium*, SE; *S. Enteritidis*, SP; *S. Pullorum*, SG; *S. Gallinarum*, SH; *S. Hadar*, SI; *S. Infantis*, EC; *E. coli* K-12, LPS; *E.coli* Lipopolysaccharide O55:B55 5 µg/ml final concentration, and non-infected control cells. Data are representative of those from three independent experiments. Asterisks indicate significance from the uninfected control cells (\* $P < 0.05$ , \*\* $P < 0.01$ ).





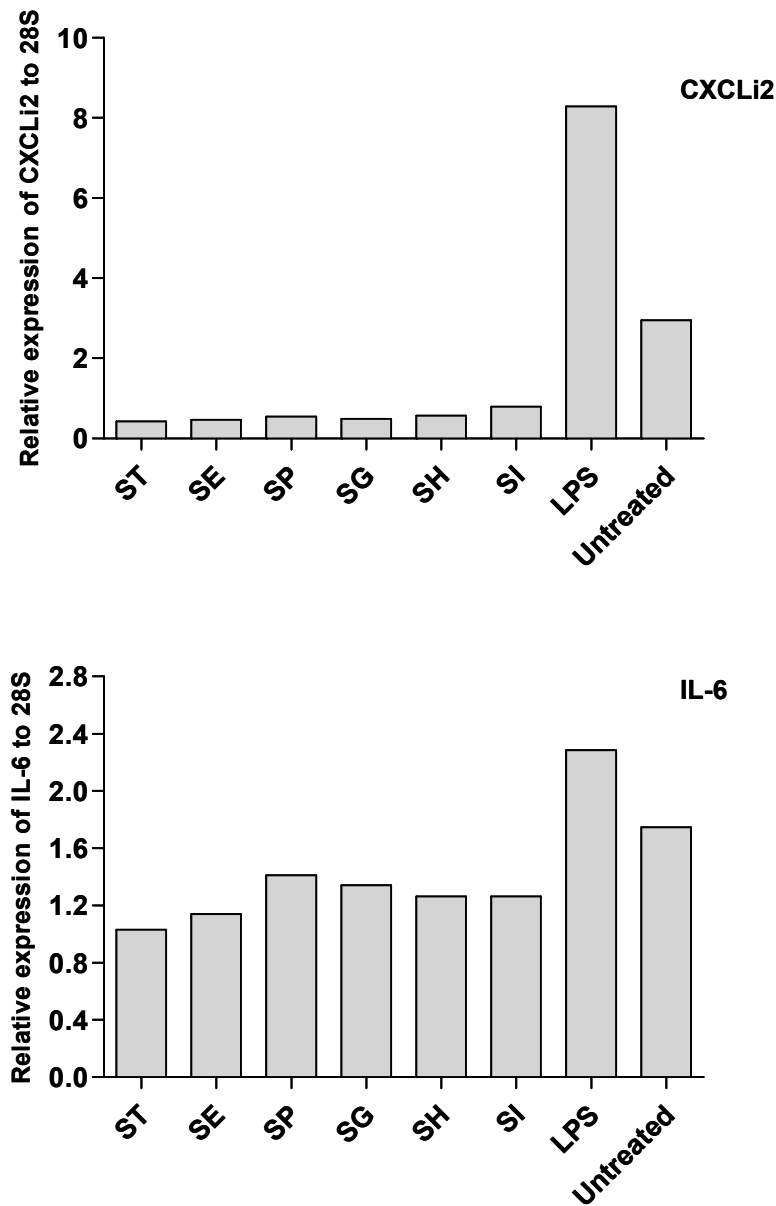
**Figure 3.7:** Cytokine gene expression of chicken lymphocytes in response to infection with *S. enterica*.

HD11 cells were infected with *S. Enteritidis* (SE) and *S. Pullorum* (SP), co-cultured with primary chicken lymphocytes and then incubated for 7 days. Duplicate experiments were performed (n=2). LPS at 1µg / ml final concentration was used as a positive control. PCR efficiencies (*E*) 28S=2.0, IL-18=1.94, IL-4=1.80.



**Figure 3.8:** Quantification of CXCLi2 and IL-6 mRNA gene expression from HD11 cells at 6 hrs after stimulation with formalized (killed) *Salmonella* serotypes.

ST; *S. Typhimurium*, SE; *S. Enteritidis*, SP; *S. Pullorum*, SG; *S. Gallinarum*, SH; *S. Hadar*, SI; *S. Infantis*, LPS; *E.coli* Lipopolysaccharide O55:B55 5 µg/ml final concentration and untreated control cells. Data shown are numerical values from a single experiment.



**Figure 3.9:** Quantification of CXCLi2 and IL-6 mRNA gene expression from CKC at 6 hrs after stimulation with formalized (killed) *Salmonella* serotypes.

ST; *S. Typhimurium*, SE; *S. Enteritidis*, SP; *S. Pullorum*, SG; *S. Gallinarum*, SH; *S. Hadar*, SI; *S. Infantis*, LPS; *E.coli* Lipopolysaccharide O55:B55 5 µg/ml final concentration and untreated control cells. Data shown are numerical values from a single experiment.

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### 3.3 Discussion

Measurement of changes in cytokine and chemokine gene expression following *Salmonella* infection remains an informative area of work for many researchers. Thus, gene expression after *Salmonella* infections in poultry has been studied in either *in vivo* (Beal et al., 2004b, Berndt et al., 2007, Fasina et al., 2008, Haghighi et al., 2008, Chappell et al., 2009) or *in vitro* work (Swaggerty et al., 2004, Kaiser et al., 2006). However, most of these studies focussed on the relative immune responses to certain serovars of *Salmonella*, particularly *S. Typhimurium* and *S. Enteritidis*, and no single study, as yet, has compared the immune responses and mechanisms surrounding *Salmonella*-associated infections with serotypes known to induce different infections and pathologies.

It has been shown that entry of *Salmonella* into the host cells is achieved through two different mechanisms, active or passive. The active mechanism (bacterial invasion) involves direct penetration of the host cell membrane as a result of secretion of certain bacterial mediators and translocated protein molecules which causes cellular membrane ruffling and then bacterial invasion (Wallis and Galyov, 2000). The other alternative pathway, the passive mechanism (bacterial uptake), which occurs as a result of direct contact and adherence of *Salmonella* to the professional phagocytic cells such as macrophages (Kramer et al., 2003). In the present study, the number of *Salmonella* and *E. coli* K-12 internalized by HD11 was higher than that of CKC. The entry of bacteria in a higher numbers into HD11 is much more likely to be as a result of both bacterial invasion and uptake (phagocytosis) while

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only invasion in CKC. As phagocytic immune cells, HD11 were able to limit the infection and survival of *Salmonella*, starting 24 h post-infection. This finding is in agreement with (Kramer et al., 2003) as infection with *S. Enteritidis* had declined at 24 h post-infection in both HD11 and MQ-NCSU chicken macrophages. It was reported that infection with *S. Typhimurium* or *S. Enteritidis* is associated with efficient colonization of the chicken gut with faecal shedding of *Salmonella* (Barrow et al., 2004, Humphrey et al., 1989) and bacterial counts detected in systemic organs such as liver were increased 24-48 hrs following oral infection with *S. Typhimurium* in newly hatched chicks (Withanage et al., 2004). It is highly evident that *Salmonella* are internalized within phagocytes, including macrophages, during the transport from the intestinal tract to systemic sites (Jones et al., 2001) and here we have shown that *Salmonella* strains are capable to persist within macrophages for 48 h post-infection. Studies on *Salmonella* resistant and susceptible chicken lines revealed a primary role for SAL1 locus encoded by primary macrophages isolated from genetically resistant lines in clearance of *S. Gallinarum*, through a strong oxidative burst pathway (Wigley et al., 2002a). In the current experiment, an increase in the production of ROS 1 h post-infection and secretion of high amounts of NO at 24 and 48 h post-infection by HD11 were detected following infection with different *Salmonella* serovars tested in this experiment. The production of ROS and NO is a well-known antimicrobial mechanism developed by activated macrophages in response to infection with intracellular pathogens. Suppression of ROS production has been found to increase *S. Typhimurium* survival or persistence in porcine macrophages (Donne et al., 2005). Compared to the mouse monocyte cell line J774A.1,

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HD11 was found to produce higher concentrations of NO in response to infection with *S. Typhimurium* and *S. Enteritidis* (Babu et al., 2006). HD11 also produced NO in response to stimulation with the TLR agonist, synthetic oligodeoxynucleotide containing unmethylated CpG-dinucleotides (CpG-ODN), through stimulation of iNOS and involvement of other common pathways, including NF- $\kappa$ B signalling pathway (He and Kogut, 2003). Moreover, chicken peripheral blood mononuclear cells have been found to produce higher amounts of NO following stimulation with CpG-ODN and *S. Enteritidis*-LPS, potent NO inducers (He et al., 2006). Nearly the same dynamic response has been reported in chicken macrophages MQ-NCSU which produce strong oxidative burst and NO in response to *S. Typhimurium*, *S. Enteritidis* and *S. Gallinarum* infection (Withanage et al., 2005a) and for murine macrophages (Vazquez-Torres and Fang, 2001a, Vazquez-Torres et al., 2008) following *Salmonella* infection.

In order to further investigate the immune responses of avian cells to *Salmonella* infection, gene expression of selected immune mediators was studied in HD11- and CKC-infected cells. CXCLi1 (K60) and CXCLi2 (IL-8) are important mediators of the innate immune system which are classified as CXC chemokines. They function as chemoattractants as their primary role is the recruitment of immune cells, such as macrophages and heterophils, to the site of infection and inflammation (Kaiser and Staheli, 2008). The data presented here showed a significant differential up-regulation of these chemokines following infection of CKC and HD11 with *S. Typhimurium* and *S. Enteritidis*. Nevertheless, *S. Pullorum* and *S. Gallinarum* failed to stimulate

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the same dynamic response from infected CKC. Interleukin-6 and iNOS mRNA gene expression was also investigated in infected CKC and HD11 cells. IL-6 and iNOS expression levels significantly increased following infection of CKC with *S. Typhimurium*, *S. Enteritidis*, *S. Hadar* and *S. Infantis*, with *S. Typhimurium* and *S. Enteritidis* showed the higher expression levels. In contrast, *S. Pullorum* and *S. Gallinarum* did not stimulate significant expression of both IL-6 and iNOS in comparison with uninfected CKC control. IL-6 is a pro-inflammatory cytokine which is produced early after infection and plays an important role in the innate immune responses and development of adaptive immune system (Kaiser and Staheli, 2008). The expression of IL-6 has been investigated *in vitro* in CKC following *Salmonella* infections (Kaiser et al., 2000). Quite similar with the current results with CKC, the expression of IL-6 was up-regulated as a result of infection with *S. Typhimurium*, *S. Enteritidis*, and *S. Dublin* while *S. Gallinarum* showed down-regulated IL-6 expression from infected CKC. Data from the current experiment showed that both *S. Typhimurium* and *S. Enteritidis* are strong inflammatory serotypes while *S. Hadar* and *S. Infantis* are less inflammatory where as *S. Pullorum* and *S. Gallinarum* are non inflammatory serotypes.

Lipopolysaccharide-induced tumour necrosis alpha factor (LITAF) did not significantly change following infection of HD11 with all *Salmonella* serotypes, in comparison with the uninfected control cells. Little is known about the biological function of LITAF in poultry. However, up-regulation of this co-stimulatory molecule has been reported *in vitro* following activation of macrophages with *S. Typhimurium* LPS and in response to treatment with

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*Eimeria* species, the aetiological agent of chicken coccidiosis (Hong et al., 2006b). In the present experiment, absence of significant changes in LITAF expression could be influenced by the time factor (6 h post-infection).

Interferon (IFN)- $\gamma$  is the hallmark of Th1 immune responses while IL-4 and IL-13 are the signature cytokines of Th2 responses. In the current experiment, no significant changes were detected in the gene expression of IFN- $\gamma$  and IL-4 in infected HD11 over non infected control cells, while IL-13 was not expressed at all. Till now, no data is available about the expression of IL-13 (Th2 cytokine) in chickens in response to *Salmonella* infection. Interleukin-10 is anti-inflammatory cytokine which thought to down-regulate the effects of IFN- $\gamma$  and to inhibit host response against infection (Kaiser and Staheli, 2008). IL-10 is described as the master regulator of immune responses which inhibits the activity of both Th1 and Th2 immune cells (Couper et al., 2008). In the present experiment, relatively mild, but significant, up-regulation of IL-10 was detected in HD11 cells in response to infection different *Salmonella* serovars. IL-10 could be produced by activated HD11 cells as an immune regulator to prevent the over expression of pro-inflammatory cytokines and inflammation, a negative feed back pathway. In this experiment, data showed changes in the gene expression of IL-18 and IL-4 cytokines in primary chicken lymphocytes co-cultured with *Salmonella*-infected HD11 cells. However, no significance was detected in the gene expression of IL-4 between infected and control cells. The most important finding is the down-regulation of IL-18 by chicken lymphocytes in response to *S. Pullorum* infection, when compared to the untreated cells. The mean value of IL-18 expression induced by *S. Enteritidis*

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infection was more than twice higher than that of the non-infected cells, though this was not statistically significant. IL-18 is inflammatory cytokine produced by activated macrophages, which has IFN- $\gamma$  inducing activities (Th1 immune mechanism). The down-regulation of IL-18 by *S. Pullorum* could indicate that *S. Pullorum* do not stimulate the Th1 immune mechanism. However, the induction of IL-18 by activated lymphocytes seems unusual. This could be influenced by the non-adherent macrophages (HD11 cells) which could be detached and released with lymphocytes into the cell medium at 7 days after infection.

In the present experiment, we demonstrated that stimulation of immune responses is triggered by infection with viable versus formalin-killed bacteria. Gene expression data from formalin-killed treated HD11 and CKC cells revealed dramatic down-regulation of CXCLi2 and IL-6. Host cells, such as macrophages, recognize pathogenic microbes through a number of innate immune receptors called pattern recognition receptors, including TLRs, which recognize the pathogen-associated molecular patterns (Fearon and Locksley, 1996). Invasion and/or uptake of live bacteria by the host cells with further secretion of bacteria products stimulate the activation of intracellular signalling pathways and production of cytokines (Nau et al., 2002). It seems that the killed bacteria lack the active contribution and the interaction with cellular immune components and, therefore, failed to stimulate the production of cytokines. This could also due to differences in interaction of cells with killed bacteria as the exposure time was 2h, compared with 6h in case of experiments performed viable bacteria.

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In this experiment, HD11 cells and CKC were used to study interaction of *Salmonella* with macrophages and epithelial cells. Cells were infected at MOI of 10 as previously described (Kaiser et al., 2000). HD11 cell line is an avian macrophage-like cell line which was produced by viral transformation with avian myelocytomatosis virus (MC-29) of chicken bone marrow cells (Beug et al., 1979). These slightly more adherent cells possess similar cultural, morphological and functional characteristics to chicken macrophages, as is the case for MQ-NCSU cells (Qureshi et al., 2000). This established, transformed cell line has proven to be very valuable and has been widely used as a fertile *in vitro* model to study the immune function of chicken macrophages (He and Kogut, 2003, Okamura et al., 2005, Smith et al., 2005, He et al., 2009, He et al., 2011). HD11 cells provide higher numbers, uniformity and viability than primary chicken macrophages (Babu et al., 2006). CKC is an epithelial cell model which has been widely used to characterize *in vitro* the invasion capabilities of, and the immune responses to, avian pathogens, including *Salmonella* (Barrow and Lovell, 1989, Henderson et al., 1999, Kaiser et al., 2000, Jones et al., 2001, Wigley et al., 2001). CKC allows the study of interaction of avian pathogens with epithelial cells, with the use of limited number of experimental birds. Compared to kidney cells, intestinal epithelial cells are adapted to different environmental conditions, including changes in pH and colonization of gut microflora as well as infection with pathogenic bacteria. However, isolation of epithelial cells from the kidney tissue reduced the potential risk of contamination from the intestinal microflora, particularly when compared with the other alternative intestinal *in vitro* systems.

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In conclusion, we have shown that the expression of cytokines and chemokines in cultured cells due to *Salmonella* infection is an active dynamic process depends primarily on the infecting serovar and the stimulation of immune responses is not directly related to the intracellular bacterial counts. This has been clearly demonstrated by the reduced induction of pro-inflammatory cytokines and chemokines by HD11 and CKC cells infected with *S. Pullorum* or *S. Gallinarum* and also the production of different levels of cytokine gene expression in CKC following infection with the weak *E. coli* K-12 weak laboratory strain. The results from the present experiment demonstrate the ability of chicken epithelial and macrophages cell lines to produce differential expression of various cytokines and chemokines in response to *Salmonella* infections. *S. Typhimurium*, *S. Enteritidis*, *S. Hadar* and *S. Infantis* seem to be more invasive and trigger the infected cells to secrete pro-inflammatory cytokines and chemokines. However, as the expression of the latter mediators was found to be much less in *S. Hadar* and *S. Infantis*-infected epithelial cells, it is concluded that these serotypes are less inflammatory in their stimulation to the avian immune system. Nitric oxide and oxygen production can be considered as important pathways for macrophages to clear infection with different serotypes of *Salmonella*. *S. Pullorum* and *S. Gallinarum* appear to be the least- or non-inflammatory serotypes and although gene expression following infection with these serotypes has now been studied, the full picture of pathogenesis and immune responses to these serotypes requires more work to be done and a comparison between what we have presented here with an *in vivo* experiment is required.

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## 4 Immune responses of avian cells to infection with *Salmonella* pathogenicity island- and flagellar assembly system-mutants of *Salmonella enterica*

### 4.1 Introduction

*S. enterica* has evolved a central strategy to interact with the host cells through the presence of major virulence determinants, *Salmonella* pathogenicity islands (SPI). The SPI-encoded TTSS play a major role in the secretion and transfer of bacterial proteins into target eukaryotic cells. The role of SPI-1 and SPI-2 mediated-TTSS-1 and TTSS-2 in the pathogenesis of *Salmonella* infection of chickens is not well understood. The majority of the previous work has focussed on and compared the colonization and invasion characteristics of mutant and parent strains of *Salmonella* but no single study, as yet, has investigated the expression profile of innate and adaptive immune responses triggered following infection with wild type and mutant *Salmonella* strains. Thus, to understand the immunologic mechanisms underlying *Salmonella* pathogenesis (colonization, invasion and survival) inside the infected host cells, the mRNA gene expression of selected cytokines and chemokines was determined in HD11 and primary CKC cells infected with either the wild type (wt) or SPI-1 and SPI-2 (TTSS-1 and TTSS-2) mutant strains of *S. Enteritidis* and *S. Pullorum*. Also, the dynamic response to infection with mutant strain of *S. Enteritidis* which is defective in flagellar assembly and motile strain of *S. Gallinarum* has been investigated.

## 4.2 Results

### 4.2.1 Invasion of HD11 and CKC by *Salmonella* serotypes

Intracellular bacterial counts were determined in infected HD11 and CKC cells at 2, 6 and 24 h following exposure to wild type (wt) and mutant strains of *Salmonella* serotypes; Enteritidis, Pullorum and Gallinarum using the gentamicin protection assay (Tables 4.1 & 4.2). As expected, intracellular survival of *S. Enteritidis* was significantly higher in both types of cells at all time points in comparison with that of *S. Pullorum* and *S. Gallinarum* ( $P < 0.01$ ). In HD11 cells, intracellular survival of wt *S. Enteritidis* was higher than that of *S. Enteritidis invA* and *fliJ* mutants at 6 h after infection while entry and survival of *S. Enteritidis ssaR* was greater than wt *S. Enteritidis* at 2, 6 and 24 h post-infection. Intracellular survival detected after infection with wt *S. Pullorum* and wt *S. Gallinarum* was not significantly changed from those of the mutant strains ( $P > 0.05$ ).

In CKC, like HD11 cells, *S. Enteritidis invA* and *fliJ* mutant strains showed a significantly reduced intracellular counts at all time points compared with wt *S. Enteritidis* ( $P < 0.001$ ), with *S. Enteritidis fliJ* showed the lower counts. Invasion and intracellular survival of *S. Enteritidis ssaR* were not significantly changed from the parent strain of *S. Enteritidis* ( $P > 0.05$ ). While *S. Gallinarum* Flg<sup>+</sup> invaded CKC in relatively higher numbers compared with wt *S. Gallinarum* at 2 h after infection ( $P < 0.05$ ), *S. Gallinarum* Flg<sup>+</sup> revealed higher intracellular bacterial counts at 6 h ( $P < 0.001$ ) and 24 h ( $P < 0.05$ ) post-infection. No significance was found in the intracellular counts between wt *S. Pullorum* and their mutants at all time points tested in this experiment ( $P > 0.05$ ).

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**Table 4.1:** Intracellular bacterial counts in HD11 cells following infection with wild type and mutant strains of *S. enterica*

Serotype	Log <sub>10</sub> inoculum counts	Intracellular bacterial counts (Log <sub>10</sub> CFU/ml)*		
		2 h	6 h	24 h
<i>S. Enteritidis</i> wt	6.922±0.04	5.80±0.22	6.40±0.19	4.13±0.34
<i>S. Enteritidis invA</i>	6.696±0.11	5.13±0.27	5.70±0.25	4.28±0.45
<i>S. Enteritidis ssaR</i>	6.938±0.05	6.40±0.36	6.93±0.21	4.95±0.22
<i>S. Enteritidis fliJ</i>	7.080±0.06	4.93±0.39	5.25±0.29	3.85±0.60
<i>S. Pullorum</i> wt	6.665±0.11	3.75±0.31	4.23±0.34	2.40±0.40
<i>S. Pullorum sipB</i>	6.739±0.08	4.10±0.28	4.00±0.30	2.85±0.43
<i>S. Pullorum spaS</i>	6.828±0.05	4.20±0.33	4.15±0.32	2.75±0.47
<i>S. Pullorum ssaU</i>	6.567±0.06	4.23±0.38	4.25±0.32	2.90±0.51
<i>S. Gallinarum</i> wt	6.799±0.06	4.63±0.22	4.60±0.28	2.90±0.56
<i>S. Gallinarum</i> Flg <sup>+</sup>	6.914±0.05	4.35±0.25	4.33±0.36	3.18±0.47

\*Values are expressed as means ± standard errors of four independent experiments. At all time points, *S. Enteritidis* was more internalized by HD11 cells than *S. Pullorum* and *S. Gallinarum* (P<0.01).

**Table 4.2:** Invasion of CKC by wild type and mutant strains of *S. enterica*

Serotype	Log <sub>10</sub> inoculum counts	Intracellular bacterial counts (Log <sub>10</sub> CFU/ml) <sup>†</sup>		
		2 h	6 h	24 h
<i>S. Enteritidis</i> wt	6.943±0.08	5.85±0.10	6.48±0.14	6.78±0.16
<i>S. Enteritidis invA</i>	7.134±0.07	4.93±0.17***	5.08±0.09***	5.63±0.18***
<i>S. Enteritidis ssaR</i>	7.124±0.05	5.90±0.17	6.40±0.10	6.73±0.17
<i>S. Enteritidis fliJ</i>	7.148±0.08	3.55±0.12***	3.45±0.09***	4.33±0.18***
<i>S. Pullorum</i> wt	7.016±0.11	2.33±0.12	2.53±0.19	2.20±0.12
<i>S. Pullorum sipB</i>	6.902±0.09	2.45±0.11	2.27±0.23	2.73±0.44
<i>S. Pullorum spaS</i>	7.233±0.20	2.60±0.00	2.08±0.07	2.00±0.00
<i>S. Pullorum ssaU</i>	6.881±0.03	2.85±0.13	2.53±0.10	2.45±0.16
<i>S. Gallinarum</i> wt	7.145±0.08	3.18±0.31	2.78±0.23	3.05±0.13
<i>S. Gallinarum</i> Flg <sup>+</sup>	7.155±0.07	3.83±0.17*	3.88±0.10***	3.75±0.13*

<sup>†</sup>Values are expressed as means ± standard errors of four independent experiments. At all time points, *S. Enteritidis* was more internalized by HD11 cells than *S. Pullorum* and *S. Gallinarum* (P<0.001). Asterisks indicate that the difference between the wild type and the mutant strain was significant (\*P<0.05, \*\*\*P<0.001).

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#### 4.2.2 Quantification of cytokine and chemokine response after infection

Gene expression of selected immune mediators was determined in both HD11 and CKC at 6 h post-infection using qRT-PCR. All *Salmonella* strains examined in this study stimulated the production of pro-inflammatory markers in infected HD11 cells at 6 h post-infection, including CXCLi1, CXCLi2, IL-6, CCLi2 ( $P<0.001$ ) and iNOS ( $P<0.05$ ) (Figure 4.1). Moreover, HD11 cells infected with wt *S. Enteritidis* stimulated higher levels of mRNA gene expression of CXCLi2 and CCLi2 (MIP-1 $\beta$ ) expression compared with *S. Pullorum*- and *S. Gallinarum*-infected cells ( $P<0.05$ ). *S. Enteritidis ssaR*-infected HD11 cells elicited significantly lower levels of CXCLi1 and IL-6 expression when compared with the parent strain of *S. Enteritidis* ( $P<0.05$ ). HD11 cells infected with *S. Pullorum sipB* mutant strain showed reduced levels of CXCLi2, IL-6 and iNOS when compared to wt *S. Pullorum*-infected cells. Compared to wt *S. Pullorum*, the *spaS* strain induced lower expression of CXCLi2. However, no significant changes were detected in the expression of pro-inflammatory cytokines and chemokines between wt *S. Pullorum* and their mutants strains ( $P>0.05$ ). Although HD11 cells infected with *S. Enteritidis invA* and *fliJ* mutants strains elicited higher levels of IL-6 cytokine production, these changes were not significantly different from that of wt *S. Enteritidis*-infected cells ( $P>0.05$ ). Infection of HD11 cells with *Salmonella* stimulated the production of IL-10 regulatory cytokine where wt *S. Enteritidis* and mutant strains *invA*, *ssaR* and *fliJ* elicited the highest levels of gene expression compared with the non-infected control cells ( $P<0.05$ ) (Figure 4.2). Infection of HD11 cells with *S. Enteritidis ssaR* induced mild, but significant, up-regulation in the gene expression of TLR4 in comparison with uninfected

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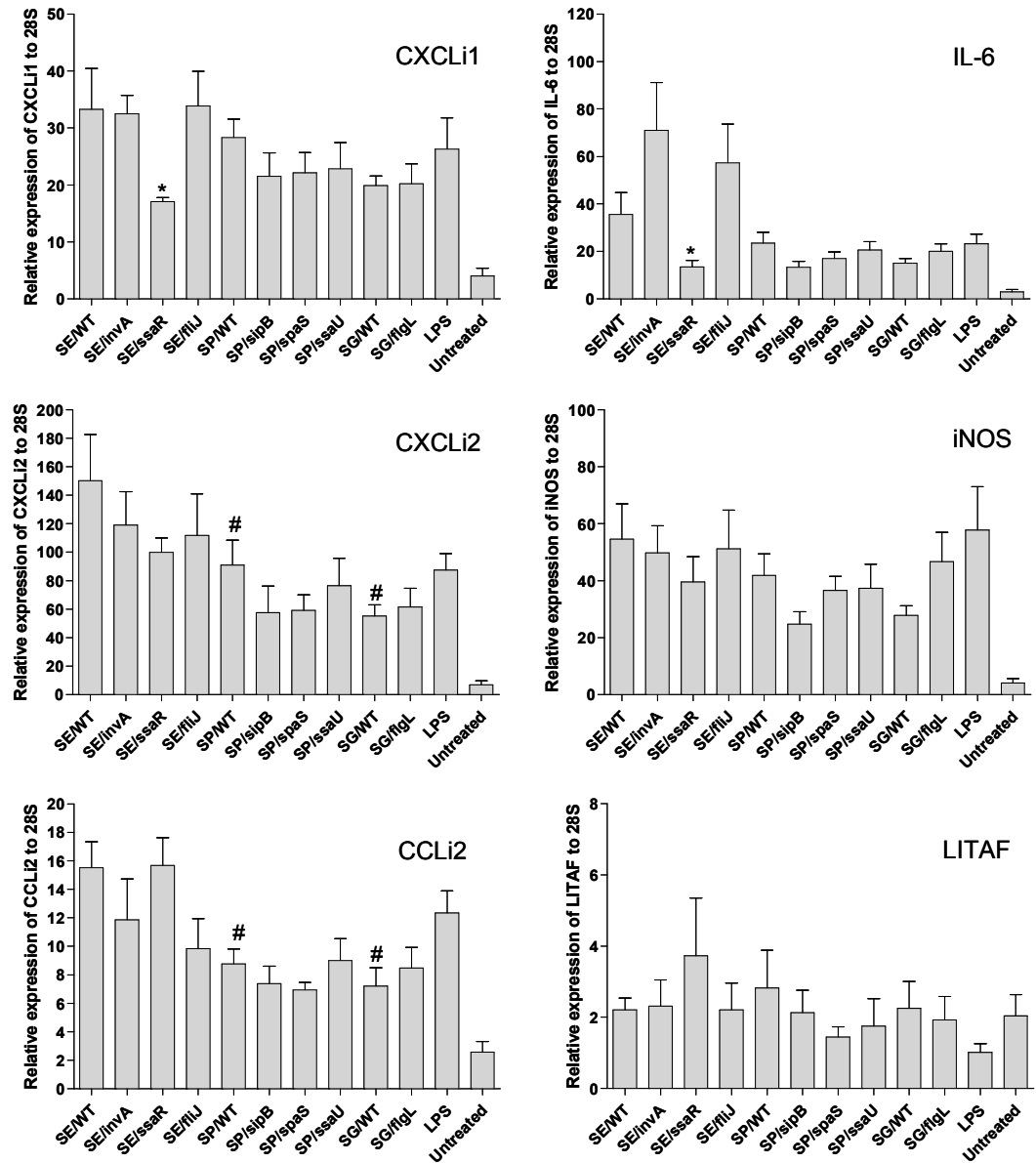
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controls ( $P < 0.05$ ) (Fig 4.3). *Salmonella* infections elicited different amounts of LITAF co-stimulatory molecule, Th1 cytokines (IL-18, IFN-  $\gamma$  and IL-12 $\beta$ ), Th2 cytokine (IL-4) and TLR5. However, no significant difference in the capacity of each strain to induce any of these mediators was detected, whereas IL-13 was not expressed by HD11 cells.

CKC infected with wild type *S. Enteritidis* expressed higher amounts of CXCLi1, CXCLi2, IL-6 and iNOS when compared with *S. Pullorum*- and *S. Gallinarum*-infected cells ( $P < 0.001$ ), which did not elicit the production of any of the immune mediators tested in this experiment (Figure 4.4). Moreover, *S. Enteritidis* wt expressed higher levels of CXCLi2 gene expression when compared to *S. Enteritidis* mutant strains (*invA*, *ssaR* and *fliJ*) and *S. Pullorum* wt ( $P < 0.001$ ). Surprisingly, the *S. Gallinarum* Flg<sup>+</sup> mutant strain expressed higher amounts of CXCLi2 and IL-6 than the wt *S. Gallinarum* ( $P < 0.05$ ). While *S. Enteritidis invA* stimulated a lower level of IL-6 compared to the wt, the IL-6 gene expression produced as a result of infection of CKC with *S. Enteritidis* wt was greater than *S. Enteritidis ssaR* ( $P < 0.05$ ) and *S. Enteritidis fliJ* ( $P < 0.01$ ). CKC infected with *S. Enteritidis* wt elicited higher levels of CXCLi1 compared with *S. Enteritidis invA*, *S. Enteritidis ssaR* ( $P < 0.05$ ) and *S. Enteritidis fliJ* ( $P < 0.001$ ). Although infection of CKC with *S. Enteritidis ssaR* revealed a relatively higher level of iNOS expression in comparison with uninfected control cells ( $P < 0.05$ ), there were no significant changes in the expression of iNOS between wt *S. Enteritidis* and *S. Enteritidis ssaR* ( $P > 0.05$ ). Furthermore, the expression of iNOS by CKC-infected *S. Enteritidis* wt was higher than *S. Enteritidis fliJ*-infected cells ( $P < 0.001$ ). The expression of TLR5

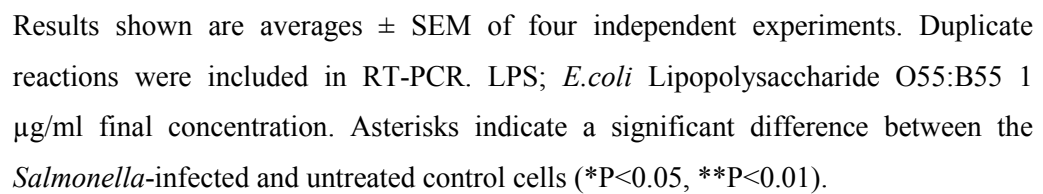
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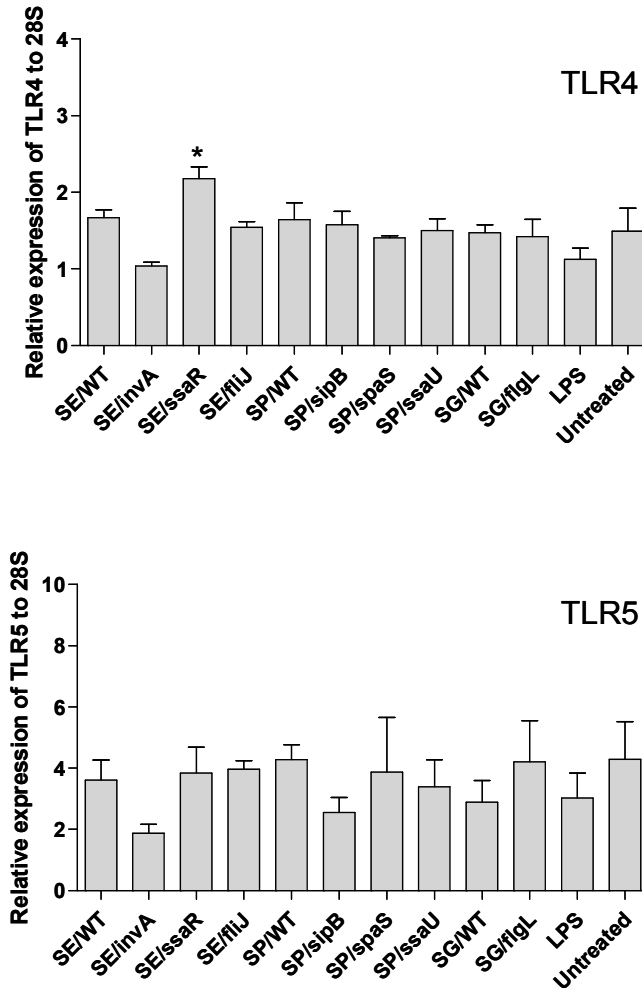
was not significantly changed in *Salmonella*-infected CKC over the non-infected control ( $P>0.05$ ).



**Figure 4.1:** Effect of *Salmonella* infection on gene expression of inflammatory mediators in HD11 cells at 6 h post-infection.

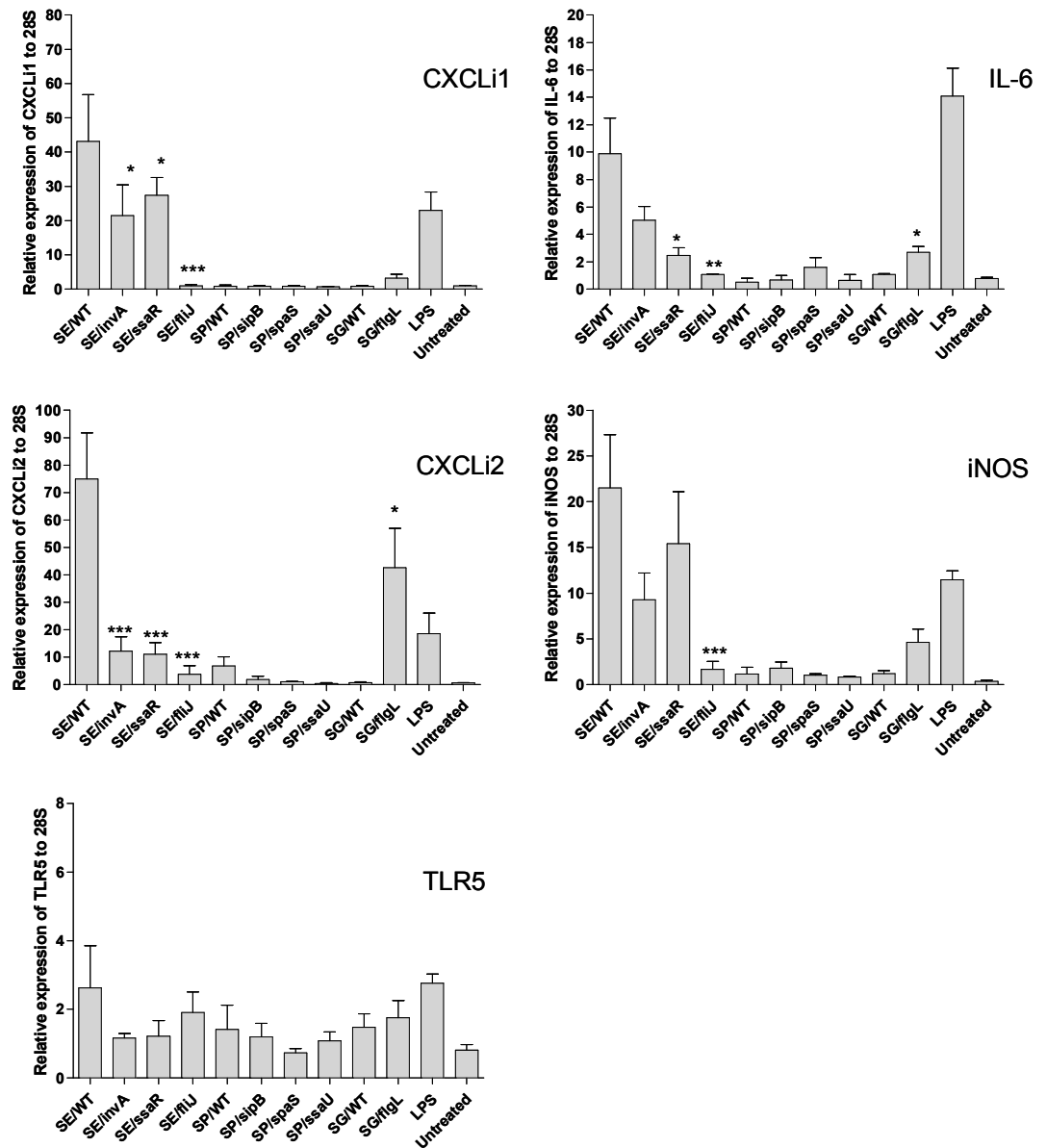
Results shown are averages  $\pm$  SEM of four independent experiments. Duplicate reactions were included in RT-PCR. LPS; *E.coli* Lipopolysaccharide O55:B55 1  $\mu$ g/ml final concentration. (\*) indicates a significant difference between the wild type and the mutant strain and (#) indicates that the difference from wt SE was significant ( $P < 0.05$ ).





**Figure 4.3:** Gene expression of TLR4 and TLR5 in *Salmonella*-infected HD11 cells at 6 h post-infection.

Results shown are averages  $\pm$  SEM of four independent experiments. Duplicate reactions were included in RT-PCR. LPS; *E.coli* Lipopolysaccharide O55:B55 1  $\mu$ g/ml final concentration. (\*) means a significant difference between the *Salmonella*-infected and untreated control cells ( $P < 0.05$ ).



**Figure 4.4:** Effect of *Salmonella* infection on gene expression of CXCLi1, IL-6, CXCLi2, iNOS and TLR5 in CKC at 6 h post-infection.

Results shown are averages  $\pm$  SEM of four independent experiments. Duplicate reactions were included in RT-PCR. LPS; *E.coli* Lipopolysaccharide O55:B55 1  $\mu$ g/ml final concentration. (\*) indicates significance between the wild type and the mutant strain (\* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001).

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### 4.3 Discussion

In order to provide further insights into the interaction between bacterial virulence determinants and the avian host at the cellular and molecular level, invasion, survival and mRNA gene expression of selected immune mediators were investigated in chicken macrophages (HD11) and primary chick kidney cells (CKC) infected with wild type and mutant strains of *S. enterica*. Here we have shown that both SPI-1 and SPI-2 (TTSS-1 and TTSS-2) and flagella are required for bacterial invasion and virulence of *S. Enteritidis* in avian cells.

Both *S. Enteritidis invA* (impaired TTSS-1) and *S. Enteritidis fliJ* (impaired flagellar assembly system) mutants showed reduced capability of invasiveness and survival in epithelial cells at 2, 6 and 24 h after *Salmonella* exposure in comparison with the wt *S. Enteritidis*. Unlike the *S. Enteritidis ssaR* mutant strain, these two mutants also showed reduced intracellular survival (persistence) in established HD11 cells at 6 h post-infection, compared to the parent strain of *S. Enteritidis*, although the persistence was retrieved at 24 h post-infection. Whilst *S. Enteritidis ssaR*, but not *S. Enteritidis invA* and *S. Enteritidis fliJ*, showed the same ability to invade and survive in CKC as the wt *S. Enteritidis*, the mRNA gene expression levels for CXCLi1, CXCLi2 and IL-6 were greatly diminished in *S. Enteritidis ssaR*-infected CKC, compared to CKC infected with the wt *S. Enteritidis*. These findings are in accordance with previous results and showed the involvement of SPI-1 and SPI-2 in the process of cellular invasion and virulence of *S. Enteritidis* in chickens (Phe et al., 2009, Li et al., 2009, Rychlik et al., 2009). New evidence has shown that *iacP* gene (SPI-1) mediates *S. Typhimurium* virulence to human intestinal epithelial cells

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(*in vitro*) and in mouse (*in vivo*), through modulating *sopA*, *sopB* and *sopD* translocated effector proteins (Kim et al., 2011). Poor invasiveness has been correlated with infection of both human intestinal epithelial (Caco-2) cells and chicken caecal and small intestinal explants with SPI-1 mutants of *S. Enteritidis* (Desin et al., 2009). With delayed detection of SPI-1 mutants, compared to the wt, from the internal organs of *S. Enteritidis*-infected one-week-old chickens, data from this study has shown that SPI-1 is important in promoting systemic infection of chickens with *S. Enteritidis* (Desin et al., 2009). In the present study, the invasiveness of the SPI-2 mutant of *S. Enteritidis* (*ssaR*) to CKC was similar to the wt, although its survival in HD11 cells was not reduced compared to the wt. However, it has been shown that the SPI-2 (*ssrA*) mutant strain of *S. Enteritidis* shows full invasion capabilities to macrophages (HD11) and T84 human colon carcinoma cells but fails to persist in HD11, while oral infection of *ssrA* in one-day-old chicks induces lower bacterial counts in the liver and spleen while the intestinal colonization remains unaltered (Bohez et al., 2008). *S. Enteritidis ssrA* is also impaired for intracellular survival in murine and porcine macrophages (Boyen et al., 2008b).

Data from the present study showed that the number of wt *S. Enteritidis* recovered from both non-phagocytic cells and macrophages was higher than that of *S. Pullorum*- and *S. Gallinarum*-infected cells. The presence of flagella and flagellar proteins (flagellins), major bacterial virulence factors, in *S. Enteritidis* could present an advantage over *S. Pullorum* and *S. Gallinarum*, and could highlight their role in the cellular invasion and pathogenesis of *S. Enteritidis* in poultry. The invasiveness and virulence of *S. Pullorum* and *S.*



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Gallinarum and their SPI mutants have been studied both *in vitro* and *in vivo* (Jones et al., 2001, Wigley et al., 2002b). Both *S. Pullorum* and *S. Gallinarum* require SPI-2 for virulence in chickens. However, the contribution of SPI-1 toward the pathogenicity of both diseases, fowl typhoid and pullorum disease, is fundamentally different (Jones et al., 2001, Wigley et al., 2002b). It has been shown that the contribution of SPI-2 toward virulence and persistence of *S. Pullorum* in chickens is more than SPI-1, which has a little influence on pathogenicity (Wigley et al., 2002b). In the same study, a SPI-1 mutant of *S. Pullorum* (*spaS*) was found to be less invasive for CKC while the invasiveness of SPI-2 mutants (*ssaU*) was not affected. In the present study, however, all the strains of *S. Pullorum* tested (both wt and SPI mutants) showed reduced invasion capabilities to CKC with no significant difference detected between the different strains. The invasiveness of *S. Gallinarum* in the *in vitro* tissue culture models was found to be the same as that of *S. Pullorum*, with the SPI-2 mutant of *S. Gallinarum* 9 (*ssaU*) failing to persist in chicken macrophages (HD11) while its invasiveness in CKC remaining unchanged from the wild type *S. Gallinarum* 9 (Jones et al., 2001). While the SPI-1 mutant of *S. Pullorum* was capable of causing disease, the SPI-2 mutant virulence was abolished for day-old chicks and was recovered from the internal organs, liver and spleen, of orally infected one-week-old chickens (Wigley et al., 2002b). Unlike *S. Pullorum*, previous findings have shown that a SPI-1 (*spas*) mutation of *S. Gallinarum* has no effect on virulence to chickens, while a *ssaU* mutant of *S. Gallinarum* shows a degree of attenuation and could not be recovered from the liver and spleen of orally infected 3-week-old chickens (Jones et al., 2001). Thus, it has been suggested that SPI-2 could enhance the persistence of *S.*

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Gallinarum in macrophages and could be involved in the transfer of *Salmonella* from the gut to the internal organs (Jones et al., 2001).

The data presented here showed that all *Salmonella* strains tested in this experiment triggered the expression of pro-inflammatory immune mediators, including CXCLi1, CXCLi2, CCLi2, IL-6 and iNOS in HD11 cells, although variable levels of expression were detected between the different strains. On the other hand, the bacterial invasion and dynamic immune response of epithelial cells (CKC) in response to the infection were clearly different from that of phagocytic (HD11) cells, with failure of *S. Enteritidis fliJ* mutant strain, in addition to *S. Pullorum* and wt *S. Gallinarum*, to stimulate immune responses in infected CKC. The production of the inflammatory immune response following infection of chicken macrophages with *Salmonella* seems, to large extent, to be affected by the amount of bacterial components present inside the infected HD11 cells while the secretion of cytokines and chemokines by CKC cells appears to be dependent on the presence of bacterial virulence factors, TTSS-1, TTSS-2 and flagella. The production of pro-inflammatory cytokines by HD11 cells infected with *S. Pullorum* parent and mutant strains seemed unusual as we and others have shown that both systemic *Salmonellae* (*S. Pullorum* and *S. Gallinarum*) failed to elicit the secretion of inflammatory responses upon infection of CKC and chickens. We suggest that stimulation of HD11 cells is influenced by the presence of bacterial components, such as and mainly by LPS, rather than bacterial virulence determinants. This could also explain the up-regulation of TLR4 gene expression by *S. Enteritidis ssaR*-infected HD11 cells. Although the number of *S. Enteritidis ssaR* recovered

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from CKC was not significantly changed from the wt *S. Enteritidis*, gene expression data revealed a statistically significant reduction in the mRNA expression levels of CXCLi1 and CXCLi2 chemokines and IL-6 inflammatory cytokine by *S. Enteritidis ssaR*-infected CKC compared to the wt-infected cells. Our data have also shown that *S. Enteritidis ssaR* (TTSS-2) stimulated the expression of lower amounts of CXCLi1 and IL-6 from HD11 while both *S. Enteritidis invA* (TTSS-1) and *S. Enteritidis ssaR* (TTSS-2) induced a significant reduction in the CXCLi1 and CXCLi2 gene expression from infected CKC. Studies with primary chicken oviduct epithelial cells (COEC) have shown that both TTSS-1 and TTSS-2 are crucial for *S. Enteritidis* invasion and virulence (Phe et al., 2009, Li et al., 2009). While both TTSS-1 (*sipA* and *sipB*) and TTSS-2 (*pipB* and *ssaV*) contribute to the reduced expression of iNOS from *S. Enteritidis*-infected COEC, TTSS-1 (*sipA* and *sipB*) deletion mutants elicit the production of lower levels of pro-inflammatory chemokines CXCLi1 and CXCLi2, compared to the wt *S. Enteritidis*. It has been also shown that TTSS-2 deletion mutants (*pipB* and *ssaV*) of *S. Enteritidis* reveal a reduction in the persistence of primary chicken peripheral blood leukocyte-derived macrophages while the survival in HD11 cells remains unaffected. It has been shown that TTSS-1-secreted proteins (SipA, SopA, SopB, SopD and SopE2) are essential for induction of CXC chemokines and intestinal pathology, PMN influx and enteritis following infection of bovine ligated ileal loops with *S. Dublin* (Galyov et al., 1997, Jones et al., 1998, Wood et al., 2000) or *S. Typhimurium* (Zhang et al., 2003). It has been reported that the up-regulation in the expression of IL-10 regulatory cytokine by *S. Typhimurium*-infected murine macrophages (Raw264.7) is

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correlated with expression of *spiC* gene (SPI-2) (Uchiya et al., 2004). In the present experiment, the expression of IL-10 was up-regulated by *S. Enteritidis*-infected HD11 cells, compared to the non-infected control cells. We suggest that the IL-10 is produced by a negative feedback mechanism to reduce the effects of inflammation resulted from over secretion of pro-inflammatory chemokines and cytokines by infected HD11 cells.

Data from non-phagocytic (CKC) cell invasion with parent and mutant strains of *Salmonella* also showed not only (i) the involvement of flagella in virulence of *Salmonella* to chickens but also (ii) the ability of *S. Gallinarum* Flg<sup>+</sup> to invade and stimulate innate immune responses. One of the most striking results is the ability of *S. Gallinarum* Flg<sup>+</sup> to stimulate the production of significant levels of CXCLi2 and IL-6, although *S. Gallinarum* Flg<sup>+</sup>, and also *S. Enteritidis*, fail to stimulate a significant change in TLR5 gene expression. In particular, *S. Gallinarum* Flg<sup>+</sup> infection of CKC stimulates the mRNA gene expression of inflammatory cytokine IL-6 and a higher level of CXCLi2. In the present experiment, although the protein expression of these immune mediators were not examined, it is reasonable to postulate that the *in vivo* relevance of this finding could be largely attributed to the expression of these inflammatory mediators, particularly CXCLi2. CXCLi2 is a chemoattractant which plays a major role in recruiting inflammatory cells, particularly heterophils, to the site of infection (Kaiser and Staheli, 2008) and previous study has shown that expression of this chemokine was linked to migration of inflammatory cells and macrophages to the caeca of *S. Enteritidis*-infected chickens (Cheeseman et al., 2008). IL-6 is an inflammatory cytokine which is involved in acute-

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phase responses, immune regulation and activation of macrophages (Kaiser and Staheli, 2008), and is down-regulated *in vitro* in response to infection with *S. Gallinarum* (Kaiser et al., 2000). It has been found that bacterial flagellin, a major protein component of bacterial flagella, is responsible for TLR5-mediated innate immune responses (Hayashi et al., 2001). Also, the role of TLR5 in the immune responses to *S. Typhimurium* infection in chickens has been described and correlated with the presence of flagella (Iqbal et al., 2005b). Thus, the non-induction of TLR5 expression by wt strains of *S. Pullorum* and *S. Gallinarum* and *S. Enteritidis fliJ* seems to be more likely due to the absence of flagella. Nevertheless, in the present experiment, none of *S. Enteritidis* wt, *S. Enteritidis invA*, *S. Enteritidis ssaR* and *S. Gallinarum* Flg<sup>+</sup> strains was able to induce the stimulation of TLR5 gene expression, although differential expression levels for CXCLi1, CXCLi2 and IL-6 were detected. The down-regulation of TLR5 gene expression has been reported following infection of two-day-old chicks with *S. Enteritidis* (MacKinnon et al., 2009). In the present experiment, however, the non-stimulation of TLR5 expression by *S. Enteritidis*-infected cells seems to be as a result of a reduced capability of HD11 and CKC cells to produce significant changes following exposure to MOI of 10 of *Salmonella*, or alternatively, manipulation of host immunity to overcome the over-stimulation of cells in response to infection, a negative feedback mechanism.

In this experiment, HD11 cells and CKC were used to elucidate the immune responses of avian cells to *Salmonella* mutants. The host-pathogen interaction was studied *in vitro* to clarify the role of bacterial virulence factors in

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mediating bacterial invasion, survival and stimulation of immune responses. Mutant strains of *Salmonella* were selected to examine the role of pathogenicity islands (*invA*, *sipB*, *spaS*, *ssaR* and *ssaU*) and flagella (*fliJ* and  $\text{Flg}^+$ ) in *Salmonella* virulence and expression of immune mediators. Here we have used different phenotypes of each individual bacterial strain. We have shown that the single mutation of each *S. Enteritidis* or *S. Gallinarum* mutant strain is in place and phenotypes of *S. Pullorum* have been confirmed previously (Wigley et al., 2001). However, it could be worth to compare the presented findings with other strains of *Salmonella* to see how far these findings are comparable with data from the circulating strains of *S. enterica*.

In summary, this experiment provides more highlights to the interaction of different *Salmonella* serotypes, inducing different diseases in poultry, with epithelial cells and macrophages, with special focus on the bacterial virulence determinants. Here we have shown that both TTSS-1 and flagellar systems are required by *S. Enteritidis* for invasion of epithelial cells while both systems, in addition to TTSS-2, are essential for the whole virulence process and initiation of inflammatory response. This experiment also demonstrates that motile (flagellated) *S. Gallinarum*, which stimulates the initiation of innate immune response, could be a promising step toward the development of a novel vaccine for *S. Enteritidis* in poultry.

#### 4.4 Acknowledgment

Many thanks should go to Oliveira O. Neto for taking part in this experiment.

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## **5 Gene expression profile and histological changes in chickens infected with *Salmonella enterica* serovars**

### **5.1 Introduction**

Changes in cytokine and chemokine gene expression following *Salmonella* infection have been studied *in vitro* following infection of epithelial cells and monocytes (Kaiser et al., 2000, Kaiser et al., 2006, Li et al., 2009) and in the avian host (Withanage et al., 2004, Withanage et al., 2005b, Berndt et al., 2007, Carvajal et al., 2008). However, the role of the caecal tonsils in the development of local and systemic immune responses to *Salmonella* is still not well-characterised. This organ is important as it seems likely that it has a major controlling influence on entry of bacterial and other pathogens into the caeca.

As chicks proceed to feed the adult diet immediately after hatch, rapid colonization of the intestine by adult-type microflora is a natural consequence (Coloe et al., 1984, Mead, 2000, Hume et al., 2003), with the possibility of infection with pathogenic bacteria. As mentioned earlier, the major site for intestinal colonization with enteric bacteria is the large intestine, particularly the two caeca. However, the development of the functional immune responses against invading bacteria during the first few days after hatching is not clear. There is evidence that intestinal innate immune responses are important in protecting chicks during the first few days after hatching (Bar-Shira and Friedman, 2006). Indeed, innate responses also differentiate between different classes of antigens and direct the adaptive responses against infection (Swaggerty et al., 2009). The role of GALT is crucial in providing the protection and initiation of immune responses against enteric pathogens

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encountered by the gut (Lillehoj and Trout, 1996). However, the role of the caecal tonsil, a major GALT in the chicken's hindgut, in the immune responses and clearance of enteric pathogens, including *Salmonella*, is not clear and data available on gene expression in the caecal tonsil in response to *Salmonella* infection in newly hatched chicks are limited (Withanage et al., 2004).

The development of more rational approaches to vaccination will require a better understanding of the GALT and their responses to diseases. Therefore, in this study we investigated the early changes in cellular composition and cytokine and chemokine expression in the caecal tonsil of newly hatched and older chickens following infection with *Salmonella* serovars known to have different biological and pathological characteristics to determine how far the immune response to these pathogens is associated with the differences in the infection biology.



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## 5.2 Results

### 5.2.1 Caecal colonization following *Salmonella* infection

The number of *Salmonella* strains was determined in the caeca of orally-infected day-old and three-week-old chickens. The tested *Salmonella* serotypes revealed differences in their ability to colonize the caeca of infected birds (Table 5.1). All the tested salmonellae were detected in the caeca one day after infection of one-day-old chicks. However, both of *S. Enteritidis* and *S. Infantis* showed a higher capability of caecal colonization, with 9.49 and 9.29 Log<sub>10</sub> counts, respectively, in comparison to *S. Pullorum* and *S. Gallinarum* (8.06 and 7.71 Log<sub>10</sub> counts, respectively) ( $P < 0.05$ ). In older chickens, both *S. Enteritidis* and *S. Infantis* were also able to colonize the caeca more efficiently with 7.3 and 7.6 Log<sub>10</sub> counts respectively when compared with *S. Pullorum* and *S. Gallinarum* which showed significantly lower counts despite the use of vancomycin (3.71 and 4.30 Log<sub>10</sub> counts, respectively) ( $P < 0.001$ ). There was no significant difference in the ability of either *S. Enteritidis* or *S. Infantis* to colonize the caeca and also between *S. Pullorum* and *S. Gallinarum* ( $P > 0.05$ ).

**Table 5.1:** Number of *Salmonella* in the caecal contents of infected newly hatched and older chickens.

<i>Salmonella</i> serotype	Bacterial count (Log <sub>10</sub> CFU/g) <sup>†</sup>	
	Infected day-old chicks	Infected three-week-old chickens
<i>S. Enteritidis</i>	9.49 ± 0.25*	7.29 ± 0.17***
<i>S. Infantis</i>	9.25 ± 0.15*	7.60 ± 0.17***
<i>S. Pullorum</i>	8.06 ± 0.12	3.71 ± 0.36
<i>S. Gallinarum</i>	7.71 ± 0.33	4.30 ± 0.29

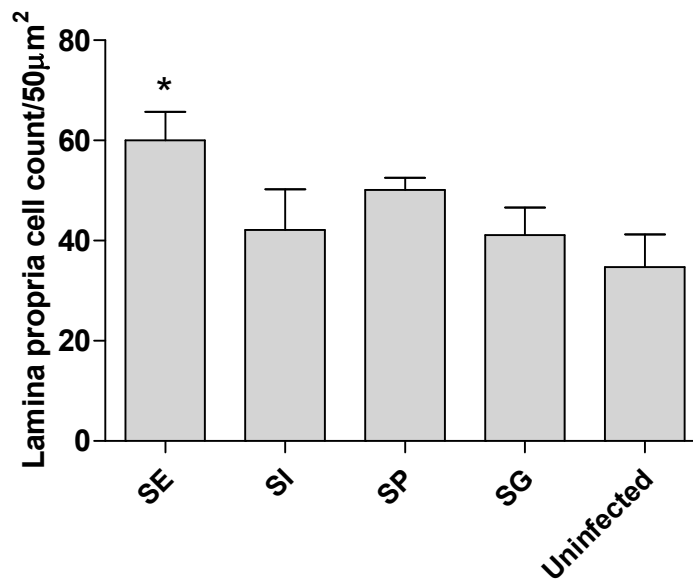
<sup>†</sup> Where mean values for the *S. Enteritidis*- and *S. Infantis*-infected birds differ significantly from *S. Pullorum*- and *S. Gallinarum*-infected birds. \*P<0.05 (n=5), \*\*\*P<0.001 (n=10).

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**5.2.2 Clinical signs, gross lesions and histopathological changes after*****Salmonella* infection**

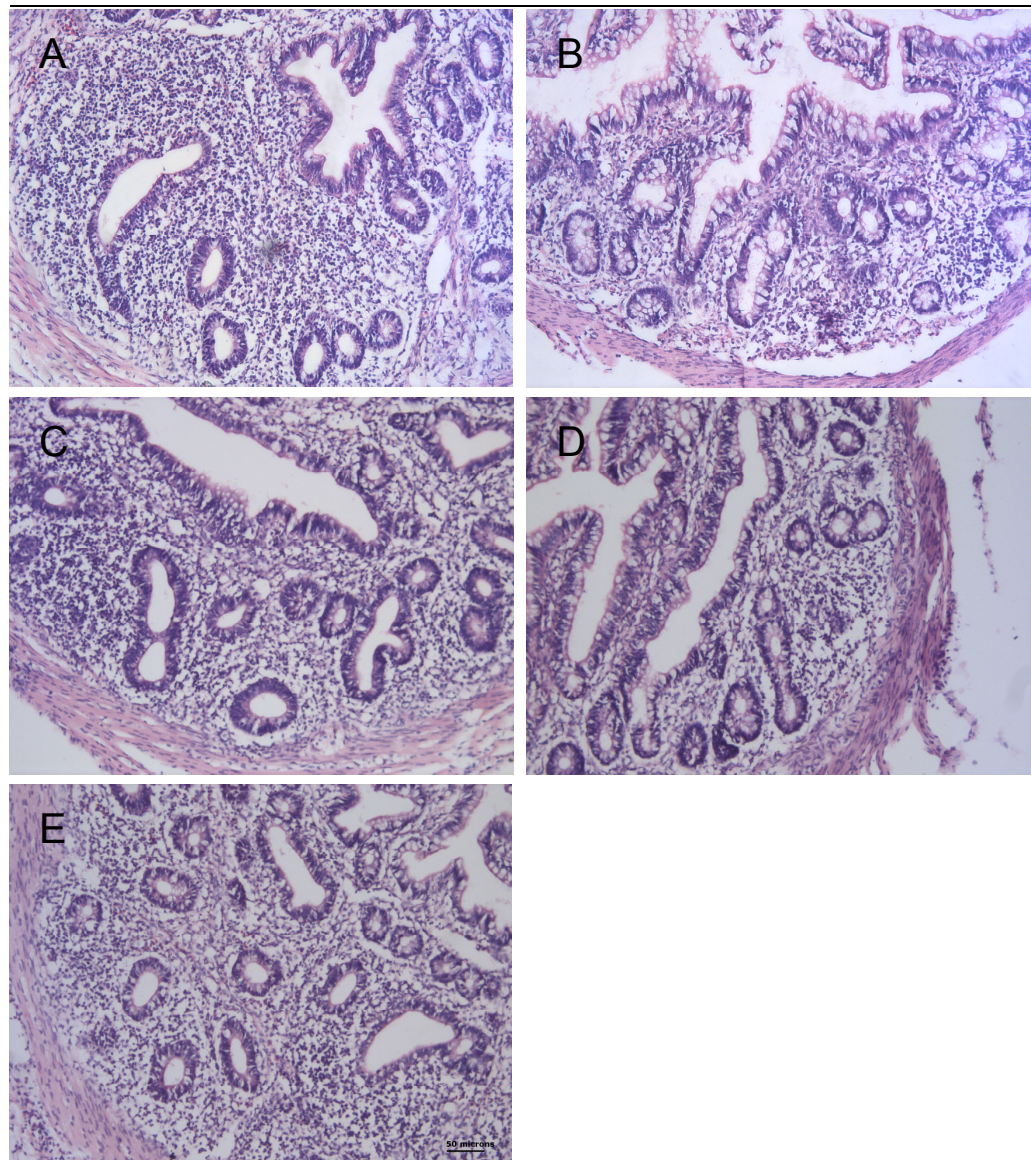
Oral infection of one-day-old chicks with *ca*  $1 \times 10^8$  CFU of *Salmonella* did not induce any apparent clinical manifestations or post-mortem lesions on the day of sampling. Microscopic examination for the identification of caecal tonsils from newly hatched chicks revealed a thickening of the sub-epithelial layer which was covered with short mucosal intestinal villi. Variable degrees of cellular (lymphocytic) infiltration were seen in the lamina propria of both infected and control birds. Caecal tonsils from *S. Enteritidis*-infected chicks showed an increase in the cellular infiltration in the lamina propria with the presence of small cellular aggregations in response to infection compared to the non-infected control birds (Figure 5.1&5.2).

Oral infection of chicks with approximately  $3 \times 10^8$  CFU of *S. Enteritidis*, *S. Pullorum* or *S. Infantis* bird did not induce any clinical signs of illness over the four days of infection. However, *S. Gallinarum*-infected birds showed mild enlargement of the spleen and presence of haemorrhage (in two birds) on the mucosal surface of the caecal tonsils at post-mortem examination. Microscopically, the presence of thickened lamina propria with massive infiltration of immune cells in the submucosal layer is a well-characteristic microscopic presentation for the caecal tonsil (del Cacho et al., 1993). In this experiment, no marked microscopic changes were noticed in either the infected or non infected birds on microscopical examination of the caecal tonsils (Figure 5.3).



**Figure 5.1:** Cellular counts in the sub-epithelial layer of the caecal tonsil one day after infection with *Salmonella* serotypes.

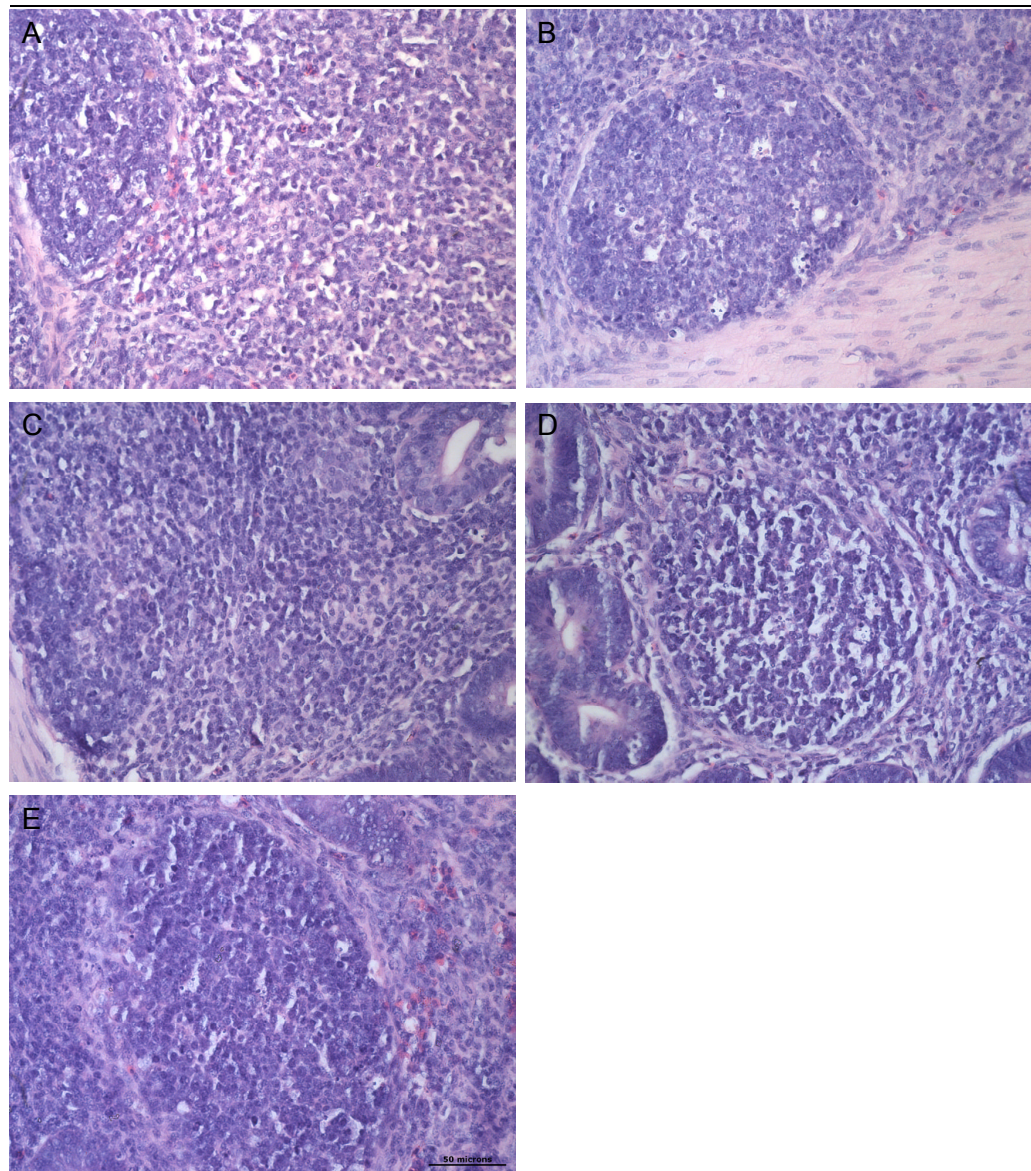
Cellular counts in the lamina propria of caecal tonsils were determined in 50 µm<sup>2</sup> using Image-Pro Plus software. Asterisks indicate a significant difference between the infected and uninfected control groups ( $P < 0.05$ ). SE; *S. Enteritidis*, SI; *S. Infantis*, SP; *S. Pullorum*, SG; *S. Gallinarum* and uninfected control birds.



**Figure 5.2:** Representative histopathological micrographs from the caecal tonsils of newly hatched chicks one day after infection with different *Salmonella* serotypes.

A; *S. Enteritidis*, B; *S. Infantis*, C; *S. Pullorum*, D; *S. Gallinarum* and E; uninfected control birds. H&E staining, magnification: X20, scale bar: 50 $\mu$ m.





**Figure 5.3:** Representative histopathological micrographs from the caecal tonsils of chickens four days after infection with different *Salmonella* serotypes.

A; *S. Enteritidis*, B; *S. Infantis*, C; *S. Pullorum*, D; *S. Gallinarum* and E; uninfected control birds. H&E staining, magnification: X40, scale bar: 50 $\mu$ m.

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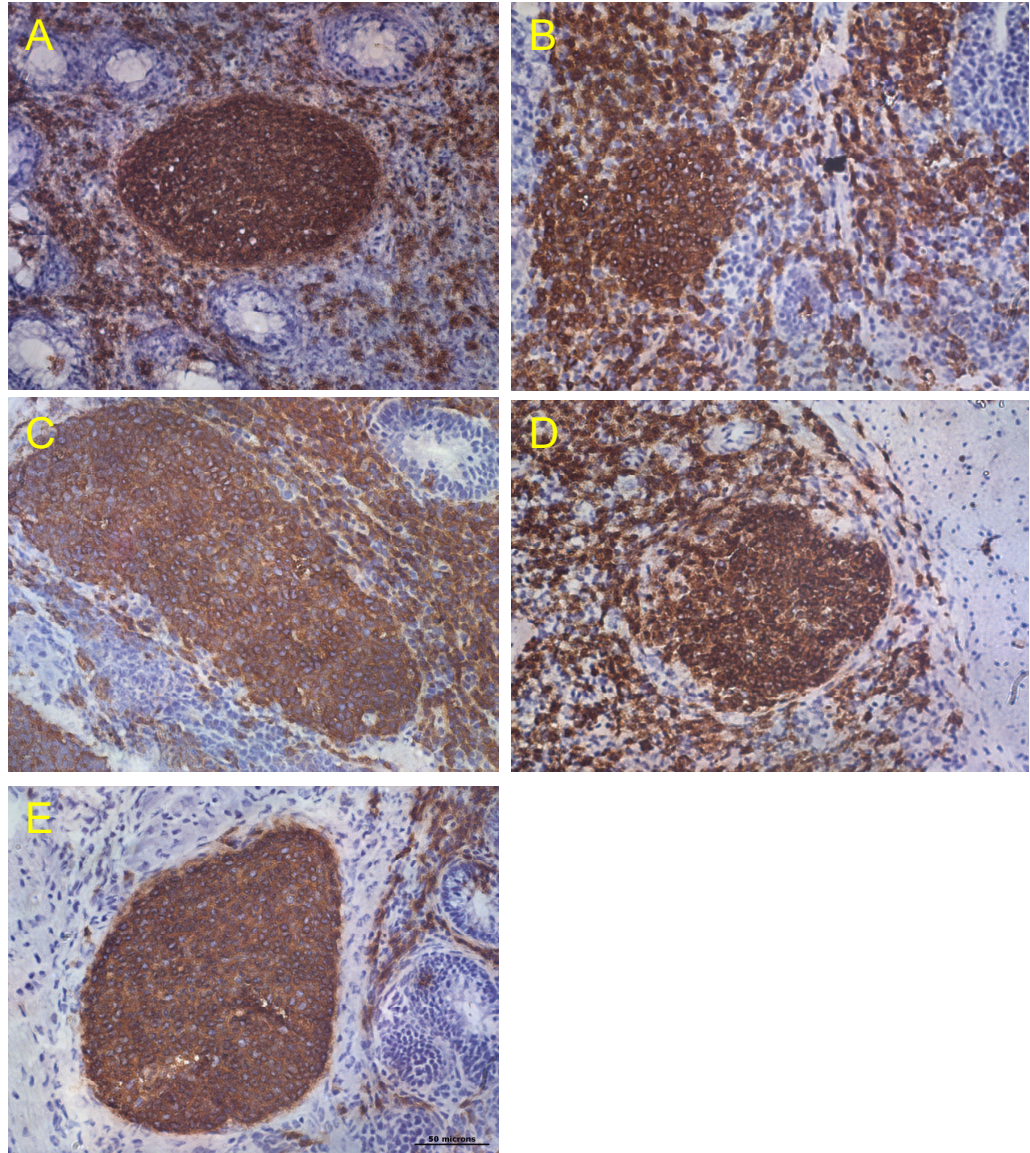
### 5.2.3 Immunohistochemical analysis

To quantify changes in the cellular composition following *Salmonella* infections, the occurrence of B-lymphocytes and macrophages was measured in the caecal tonsil tissues using immunohistochemistry (Figure 5.4 & 5.5). After infection, there was a significant increase in the percentage of B-cells and macrophages in the caecal tonsils when compared to non-infected control birds ( $P < 0.05$ ). Compared to non-infected control birds, *S. Pullorum*-infected birds showed the highest significant increase in the percentage of B-lymphocytes ( $P < 0.001$ ) followed by *S. Gallinarum* ( $P < 0.01$ ), *S. Enteritidis* and *S. Infantis* ( $P < 0.05$ ). The percentage of macrophages in the caecal tonsils of infected birds was significantly higher than that of the uninfected controls ( $P < 0.01$  for *S. Enteritidis* and  $P < 0.05$  for *Salmonella* serotypes *Infantis*, *Pullorum* and *Gallinarum* (Figure 5.6).

### 5.2.4 Flow cytometry

Cell viability determined using trypan blue assay was extremely diminished in most of the samples. We proposed that treatment of the caecal tonsils with Griffith's tubes had destroyed the cell populations as a resulting of excessive homogenization. Thus, the determination of T-lymphocyte cell populations was not possible in the caecal tonsils using flow cytometry.

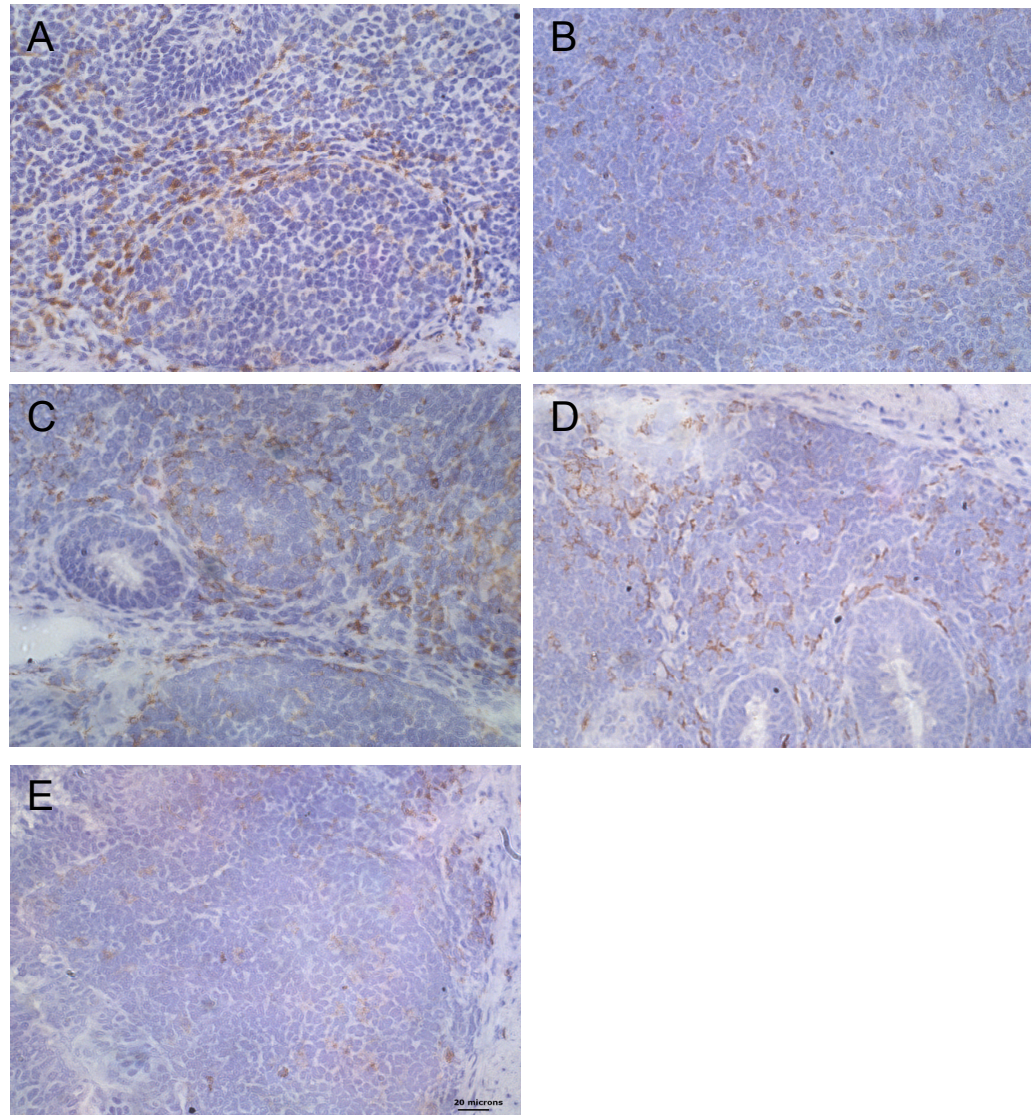




**Figure 5.4:** Representative immunohistochemistry micrographs of the caecal tonsils for the presence of B-lymphocytes four days post-infection of three-week-old chickens with *S. enterica* (A-E).

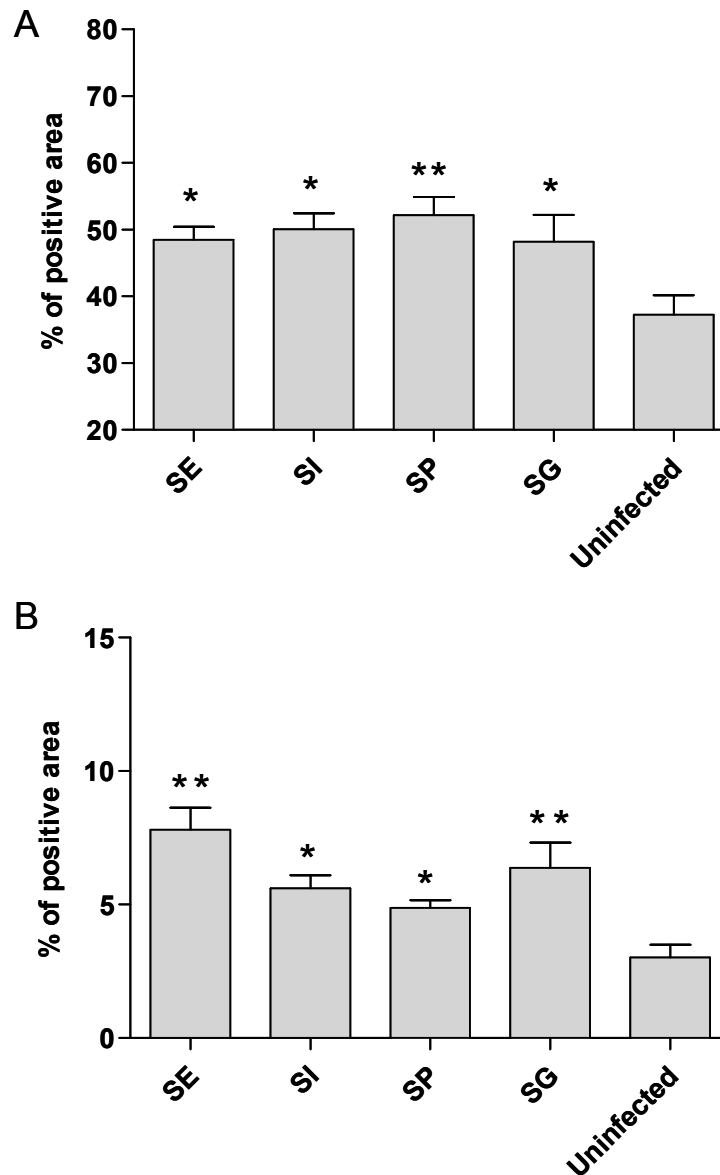
A, *S. Enteritidis*; B, *S. Infantis*; C, *S. Pullorum*; D, *S. Gallinarum*; E, uninfected control birds. Anti-AV20 immunostaining (brown colour), counterstaining with Haematoxylin (blue colour), magnification, X40, scale bar, 50 $\mu$ m.





**Figure 5.5:** Representative immunohistochemistry micrographs of the caecal tonsils for the presence of macrophages four days post-infection of three-week-old chickens with *S. enterica* (A-E).

A, *S. Enteritidis*; B, *S. Infantis*; C, *S. Pullorum*; D, *S. Gallinarum*; E, uninfected control birds. Anti-KUL01 immunostaining (brown colour), counterstaining with Haematoxylin (blue colour), magnification, X40, scale bar, 20µm.



**Figure 5.6:** Occurrence of B-lymphocytes (A) and macrophages (B) in the caecal tonsils four days after infection of three-week-old chickens with *S. enterica*.

Error bars present the standard deviations and errors for five samples from five birds. Anti-AV20 (B-cells) and anti-KUL01 (macrophages) immunostaining. SE, *S. Enteritidis*; SI, *S. Infantis*; SP, *S. Pullorum*; SG, *S. Gallinarum* and non-infected controls. \* $P < 0.05$ , \*\* $P < 0.01$ .

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### **5.2.5 Gene expression profile of the caecal tonsils of newly hatched chicks in response to *Salmonella* infection**

The mRNA gene expression of selected cytokines and chemokines was measured using qRT-PCR. *Salmonella* infection of newly hatched day-old chicks differentially modulates the gene expression of selective immune mediators examined in this study. There was a significant up-regulation in the gene expression levels of CXCLi1 (< 3.13 fold change,  $P < 0.05$ ) and CXCLi2 (< 4.79 fold change,  $P < 0.01$ ) chemokines in the caecal tonsils of *S. Enteritidis*-infected birds when compared to uninfected controls (Figure 5.7). Further, CXCLi1 expressed in response to *S. Enteritidis* infection was greater than that induced in response to *S. Pullorum* infection ( $P < 0.05$ ), while CXCLi2 expressed as a result of *S. Enteritidis* infection was higher than that produced in response to infection with any of the other tested serovars ( $P < 0.01$ ). Further, *Salmonella* infection induced the expression of LITAF from the caecal tonsils of infected chicks, with *S. Gallinarum* stimulated the expression of higher levels (< 3.00 fold change) of this particular molecule ( $P < 0.01$ ). No significant changes were detected in the expression levels of IL-6, iNOS, IFN- $\gamma$  and IL-4 genes in response to infection with any of *Salmonella* serotypes tested in this study ( $P > 0.05$ ), while IL-10 and IL-13 were not expressed.

### **5.2.6 Gene expression of the caecal tonsils and spleen of older chickens in response to *Salmonella* infection**

The expression of cytokines and chemokines in the caecal tonsils and spleen of *Salmonella*-infected three-week-old chickens at four days after infection was also determined using qRT-PCR. In the caecal tonsils, the presence of mild but significant up-regulation in the gene expression of CXCLi2 (< 2.9 fold

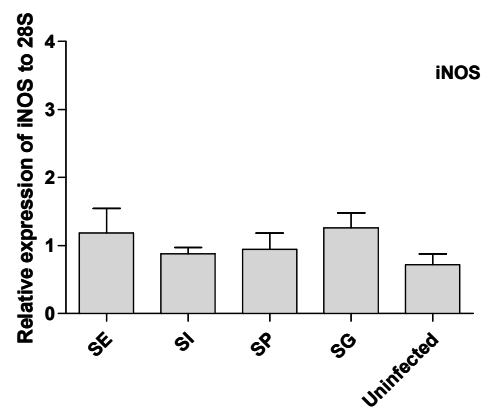
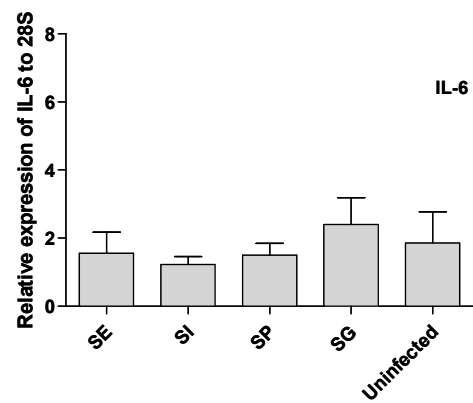
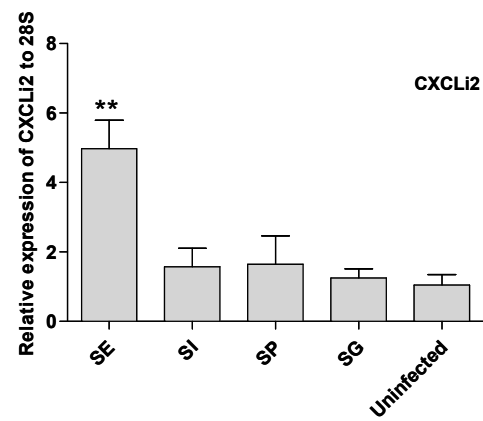
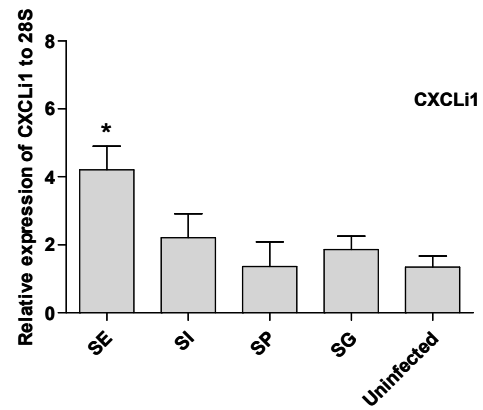
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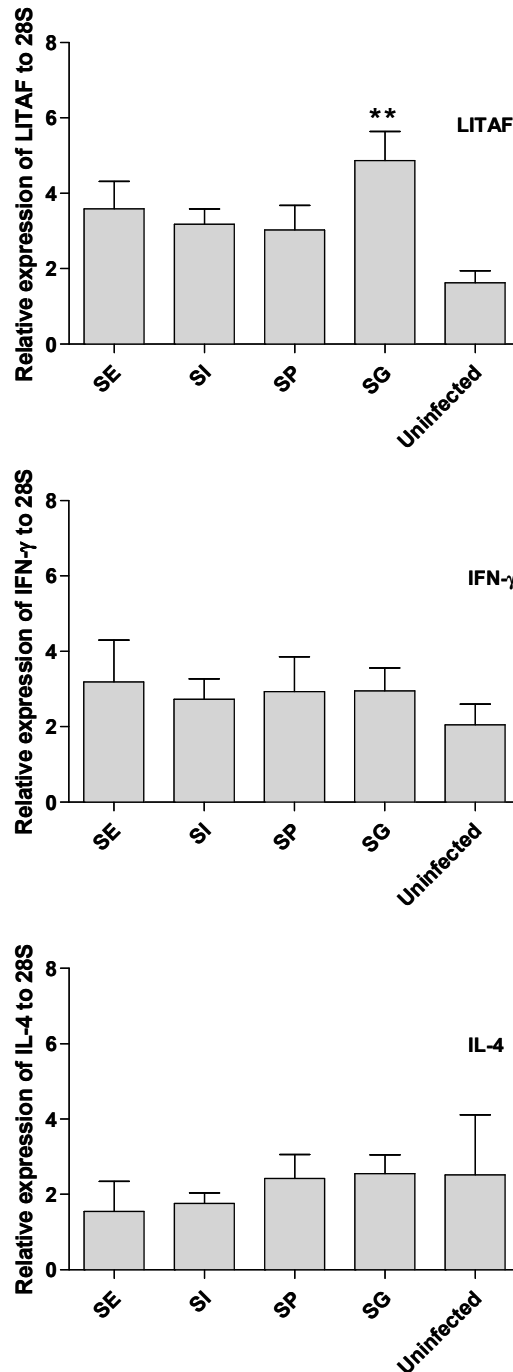
change), iNOS (< 3.1 fold change), LITAF (< 1.49 fold change) and IL-10 (< 2.25 fold increase) was correlated with *S. Enteritidis* infection when compared to non-infected controls ( $P < 0.05$ ). In contrast, *S. Pullorum* down-regulated CXCLi1 mRNA expression (<0.5 fold change) when compared with non infected controls ( $P < 0.05$ ) (Figure 5.8 and 5.9). Further, the expression of CXCLi2 and LITAF by *S. Enteritidis*-infected birds was significantly higher than that of *S. Pullorum*- and *S. Gallinarum*-infected birds ( $P < 0.05$ ). However, the cytokine and chemokine gene expression was not significantly changed in response to *S. Infantis* or *S. Gallinarum* infections in comparison with the uninfected controls ( $P > 0.05$ ). No significant changes have been detected in the gene expression levels of IFN- $\gamma$ , IL-4, IL-13 from any of the *Salmonella* infected groups when compared with non-infected birds ( $P > 0.05$ ). In spleen, CXCLi2 expression was not changed in response to infection with any of the tested serotypes ( $P > 0.05$ ), while the expression of iNOS, IL-6, LITAF, IFN- $\gamma$  and IL-4 genes was down-regulated at four days following infection with all *Salmonella* serotypes in comparison with non infected controls ( $P < 0.05$ ) (Figure 5.10). CXCLi1, IL-10 and IL-13 were not expressed by the splenic tissue at four days post-infection.

Results from the present study demonstrate the changes in the cellular composition and in the cytokine and chemokine expression in the caecal tonsils in response to infection of three-week-old chicks with *Salmonella* serovars, Enteritidis, Infantis, Pullorum and Gallinarum. *S. Enteritidis* is a strongly inflammatory serotype inducing the production of iNOS and CXCLi2 chemokine when compared to the other serotypes tested in this study. Whilst *S.*

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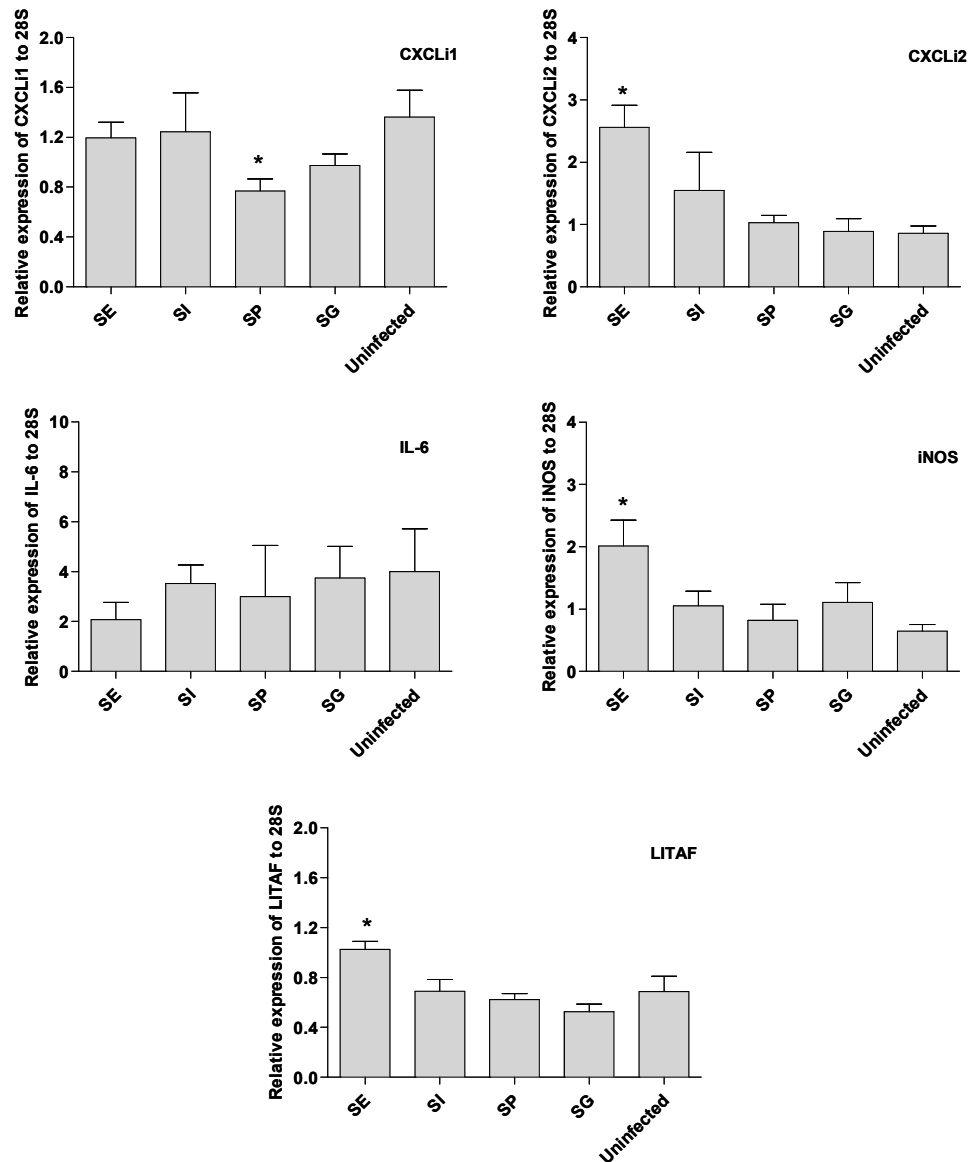
Infantis showed a high level of caecal colonisation it did not stimulate the production of significant changes in the level of CXCLi2 and iNOS compared to *S. Enteritidis* suggesting that the bacterial load in the caeca is not directly related to the induction of cytokines. The presented data also shows that *S. Pullorum*, unlike *S. Enteritidis*, is a less-inflammatory serotype which fails to stimulate an inflammatory response in the intestine where infection usually progresses to systemic spread into the internal organs. Although *S. Gallinarum* infection hardly stimulates inflammatory responses in this study, our data has shown that LITAF is up-regulated in the caecal tonsils during the early stages of *Salmonella* infection, particularly the systemic serovar, *S. Gallinarum*.





**Figure 5.7:** The mRNA gene expression from caecal tonsils one day following infection of one-day-old chicks with different serotypes of *Salmonella* serovars.

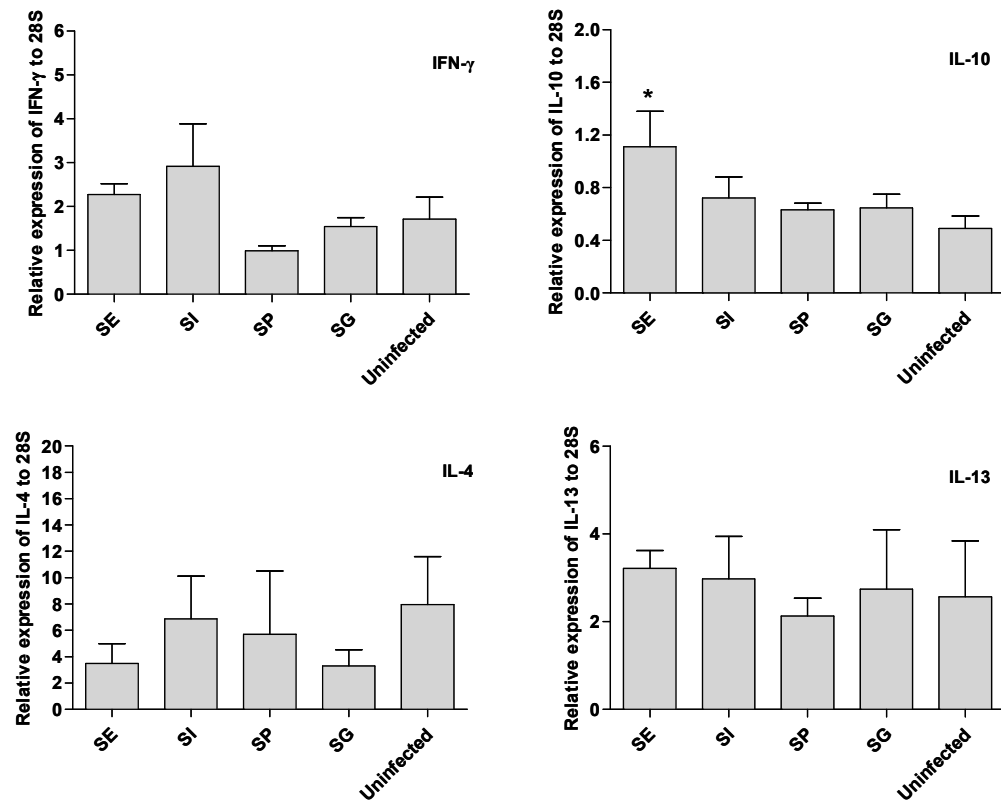
Data are presented as changes in the cytokine or chemokine gene expression levels for *Salmonella*-infected birds and uninfected controls. The error bars present the standard error for five samples from five birds. SE, *S. Enteritidis*; SI, *S. Infantis*; SP, *S. Pullorum* and SG, *S. Gallinarum*. Asterisks indicate significance from uninfected control birds (\* $P < 0.05$ , \*\* $P < 0.01$ ).



**Figure 5.8:** Gene expression of pro-inflammatory chemokines, IL-6, iNOS and LITAF from the caecal tonsils four days after infection of three-week-old chickens with *S. enterica*.

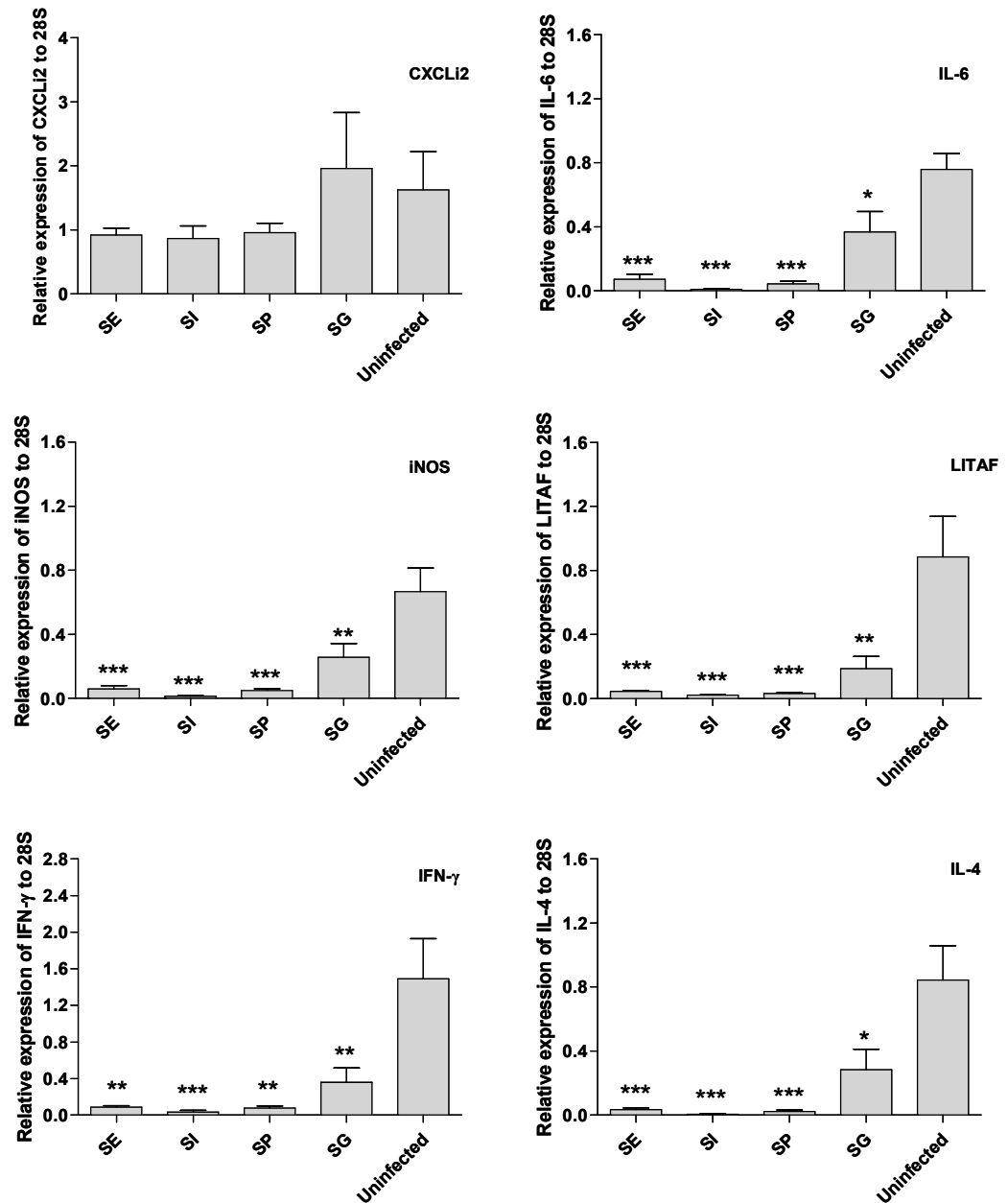
Data are presented as changes in the cytokine or chemokine mRNA expression levels for *Salmonella*-infected birds and uninfected controls. The error bars present the standard error for five samples from five birds. SE, *S. Enteritidis*; SI, *S. Infantis*; SP, *S. Pullorum* and SG, *S. Gallinarum*. Asterisks indicate significance from uninfected control birds (\*P<0.05).





**Figure 5.9:** Gene expression of Th1/Th2 cytokines from the caecal tonsils four days after infection of three-week-old chickens with *S. enterica*.

Data are presented as changes in the cytokine or chemokine mRNA expression levels for *Salmonella*-infected birds and uninfected controls. The error bars present the standard error for five samples from five birds. SE, *S. Enteritidis*; SI, *S. Infantis*; SP, *S. Pullorum* and SG, *S. Gallinarum*. Asterisks indicate significance from uninfected control birds (\*P<0.05).



**Figure 5.10:** Gene expression of cytokines and chemokines, iNOS and LITAF from spleens four days after infection of three-week-old chickens with *S. enterica*.

Data are presented as changes in the cytokine or chemokine mRNA expression levels for *Salmonella*-infected birds and uninfected controls. The error bars present the standard error for five samples from five birds. SE, *S. Enteritidis*; SI, *S. Infantis*; SP, *S. Pullorum* and SG, *S. Gallinarum*. Asterisks indicate significance from uninfected control birds (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ).

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### 5.3 Discussion

An understanding of the immunological mechanisms undertaken by the GALT will provide valuable insights into host and pathogen interaction at the cellular level and will help to improve the current scientific knowledge from the perspective of vaccinology. In this study, four different *Salmonella* serovars, known to produce different pathological conditions in chickens, were compared in terms of their ability to colonize chicken gut and to elicit the immune responses in the caecal tonsils, a major GALT in the avian hind gut.

The caeca are the main site of intestinal colonisation where the highest bacterial counts are attained. It also seems likely that the caecal tonsil in the mature bird has some element of physical control of entry of material and perhaps immunological control of entry of bacterial and other pathogens. Data from the present experiment has shown that both *S. Enteritidis* and *S. Infantis* were able to colonize avian gut much better than *S. Pullorum* and *S. Gallinarum*, where lower bacterial counts were detected in the caeca of both newly hatched and older chickens. Based on previous findings by (Barrow, 1989) and (Stern, 2008), administration of vancomycin was used to help with increasing colonisation by those serovars by reducing the inhibitory effects of the gut flora. However, administration of vancomycin one day prior to infection of older chickens with *Salmonella* seems not to enhance caecal colonization with systemic serovars as the caecal counts following *S. Pullorum* and *S. Gallinarum* remained relatively low in comparison with *S. Enteritidis* and *S. Infantis*. The removal of bacteria which are inhibitory to the colonisation of serovars such as *S. Typhimurium* clearly does not affect *S. Gallinarum*

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indicating that the latter's inability to colonise the gut is not related to the inhibitory effects of these gut commensals. It has been shown *in vitro* using epithelial cells that *S. Gallinarum* induced a significant changes in the cytokine expression, including down-regulation of IL-1 $\beta$ , whilst its invasiveness was similar to that of *S. Enteritidis* (Kaiser et al., 2000). In the current study, we reported cellular changes in the caecal tonsils following infection with both *S. Pullorum* and *S. Gallinarum*, including significant increase in the populations of B-lymphocytes and macrophages. Moreover, although infection with the latter serovars revealed lower bacterial counts in the caeca, it induced changes in the cytokine and chemokine expression in the gut (down regulation of CXCLi1 in response to *S. Pullorum* infection) and in the spleen, including down regulation of iNOS, IL-6, LITAF, IFN- $\gamma$  and IL-4 mRNA. These findings indicate that bacterial numbers in the gut was obviously not a major factor in determining the host response against infection.

The data presented here has shown that *S. Infantis* fails to stimulate the caecal tonsils to produce significant changes in any of the cytokines and chemokines tested in this study. In the present experiment, however, *S. Enteritidis* infection stimulated the expression of both CXCLi1 and CXCLi2 chemokines from the caecal tonsils of infected day-old chicks. Further, our data have indicated that *S. Enteritidis* infection stimulated CXCLi2 and iNOS production while *S. Pullorum* down-regulated the expression of CXCLi1 in the caecal tonsils of three-week-old chickens. These findings are in accordance with those of (Kaiser et al., 2000, Chappell et al., 2009) and illustrate that the nature of the innate immune response is differentially dependent of the

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infecting *Salmonella* serotype (or pathotype). Both CXCLi1 and CXCLi2 are inducible inflammatory chemokines which regulate the process of recruitment of inflammatory cells to the site of infection. It is suggested that CXCLi1 is more efficient in recruiting heterophils while CXCLi2 induces migration of both monocytes and heterophils, suggesting that the latter might have an immunoserveillance function (Kaiser and Staheli, 2008). The expression of CXCLi1 and CXCLi2 was found to be up-regulated in the chicken gut following infection of chicks with *S. Typhimurium* (Withanage et al., 2004) and *S. Enteritidis* (Cheeseman et al., 2008). In the present study, the differential expression of chemokines and cellular changes in the caecal tonsils following infection with both *S. Enteritidis* and *S. Pullorum* clearly illustrate differences in pathogenesis between these two related serotypes.

The presence of nodular and diffuse lymphocytic aggregations in the lamina propria and submucosa near the opening of each caecum with the large intestine is a well characterized microscopic image for the caecal tonsil. Tissues of the caecal tonsils are dense and impacted with lymphocytes and other immune cells. It has been reported that infection of chickens with *Salmonella* induces infiltration of immune cells, such as B-lymphocyte, and macrophages, into the caeca (Berndt and Methner, 2004). Changes in cellular composition in the intestinal tissues following *Salmonella* infection have been reported, including the caeca (Van Immerseel et al., 2002a), and caecal tonsils of laying chickens (Holt et al., 2010). In the present study, although both *S. Enteritidis* and *S. Infantis* colonized the caeca in a similar way, caecal tonsils from *S. Enteritidis*-infected birds showed an increase in the cellular matrix of

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the sub-mucosal layer when compared to the non infected control birds. This finding could be as a result of recruitment of immune cells, such as macrophages and T-cells, to the lamina propria of the caecal tonsils. It has been shown that macrophages and T-cells are recruited to the caeca of orally-infected-two-day-old chicks as early as 24 h following infection with *S. Enteritidis* (Van Immerseel et al., 2002a). It has been also reported that changes in the cellular populations of caecal mucosa, including heterophils and T-lymphocytes, were correlated with the invasion capabilities of *Salmonella* serotype being infected (Berndt et al., 2007). The absence of significant changes in the gene expression of immune mediators and cellular changes following infection of newly hatched chicks with *S. Infantis* is very likely to be as a result of weak invasion capabilities of *S. Infantis* in chickens.

Cytokine and chemokine expression of chicken caeca has been also reported following *S. Typhimurium* infection (Fasina et al., 2008) and *S. Enteritidis* infection (Cheeseman et al., 2008). Most of avian cytokines have been cloned in chickens (Kaiser et al., 2000) while tumor necrosis factor- $\alpha$  (TNF) has not been identified in avian species. However, LITAF, which may stimulate the expression of TNFSF15 (a member of TNF super-family), has been identified (Hong et al., 2006b). The data presented here showed that the innate immune system can recognize different classes of *Salmonella* via production of serotype-specific cytokines and chemokines and through cellular changes after infection. An increase in the gene expression level of CXCLi2 cytokine and iNOS and LITAF in response to *S. Enteritidis* infection was correlated with an increase in macrophage positive staining in the caecal tonsils four days after

infection. It is suggested that LITAF plays an important role in driving inflammatory responses and bacterial clearance. Although little is known about the biological function of LITAF in poultry, it has been reported that the expression of this co-stimulatory molecule was up-regulated in *S. Typhimurium* LPS-stimulated macrophages and in response to treatment with *Eimeria* species, the causative agent of chicken coccidiosis (Hong et al., 2006b). Moreover, the expression of higher levels of LITAF in the caeca of newly hatched chicks has been reported in response to infection with *S. Enteritidis*, suggesting a role in the inflammatory responses and clearance of *Salmonella* (Berndt et al., 2007). Data from the present experiment has shown up-regulation of LITAF in the caecal tonsils following infection of newly hatched chicks with *Salmonella*, particularly with *S. Gallinarum*, pointing out the importance of this lymphoid tissue in the pathogenesis and immune response surrounding *Salmonella*-associated infections in poultry. The production of iNOS has been correlated with the host resistance of mice against *S. Typhimurium* infection (Rosenberger and Finlay, 2002) and with the caecal responses to infection with *S. Typhimurium*, *S. Enteritidis* and *S. Hadar* in newly hatched chicks (Berndt et al., 2007). In the current experiment, the expression of iNOS by the caecal tonsils in response to *S. Enteritidis* infection indicates the central role of macrophages in clearance of this pathogen.

In order to further investigate the avian immune responses developed in response to *Salmonella* infections, the expression of several genes involved in acquired immunity was measured. Although no significant changes were detected in the gene expression levels of T-cell mediated cytokines IFN- $\gamma$ , IL-4

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and IL-13 in the caecal tonsils following *Salmonella* infection, the mRNA gene expression level of IL-10 was up-regulated in the caecal tonsils of *S. Enteritidis*-infected three-week-old chickens. However, the down-regulation of IL-10 has been reported 10 days following infection of commercial newly hatched chicks with *S. Typhimurium* (Fasina et al., 2008). In another study, no significant changes were observed in the gene expression of IL-10 in the caecal tonsils of newly hatched chicks infected with *S. Typhimurium* following treatment with probiotics (Haghighi et al., 2008). It is believed that IL-10 is an anti-inflammatory mediator and produced to down-regulate the effects of IFN- $\gamma$  and to prevent over-expression of host defences against infection (Kaiser and Staheli, 2008). In the present study, however, the level of IFN- $\gamma$  expression was measured at four days after infection, and did not differ significantly between the infected and non-infected birds, which could be influenced by the time points tested in this experiment. Nevertheless, the role of the Th1 key cytokine IFN- $\gamma$  in *Salmonella* infections of chickens has been studied (Withanage et al., 2005b, Berndt et al., 2007). This Th1 key cytokine IFN- $\gamma$  is up-regulated in the caeca of newly hatched chick SPF chicks infected with *S. Typhimurium* and *S. Enteritidis* (Berndt et al., 2007). It has been shown that IFN- $\gamma$  expression in the caecal tonsils starts as early as 3 days following infection of one-week-old SPF chickens (Withanage et al., 2005b). In the present experiment, the absence of significant changes in IFN- $\gamma$  expression could be due to differences in age and breed of infected chickens as well as the infecting serotypes and samples examined. Till now, no data has been available on the expression of IL-13 (a Th2 cytokine) in chickens in response to *Salmonella* infection. However, previous work has shown that IL-13 is expressed in the liver of turkeys

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following the exposure to the protozoan *Histomonas meleagridis*, the causative agent of blackhead disease (Powell et al., 2009). The absence of statistically significant changes in the gene expression of IFN- $\gamma$ , IL-4 and IL-13 could be a result of the early sampling time conducted in this experiment (four days post-infection). The immune regulatory function of IL-4 in poultry is unclear. However, previous finding by (Chappell et al., 2009) demonstrated that *S. Pullorum* can modulate immune responses away from Th1 response where data from spleen showed up-regulation of IL-4 at 14 days after infection. The up-regulation of IL-4 has been reported after infection with Marek's disease virus and *Eimeria* protozoan (Hong et al., 2006a, Heidari et al., 2008). In the present study, however, data from spleen revealed down regulation in the gene expression of most of tested cytokines, including IL-4. These results are in the line with what have been reported by (Withanage et al., 2004, Withanage et al., 2005b) in response to *S. Typhimurium* infection. These findings in the splenic tissue can be interpreted in the light of the observations by (Beal et al., 2004a) following *S. Typhimurium* infection when proliferation of T-lymphocytes was diminished while recruiting T cells from the spleen to the intestine. This paradoxical phenomenon in *Salmonella* immunity has been also observed after *Salmonella* infection in mice (Eisenstein, 2001). Moreover, new evidence suggested that SPI-1-mediated TTSS has an immunosuppressive function through the interference with *S. Typhimurium* and *S. Enteritidis* recognition by infected porcine alveolar macrophages and suppression of cytokine signalling, including IL-1 $\beta$ , IL-8 and TNF $\alpha$  (Volf et al., 2010). In chickens, the same biological dynamic response was seen when, in contrast to *S. Enteritidis* and *S. Typhimurium*, *S. Infantis* and *S. Hadar* did not suppress the expression of SPI-1

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genes in chicken macrophages, and in this case they may conceivably have a greater immunosuppressive effect than *S. Typhimurium* and *S. Enteritidis* (A. Imre, et al., unpublished findings). While no significant changes were seen in IL-4 gene expression in the caecal tonsils following *S. Pullorum* infection four days after infection, data from immunohistochemical staining revealed an increase in the amount of B-lymphocyte-positive staining in the caecal tonsils of *S. Pullorum*-infected birds over non infected controls. The latter has been observed with down regulation in CXCLi1 gene expression following infection. These findings could indicate the potential role of humoral immunity in the pathogenesis of *S. Pullorum* infection through the development of the carrier state, a possible Th2-driven pathway.

...In the present experiment, one-day-old chicks and three-week-old chickens were used to study the immune responses to *S. enterica* serotypes before and after the development of gut adaptive immune responses. In the first experiment, the gene expression of the caecal tonsils were determined one day following infection of newly hatched chicks to study the early immune responses that develop to *Salmonella* infection. In older chickens, the time point was selected (four days post-infection) to study the Th1/Th2 paradigm following infection with *Salmonella*. Previous studies have shown that most of the avian immune mediators are better expressed at this time point (Withanage et al., 2005b, Berndt et al., 2007). However, inclusion of longer experiments with more time points could strengthen the results and the conclusion drawn. As mentioned, changes in macrophages and B-cells were measured in the caecal tonsils using IHC. Cellular changes were measured as a percentage of

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the total area of staining positive for the examined cells (Cheeseman et al., 2008). Although the exact number of cell populations was not determined in the present experiment, the present findings provide further insights on the interaction of *Salmonella* with the GALT (caecal tonsils).

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## 6 General discussion

The aim of this study was to elucidate and compare the immune responses induced in response to infection of poultry with poultry-specific or non-host-adapted serovars of *S. enterica*. The gene expression profiles and cellular changes were recorded following infection with different serotypes of *Salmonella*. The interaction of *Salmonella* with chicken macrophages and epithelial cells was also studied with special focus on the role of SPI and flagella in *Salmonella*-associated infections and the immune response. The principle objectives of the present study were, therefore, to provide further insights into the interaction of *Salmonella*, at the cellular level, with the avian host, both *in vivo* and *in vitro*. Here we identified that differences in immune responses are related to the infecting strain, and involve the contribution of TTSS and flagella and perhaps other factors.

It is clear from the experimental work carried out in this study that exposure of avian cells and chickens to live *Salmonella* induces a differential expression of various biological and immunological parameters, as summarized in Tables 6.1 & 6.2.

**Table 6.1:** Immune responses of cultured avian cells to infection with typhoid and non-typhoid *Salmonella* serovars

Bacterial stimulus	<i>In vitro</i> invasiveness	Response of macrophages	Epithelial response to infection	Response of lymphocytes
<i>S. Typhimurium</i>	++	↑ NO, ROS ↑↑ Inflammatory response ↑↑ CXCLi2, ↑↑ iNOS	Strong inflammatory response ↑↑ CXCLi1, CXCLi2, IL-6, iNOS	N/D
<i>S. Enteritidis</i>	++	↑ NO, ROS ↑↑ CXCLi2, CCLi2, IL-6, iNOS ↑↑ Inflammatory response ↑ IL-10,	Strong inflammatory response ↑↑ CXCLi1, CXCLi2, IL-6, iNOS <i>fliJ</i> → CXCLi1, CXCLi2, IL-6, iNOS SPI-1 & SPI-2 ↑ CXCLi1, CXCLi2	↑ IL-18 in chicken blood lymphocytes
<i>S. Hadar</i>	++	↑ NO, ROS ↑↑ CXCLi2	Moderate inflammatory response ↑ CXCLi1, CXCLi2, IL-6, iNOS	N/D
<i>S. Infantis</i>	++	↑ NO, ROS ↑ CXCLi2	Moderate inflammatory response ↑ CXCLi1, CXCLi2, IL-6, iNOS	N/D
<i>S. Pullorum</i>	+	↑ NO, ROS ↑ CXCLi2, CCLi2, IL-6, iNOS	No inflammatory response → CXCLi1, CXCLi2, IL-6, iNOS	↓ IL-18 in chicken blood lymphocytes
<i>S. Gallinarum</i>	+	↑ NO, ROS ↑ CCLi2, iNOS	No inflammatory response → CXCLi1, CXCLi2, IL-6, iNOS Flg <sup>+</sup> ↑ CXCLi2, IL-6	N/D

↑↑ High increase, ↑ moderate increase, ↓ decrease, → no change, N/D, not done

**Table 6.2:** Immune responses of chickens to infection with typhoid and non-typhoid *Salmonella* serovars

Bacterial stimulus	Caecal colonization	Cellular changes in caecal tonsils	Immunological parameters of caecal tonsils
<i>S. Enteritidis</i>	++	↑↑ MØ ↑ B-cells	↑ CXCLi1, ↑ LITAF, ↑↑ CXCLi2 in newly hatched chicks ↑ CXCLi2, iNOS, LITAF, IL-10 in older chickens
<i>S. Infantis</i>	+++	↑ MØ ↑ B-cells	Weak inducer of immune responses → CXCLi1, CXCLi2, IL-6, iNOS ↑ LITAF in newly hatched chicks
<i>S. Pullorum</i>	±	↑ MØ ↑↑ B-cells	↑ LITAF in newly hatched chicks ↓ CXCLi1 in older chickens
<i>S. Gallinarum</i>	±	↑↑ MØ ↑ B-cells	↑↑ LITAF in newly hatched chicks

+++ High colonizing, ++ colonizing, ± poor colonization

↑↑ High increase, ↑ moderate increase, ↓ decrease, → no change

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## 6.1 Immunology of avian systemic salmonellosis

Data presented in this study showed that the poultry-restricted serotypes, *S. Pullorum* and *S. Gallinarum*, failed to induce an inflammatory response from the infected epithelial cell model (CKC). Indeed, epithelial cells infected with these systemic *Salmonella* serotypes did not express any of the pro-inflammatory markers tested in this study, including CXCLi1 and CXCLi2 chemokines, IL-6 and iNOS. In this study, the same dynamic response has been observed in the caecal tonsils following infection in chickens (with the exception of up-regulation of LITAF in caecal tonsils of newly hatched chicks). Interestingly, the expression of CXCLi1 was down-regulated in the caecal tonsils of *S. Pullorum*-infected chickens. Colonization of human intestinal epithelial cells by human enteritis-producing *Salmonella* serovars (*S. Typhimurium* and *S. Enteritidis*) induces transepithelial signalling and transepithelial migration of PMNs when compared with serovars causing either typhoid fever (*S. Typhi* and *S. Paratyphi*) or no disease (*S. Pullorum*) in humans, with reduction in the production of IL-8 in response to non pathogenic strains of *Salmonella* when compared with the wild-type serovars (McCormick et al., 1993, McCormick et al., 1995a, McCormick et al., 1995b). Infection with the two non-motile poultry serotypes, causing typhoid symptoms in poultry, was shown to induce reduced or no inflammatory response at all with equivalent avian cell models or chickens. *In vitro* infection of epithelial cells with *S. Gallinarum* did not trigger the production of IL-6 and down-regulated the expression of IL-1 $\beta$  (Kaiser et al., 2000). Previous work has also shown that *S. Pullorum* infection down-regulates the expression of CXCLi1 and CXCLi2 in the ileum of infected chickens (Chappell et al., 2009). The up-

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regulation of CXCLi1 and CXCLi2 in the chicken gut has been shown following infection of chicks with *S. Typhimurium* and *S. Enteritidis* (Withanage et al., 2004, Cheeseman et al., 2008). The data shown here are in accordance with this reported by (Chappell et al., 2009) which shows up-regulation of CXCLi1 and CXCLi2 up to 24 h in the ileum of day-old SPF White Leghorn chicks due to *S. Enteritidis* infection compared with *S. Pullorum*. This finding is consistent with the data available on the infection biology of these *Salmonella* serotypes in poultry hosts. Indeed and with the exception of very young chicks, infection with *S. Typhimurium* and *S. Enteritidis* in poultry is usually limited to the gut as infection is usually result in intestinal colonization (non-systemic) due to the presence of strong inflammatory response. Nonetheless, *S. Pullorum* and *S. Gallinarum* induce less inflammatory cytokines and chemokines and infection may progress to systemic spread due to the absence of intestinal inflammation. It has been postulated that, unlike *S. Pullorum* and *S. Gallinarum*, motile *Salmonella* such as *S. Typhimurium* may invade the intestinal epithelium with stimulation of TLR-5 (Iqbal et al., 2005b). Thus, chickens infected with systemic serovars are unlikely to mount a strong inflammatory response in the gut and, therefore, early systemic infection is often inevitable with systemic spread of bacteria to the internal organs, including liver and spleen.

The data presented here showed that both *S. Pullorum* and *S. Gallinarum* are invasive to both phagocytic and non-phagocytic cell models, though their invasiveness is reduced when compared with the broad-host-range serotypes. It has been shown that *S. Gallinarum* is invasive to chicken epithelial cells (CKC)

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(Kaiser et al., 2000) and taken up by primary blood monocytes-derived macrophages (Wigley et al., 2006), though its intracellular counts were reduced when compared with *S. Typhimurium*. *S. Pullorum* also expresses some degree of invasiveness to CKC (Barrow and Lovell, 1989, Wigley et al., 2002b). In this study, the systemic serovars also show a reduced capability to colonize the caeca of infected birds, when compared to the non-host-adapted serotypes. Thus, it is obviously evident that *S. Pullorum* and *S. Gallinarum* are poor colonizers of the chicken gut. Following infection with a systemic serovar, it seems much more likely that a proportion of the intestinal bacteria is capable of invading the intestinal epithelia into the deeper tissues and, consequently, infection is often spread systemically and without stimulation of gut immune responses. This epithelial cell invasion is highly anticipated to occur primarily through the GALT, mainly at the caecal tonsils, since infection induces cellular changes and cytokine expression in the caecal tonsils. Indeed, it is highly anticipated in mammals as well as in birds that *Salmonella* can invade the intestinal lumen into systemic sites via specialized antigen-sampling cells (M cells) of the FAE of GALT (Barrow et al., 2000, Jepson and Clark, 2001).

## **6.2 Immunology of fowl paratyphoid infection**

The data presented here have demonstrated that infection with the non-host-adapted serovars is characterised by efficient colonization of the caeca. Both newly hatched chicks and older chickens infected with *S. Enteritidis* or *S. Infantis* showed high bacterial counts in the caeca. This finding is in accordance with (Methner et al., 2010), who reported that oral infection of day-old-chicks with *S. Enteritidis* or *S. Infantis* results in a high level of caecal colonization. Indeed, infection with *S. Typhimurium* or *S. Enteritidis* in

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chickens older than 3 days of age results in disease-free gut colonization (Barrow et al., 1987b, Humphrey et al., 1989, Withanage et al., 2004). In the present study, infection with broad-host-range serotypes, such as *S. Typhimurium*, *S. Enteritidis*, *S. Hadar* and *S. Infantis*, also induces the expression of higher levels of pro-inflammatory cytokines and chemokines, including CXCLi1, CXCLi2 and IL-6, and iNOS in the infected tissue culture models. Furthermore, *S. Enteritidis* or *S. Typhimurium* infection of chicken epithelial cells triggers the expression of higher levels of inflammatory mediators in comparison with both *S. Hadar* and *S. Infantis*, though they express the same degree of invasiveness. It has been shown recently that SPI-1-mediated TTSS has an immunosuppressive function through the interference with *S. Typhimurium* and *S. Enteritidis* recognition by infected porcine alveolar macrophages and suppression of cytokine signalling (Volf et al., 2010). In chickens, it seems very likely that *S. Infantis* and *S. Hadar* did not suppress the expression of SPI-1 genes in chicken macrophages and, hence, possess a greater immunosuppressive effect than *S. Typhimurium* and *S. Enteritidis* (A. Imre, et al., unpublished findings). Previous work has shown that *S. Enteritidis* or *S. Typhimurium* infection of CKC triggers the expression of the inflammatory cytokine, IL-6 (Kaiser et al., 2000). *S. Typhimurium* infection of newly hatched chicks induces enteropathogenic responses typified by the up-regulation of the gene expression of inflammatory cytokines and chemokines CXCLi1, CXCLi2, MIP-1 $\beta$  and IL-1 $\beta$  (Withanage et al., 2004). Accordingly, these combined data strongly suggest that *S. Typhimurium* and *S. Enteritidis* are strong inflammatory serotypes when compared with *S. Hadar* and *S. Infantis*. In the present study, while *S. Infantis* infection revealed high

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capability of caecal colonization, but stimulates limited changes in the cytokine response of the caecal tonsils. (Berndt et al., 2007) have also shown that *S. Infantis* is a weak immune stimulator to the caeca of orally infected day-old chicks, when compared with *S. Typhimurium*, *S. Enteritidis* and *S. Hadar*. The data presented here has indicated that *S. Enteritidis* infection of newly hatched chicks induces significant up-regulation of CXCLi1 and CXCLi2 chemokines in the caecal tonsils one day later. In addition, *S. Enteritidis* infection triggers the up-regulation in gene expression of CXCLi2 chemokine, LITAF and iNOS in the caecal tonsils four days after infection of three-week-old chickens, again pointing out the high capability of *S. Enteritidis* to stimulate gut inflammatory responses and migration of immune cells to the site of infection and inflammation. The differential expression of iNOS has been reported after infection of one-day-old chicks with *Salmonella* serovars, *S. Typhimurium*, *S. Enteritidis*, *S. Hadar* and *S. Infantis*, with *S. Enteritidis* showed the higher expression level with up to 298-fold change (Berndt et al., 2007). It has been shown that infection with the non-host-adapted serovars, including *S. Typhimurium* or *S. Enteritidis*, induces gut inflammation characterized by the expression of a wide range of inflammatory and immune mediators, including IL-1 $\beta$ , IL-6, CXCLi1, CXCLi2, IFN- $\gamma$ , LITAF and iNOS (Withanage et al., 2004, Withanage et al., 2005b, Berndt et al., 2007, Cheeseman et al., 2008, Fasina et al., 2008). The Th1 key cytokine, IFN- $\gamma$ , has been shown to be highly up-regulated in the caeca of *S. Typhimurium* and *S. Enteritidis*-orally-infected day-old chicks (Berndt et al., 2007). Moreover, the expression of IFN- $\gamma$  has been found to be up-regulated *in vivo* in spleens, livers and caecal tonsils up to 14 days after infection of one-week-old SPF chicks with *S. Typhimurium*,

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suggesting a role for Th1 immune responses in clearance of *Salmonella* (Withanage et al., 2005b). Evidence of IFN- $\gamma$  up-regulation has been also found to be associated with the clearance of *S. Typhimurium* in chickens (Beal et al., 2004a). In the present study, however, no significant changes in the gene expression of IFN- $\gamma$  from infected macrophages or chicken caecal tonsils were observed. This could be a result of many factors involved in the experimental infections, including the sampling time, age of chicken at the time of infection and/or breed genetics. In the present study, *S. Enteritidis* infection resulted in the induction of the anti-inflammatory cytokine IL-10, both *in vivo* (caecal tonsils) and *in vitro* (HD11 cells). The expression of IL-10 by murine macrophages has been reported in response to *S. Typhimurium* infection (Uchiya et al., 2004). Also, *S. Enteritidis* infection of COEC triggers the expression of IL-10 (Li et al., 2009). Taken together, this observation contradicts with previous finding by (Fasina et al., 2008) who reported down-regulation of IL-10 in the gut of *S. Typhimurium*-orally-infected chickens. As already stated, it is suggested that IL-10 is a regulatory cytokine produced to reduce the inflammation resulting from over-secretion of inflammatory mediators and IFN- $\gamma$ .

The present study has shown that LPS-induced TNF- $\alpha$  factor (LITAF) is up-regulated in the caecal tonsils as early as 24 h following infection of day-old-chicks with typhoid and non-typhoid *Salmonella* serovars, particularly with *S. Gallinarum* infection. In mice, TNF- $\alpha$  is induced by murine macrophages in response to LPS treatment or *Salmonella* infection and has been shown to play a central role in clearance of and controlling *Salmonella* infection in mammals

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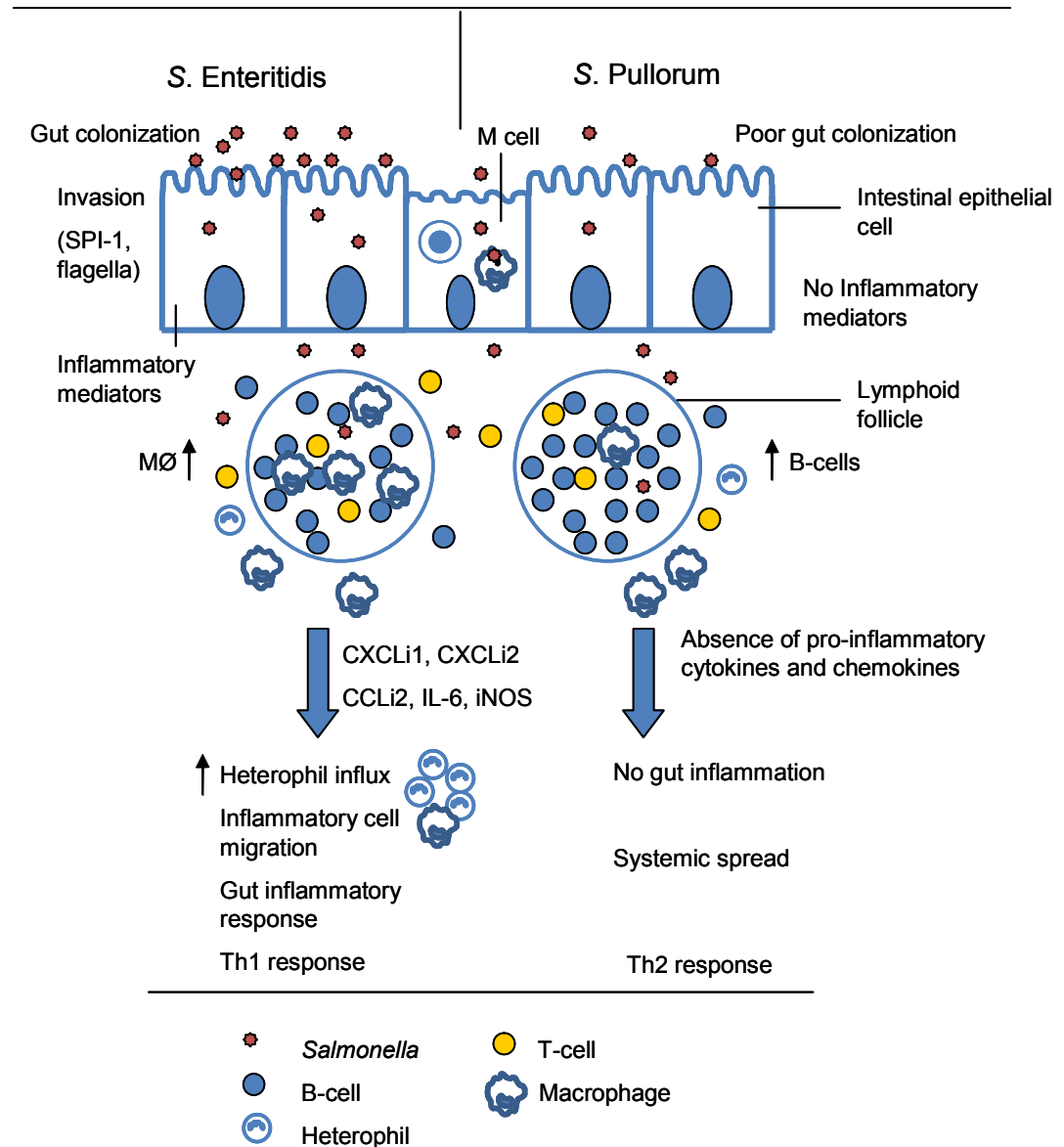
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(Nauciel and Espinasse-Maes, 1992, Morrissey et al., 1995, Gulig et al., 1997, Royle et al., 2003, Talbot et al., 2009). The role played by LITAF in the immune responses against poultry pathogens is not fully understood. Nonetheless, it is suggested that observed cellular changes that induced in the caecal tonsils following *Salmonella* infection might be influenced by the up-regulation of LITAF. Here we have shown that LITAF is up-regulated in response to *Salmonella* infection in newly hatched chicks. It seems that this TNF- $\alpha$  plays an important role in immunity to *Salmonella* during the first few days after hatch. Recent evidence has shown that TNF- $\alpha$  possess a regulating function and controlling the expression of *Salmonella* effector proteins (Ma et al., 2010). We proposed that the role of TNF- $\alpha$  could extend to include, and regulate, the expression *Salmonella* effector protein, AvrA. It is suggested that AvrA inhibits the host inflammatory response against infection, via acting on MAPK, Jun N-terminal kinase (JNK) and NF- $\kappa$ B signalling pathways (Jones et al., 2008). AvrA blocks I $\kappa$ B degradation and ubiquitination which interferes with the nuclear translocation of active NF- $\kappa$ B, leads to inhibition of NF- $\kappa$ B signalling and inhibition of induction of inflammatory cytokines and chemokines, including IL-6, IL-8, IL-12, IFN- $\gamma$  and TNF- $\alpha$  (Collier-Hyams et al., 2002, Ye et al., 2007, Lu et al., 2010). In the present experiment, the differential expression of cytokine and chemokine response between systemic and enteric serovars, as exemplified by *S. Pullorum* and *S. Enteritidis*, could be influenced by translocation of bacterial AvrA. Although the role AvrA in either *S. Pullorum* or *S. Gallinarum* infection in poultry has not yet been identified, this effector protein appears to play an important role in avian systemic salmonellosis, mediating the down-regulation of inflammatory cytokines,

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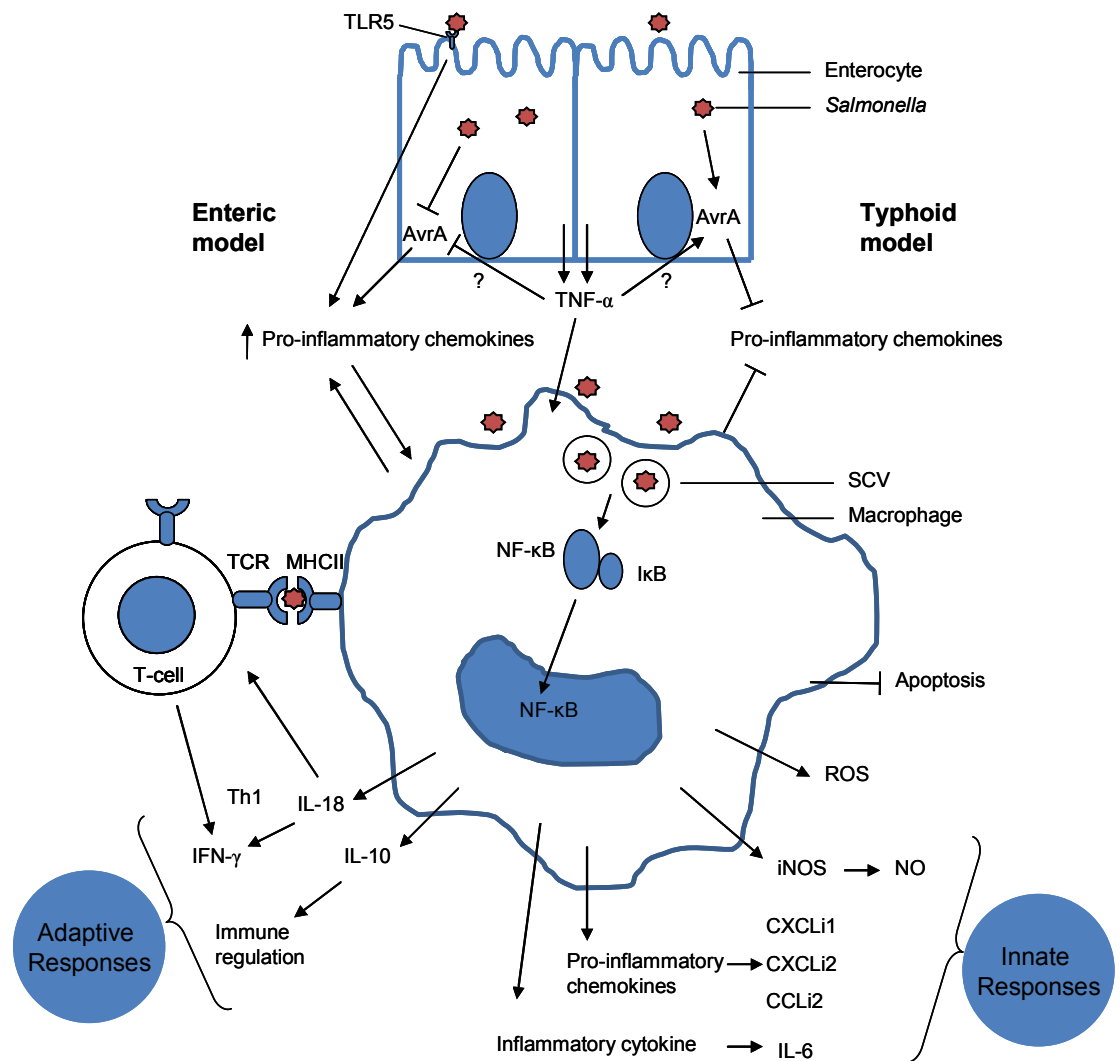
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including IL-18 (IFN- $\gamma$  promoter). Indeed, the up-regulation of gut inflammatory cytokines and chemokines CXCLi1, CXCLi2, MIP-1 $\beta$ , IL-1 $\beta$  and IFN- $\gamma$  has been shown following infection with *S. Typhimurium* in one-day-old chicks (Withanage et al., 2004) and one-week-old chickens (Withanage et al., 2005b). Figures 6.1 and 6.2 are schematic representations which show the possible outcomes and cellular responses to infection with typhoid and non-typhoid *Salmonella* serovars.



**Figure 6.1:** *Salmonella* infection in poultry.

Schematic representation shows the immunological pathways following oral infection of chickens with typhoid and non-typhoid *Salmonella* serovars. Unlike *S. Pullorum* and *S. Gallinarum*, infection with the non-host adapted serovars, including *S. Typhimurium* and *S. Enteritidis*, is characterized by production of pro-inflammatory cytokine and chemokine response which subsequently stimulates the migration of PMNs into the intestinal tract (Henderson et al., 1999, Withanage et al., 2004).



**Figure 6.2:** Interactions of intracellular *Salmonella* with host cells.

In the intracellular environment, *Salmonella*-containing vacuole (SCV) enables *Salmonella* to persist inside the infected cells and tolerate the intracellular killing mechanisms (Eswarappa et al., 2010). Processed antigens are expressed on the surface of phagocytes bound to the MHC class II and exposed to effective cells of the adaptive immune system (lymphocytes). *Salmonella* infection leads to activation of nuclear factor- $\kappa$ B which promotes transcriptional changes of genes involved in both innate and adaptive immune responses. *S. Pullorum* infection of HD11 cells induces lower levels of inflammatory cytokine and chemokine response when compared with *S. Enteritidis*. The cross-talk between the type of *Salmonella* serovar and expression of TNF- $\alpha$  could be bi-directional and might involve a role for bacterial effector protein, AvrA.



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**6.3 Cellular changes following *Salmonella* infection**

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The dynamics of infiltration of immune cells, macrophages and B lymphocytes, have been studied in the caecal tonsils at four days post-infection of three-week-old chickens. It appears that caecal tonsils are targeted by *Salmonellae*, since the enlargement and haemorrhages of the caecal tonsils occurred following infection with *S. Gallinarum* and increase in macrophages and B-cell populations of the caecal tonsil in response to infection with typhoidal and non-typhoidal serotypes. In the present study, data from immunohistochemistry staining of the caecal tonsils have shown that *S. Enteritidis* infection increases the total area staining positive for macrophages while *S. Pullorum* infection increases the area positive for B-cells in the caecal tonsils of infected three-week-old chickens. It has been shown that *S. Enteritidis* infection increases CD4<sup>+</sup> T-cells and IgG<sup>+</sup> B-cells in the caecal tonsils at 4-6 days following infection of 16-day-old chickens (Sasai et al., 2000). It has also been shown that *S. Enteritidis* infection or vaccination of young chicks induced cellular changes in the T- and B-cells, macrophages and heterophils in the caecal mucosa as early as 24 h post challenge (Van Immerseel et al., 2002a, Van Immerseel et al., 2002b). In the present study, the presence of an increase of macrophage cells (as measured by total area staining positive for these cells) in the caecal tonsils is associated with the expression of CXCLi2, iNOS and LITAF in older chicken infected with *S. Enteritidis*. However, as already described *S. Pullorum* infection down-regulated CXCLi1 expression and increased the B-cell population in the caecal tonsils. This fundamental difference between the response of caecal tonsils to infection with systemic and paratyphoid serotypes is immensely important, and also pointing

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out that differences in immune responses are dependent on the infecting *Salmonella* serotype. The present study has also shown that *S. Pullorum* infection down-regulates the expression of IL-18 (IFN- $\gamma$  inducer) *in vitro*. This evidence strongly suggests that, unlike infection with *S. Enteritidis*, *S. Pullorum* infection is more likely to suppress the IL-18/IFN- $\gamma$  axis and, therefore, is not associated with Th1 immune pathway, but immune response is more likely to be Th2-driven, as also suggested by (Chappell et al., 2009). In contrast to *S. Enteritidis*, the tendency of *S. Pullorum* to mediate humoral immune responses rather than cellular responses could highlight the importance of killed vaccine in mediating immunological memory against *S. Pullorum* and controlling pullorum disease in birds.

#### **6.4 *Salmonella* survival in macrophages and infection of epithelial cells**

The ability of *Salmonella* to survive in the intracellular environment is crucial to pathogenesis of *Salmonella* in different hosts. It is also suggested that systemic *Salmonella* use phagocytes as a mean of transport for translocation from the gut to the internal organs of poultry (Jones et al., 2001). The data presented here has shown that *Salmonella* can persist in the infected macrophages for at least 48 h post-infection, though survival of bacteria is reduced over time. This reduction in intracellular bacterial numbers is correlated with the production of the antimicrobial NO and ROS. It is, therefore, unclear whether *Salmonella* are able to survive within phagocytes long enough while translocating from the gut to the internal organs. It is suggested that *S. Dublin* is extracellular during transfer from the gut of the calf (Pullinger et al., 2007), and whether this mechanism is involved in the course of *Salmonella* infection in poultry remains unclear. As already mentioned,

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infection of epithelial cells with *S. Pullorum* or *S. Gallinarum* does not stimulate the production of an inflammatory cytokine response. However, the expression of a relatively lower, but significant, levels of pro-inflammatory cytokine response by HD11 cells as a result of infection with systemic serovars was observed. This seems unusual since our *in vivo* data have shown that the absolute non-induction or even down-regulation of inflammatory response (CXCLi1) is a solid feature to infection with systemic poultry serotypes. Accordingly, it could also be proposed that, following epithelial invasion and while surviving with macrophages, systemic *Salmonella* might stimulate the expression of certain levels of inflammatory mediators to recruit more immune cells into the site of infection to promote the bacterial survival, migration and invasion of systemic organs. Or, alternatively, production of pro-inflammatory cytokine and chemokines by HD11 cells in response to infection with either *S. Pullorum* or *S. Gallinarum* could also be influenced by the MOI (1:10) used in this study.

The present study did not include infection of chickens with *S. Typhimurium* or *S. Hadar* and, hence, determination of immune responses of chickens to infection with these serotypes was not performed. In the present study, *Salmonella*-mediated immune responses of the caecal tonsils (*in vivo*) appear to be correlated to infection of primary epithelial cells (CKC), pointing out that initial, early interaction of *Salmonella* with epithelial cells might contribute to most of the interaction of *Salmonella* with the caecal tonsil. Accordingly, it is highly predicted that infection of chickens with *S. Typhimurium* or *S. Hadar* could stimulate a comparable levels of immune responses as *S. Enteritidis* and *S. Infantis*, respectively. Previous studies have shown that *S. Typhimurium*

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infection in chickens induces gut enteropathogenic responses (Withanage et al., 2004, Withanage et al., 2005b) while *S. Hadar* is regarded as less-invasive and of moderate immune stimulating characteristics when compared with *S. Enteritidis* (Berndt et al., 2007).

### **6.5 *Salmonella* TTSS- and flagella-associated virulence**

It has been shown that *S. Typhimurium* requires both SPI-1 and SPI-2 for gastrointestinal colonization and systemic spread in chickens (Jones et al., 2007). Using a mixed infection approach, recent evidence indicates that the contribution of SPI-1 in the colonization of gut and spleen of chickens is greater than that of SPI-2 (Dieye et al., 2009). In a previous study which was conducted to investigate the potential role of different SPI (1 to 5) in the colonization of *S. Enteritidis* of orally infected chickens, it has been shown that both SPI-1 and SPI-2 are known to be required by *S. Enteritidis* for colonization of internal organs (liver and spleen), while the influx of PMNs into the caecum is SPI-1 dependent (Rychlik et al., 2009). Moreover, it has been found that SPI-2 is required for invasion and systemic spread of *S. Enteritidis* following infection of one-week-old chickens (Wisner et al., 2010). It has been also shown that SPI-2 is important for reproductive tract colonization by *S. Enteritidis* (Bohez et al., 2008) while SPI-1 is required for caecal colonization of chickens by *S. Enteritidis* (Bohez et al., 2006). While a *ssrA* (SPI-2) mutant of *S. Enteritidis* poorly colonizes the ovaries and oviducts and induces less pathology in the reproductive organs, compared the wt *S. Enteritidis*, of laying hens infected intravenously (Bohez et al., 2008), a *hilA* (SPI-1) mutant strain of *S. Enteritidis* produces a strong colonization inhibition effect in the caecum and internal organs of newly hatched chicks when

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administered 24 h before the challenge with the wt strain of *S. Enteritidis* (Bohez et al., 2007). New evidence suggests that SPI, other than SPI-1 and SPI-2, are important for colonization of *S. Gallinarum* in chickens and could provide further explanations regarding the contribution of SPI to the host specificity of *S. Gallinarum* to chickens. Indeed, It has been recently shown that the newly identified SPI-19 (encode T6SS) plays a vital role in the colonization of *S. Gallinarum* in chickens, where a SPI-19 deletion mutant showed a severe reduction in colonization of the intestinal tract (ileum and caecum) and the internal organs (liver and spleen) compared with the wt *S. Gallinarum* (Blondel et al., 2010).

The present study also demonstrates the important role played by SPI-encoded TTSS and flagella in the pathogenesis and immune responses to *Salmonella*. TTSS-1 (*invA*) and non-motile (*fliJ*), but not TTSS-2 (*ssaR*), mutants of *S. Enteritidis* have shown a reduced capability to invade, and survive in, the chicken epithelial cells, in comparison with the wt *S. Enteritidis*. Moreover, while *fliJ* non-motile strain of *S. Enteritidis* was a non-inducer of pro-inflammatory immune markers, such as CXCLi1, CXCLi2, IL-6 and iNOS, from infected epithelial cells, infection with TTSS-1 (*invA*) or TTSS-2 (*ssaR*) deficient mutants of *S. Enteritidis* is typified by lower induction of inflammatory mediators, when compared with the parent strain of *S. Enteritidis*. It is clearly evident that TTSS mutants of *S. Enteritidis* are expressing a certain degree of immune stimulation, although they can be regarded as less virulent in the context of invasion and stimulation of immune response. Compared to the parent strain of *S. Gallinarum*, the motile strain of *S.*

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*Gallinarum* (Flg<sup>+</sup>) has shown an increased degree of invasiveness and inflammatory cytokine immune response (CXCLi2 and IL-6) in the infected epithelial cells. These findings show the important role of flagella in invasion and induction of immune responses during the course of *Salmonella* infection in poultry. Mice infected with non-flagellate mutants of *S. Typhimurium* failed to express a robust, initial intestinal inflammatory response and developed a severe systemic form of the disease two days following infection (Vijay-Kumar et al., 2006). Genome analysis has indicated that *S. Enteritidis* and *S. Gallinarum* are recently diverged clones, with the latter genome has undergone extensive functional gene loss and pseudogene formation which is potentially involved in virulence and host adaptation (Thomson et al., 2008). This could also provide an evolutionary reason for *S. Gallinarum* and *S. Pullorum* losing motility and suggesting that the role of TLR5 may be important in chickens than mammals since the taxonomically related serovars (*S. Enteritidis* and *S. Dublin*) remain motile. This is also support the contention that, in contrast to *S. Enteritidis*, the absence of flagella by the poultry-specific serovars would enable these serotypes to invade the intestine without the stimulation of an inflammatory response from the host intestine (Kaiser et al., 2000, Iqbal et al., 2005b). Taken together, these findings also represent a major shift toward the production of novel vaccine to control *Salmonella* infection in poultry. Further research is required to evaluate the pathogenicity, immunogenicity and efficacy of protection of this strain in chickens (*in vivo*).

In conclusion, the present study provides more insights on the mucosal immune response to *Salmonella* as well as their interaction with the innate cellular components. Here we have shown that avian immune responses are

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differentially modulated by *Salmonella* and in a way that is dependant on the infecting serovar. While *S. Enteritidis* and *S. Typhimurium* expressed a higher magnitude of inflammatory immune response, *S. Pullorum* and *S. Gallinarum* did not.

## 6.6 Future work

No doubt that there is a need to clarify some points of interest based on the findings gained from this study.

1- Future work might involve the use of primary blood-derived or tissue (spleen) macrophages to confirm and characterize the immune pathways involved in both typhoid and non-typhoid *Salmonella* infection.

2- Future work will study the role of, and relation between, host LITAF and bacterial AvrA in enteric and typhoid *Salmonella* infection in poultry. This can be performed by using *Salmonella* AvrA mutants and study the immune responses of poultry to infection with or without the administration of TNF- $\alpha$  neutralizing antibodies.

3- Future research will also be conducted to study changes in T-cell population in response to infection with typhoid and non-typhoid *Salmonellae* in poultry. It is clear now that development of effective *Salmonella* vaccines in poultry requires more comprehensive analysis and study of the avian immunology and host responses to infection. This should take into account the interaction of *Salmonella* with T-cells and study of cellular changes in T-cell composition following infection.

4- Future work will involve an analysis of additional time points to support the data and conclusions.

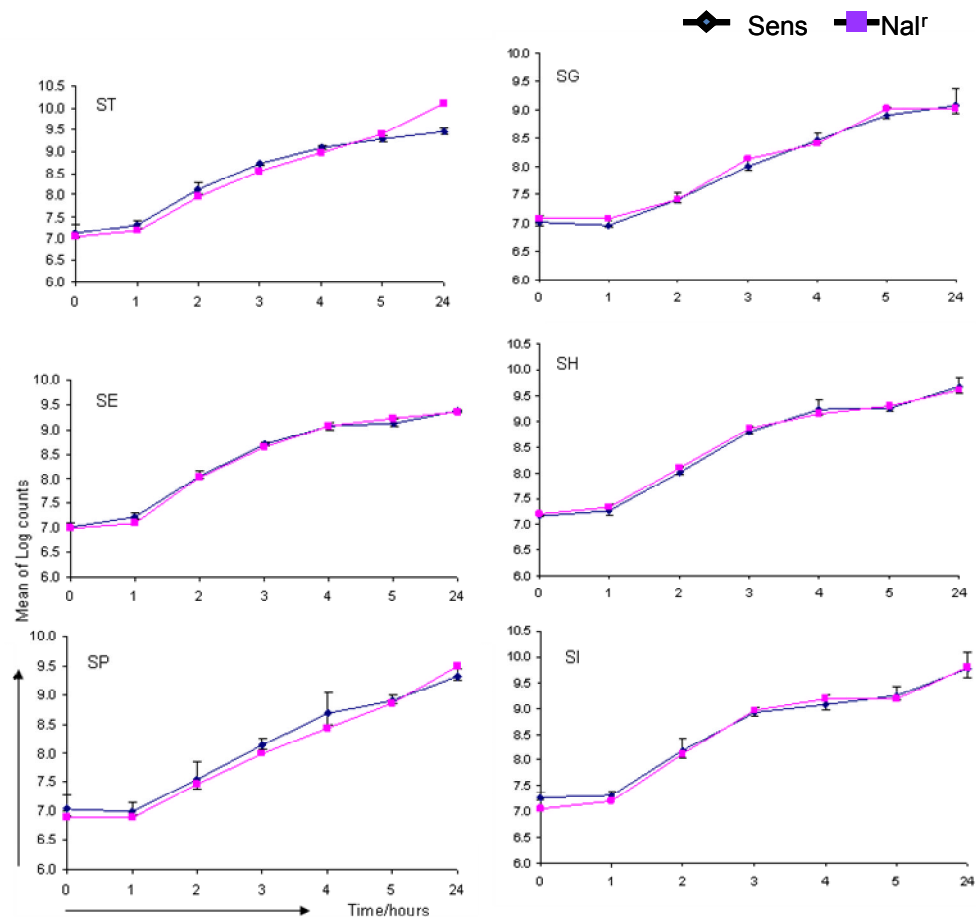
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## 7 Appendices

### 7.1 Growth curves of different *Salmonella* serotypes and determination of exponential growth (Log) phase

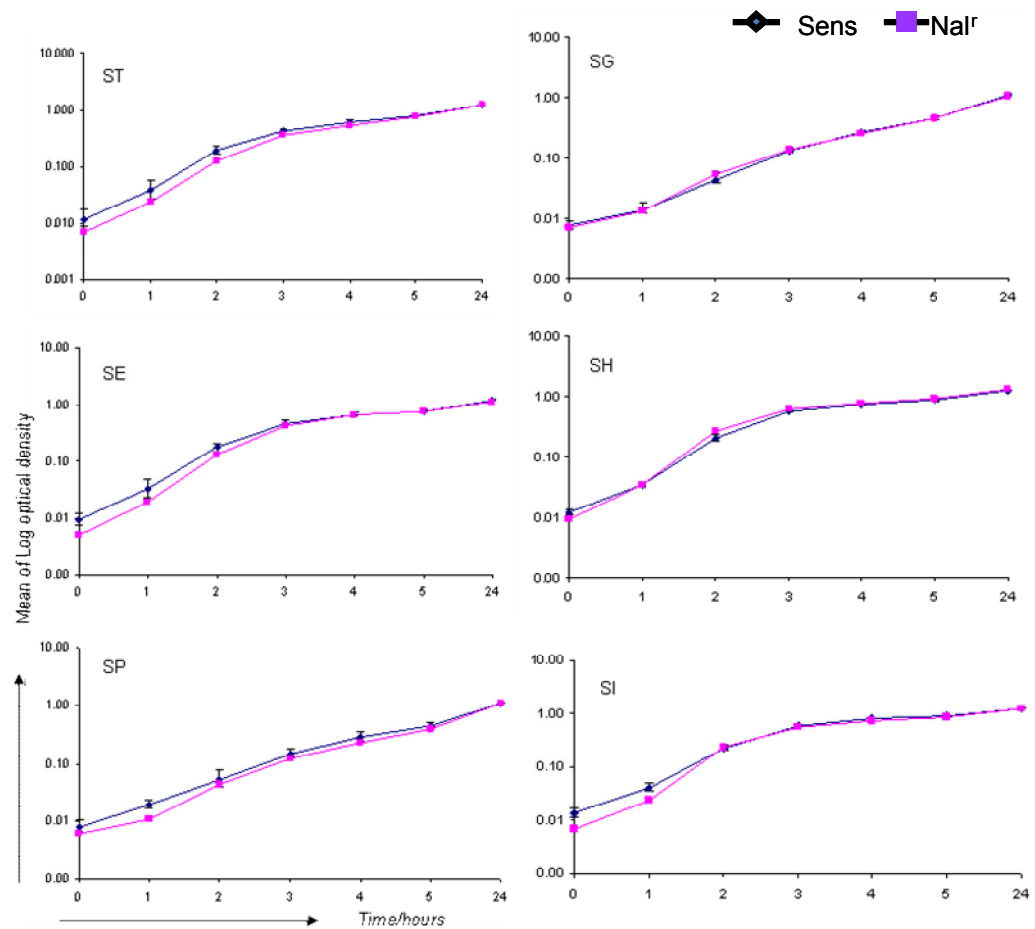
Nal<sup>r</sup> resistance was performed as described earlier for all *Salmonella* serotypes examined in this study. For infection experiments and to determine the exponential growth phase of each strain, growth patterns for different serotypes were determined using the serial dilution (viable colony count) and spectrophotometric methods (OD) (Figures 7.1 & 7.2). Results indicated that the mid-log phase for different serotypes is located between 1 and 3 hours post incubation. Both *S. Pullorum* and *S. Gallinarum* showed a slower growth in comparison with the other strains tested in this experiment. Both parent and Nal<sup>r</sup> resistant serotypes were compared in terms of their growth pattern and the obtained results demonstrated that the growth rate was not changed by the nalidixic acid resistance (Figures 7.1 & 7.2). The exponential growth rate ( $\mu$ ) remained unaltered by the nalidixic acid resistance. A calibration graph was designed between the log<sub>10</sub> counts and the optical density and further used to estimate the infection dose (1:10) for each individual strain ((Figures 7.3 & 7.4).



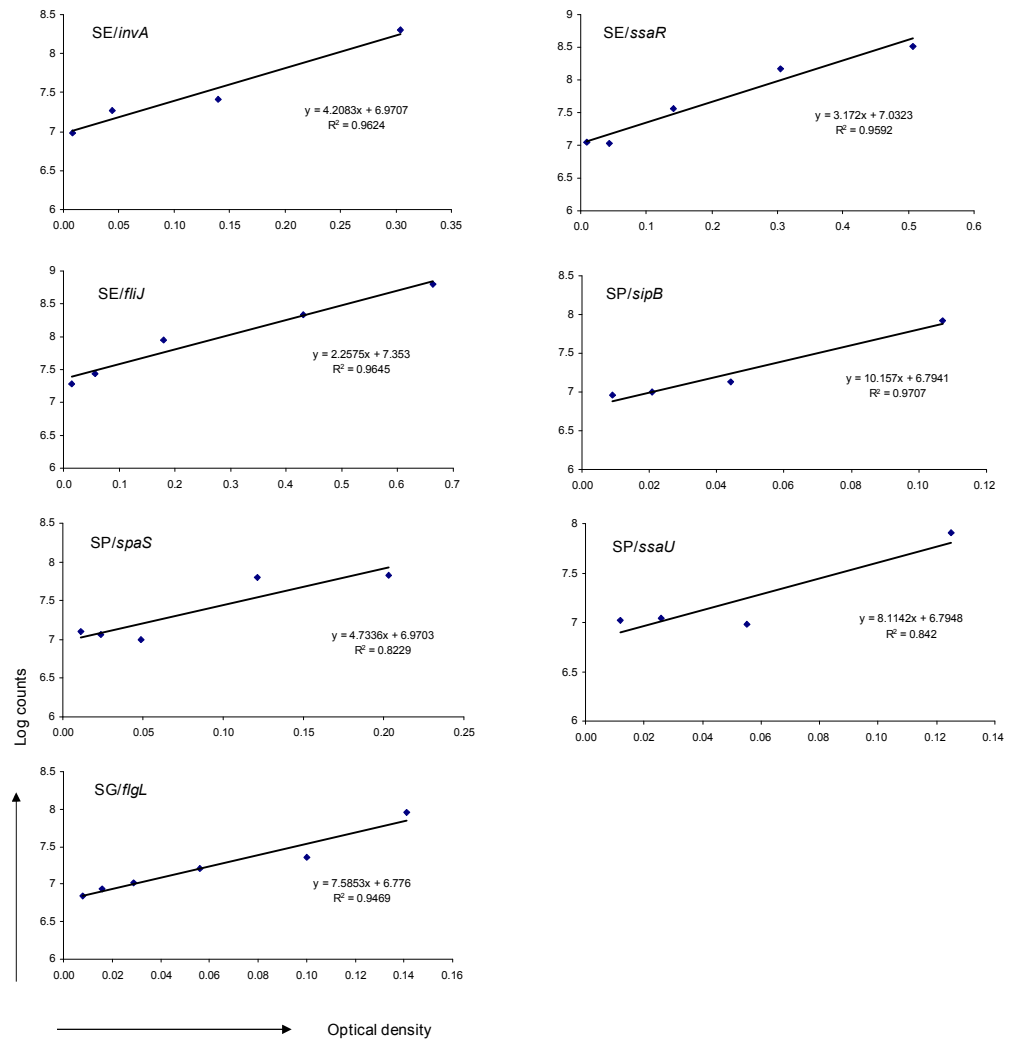


**Figure 7.1:** Growth curves of different *Salmonella* serovars based on VCC.

(ST) *S. Typhimurium* ( $\mu=0.6$ ), (SE) *S. Enteritidis* ( $\mu=0.6$ ), (SP) *S. Pullorum* ( $\mu=0.5$ ), (SG) *S. Gallinarum* ( $\mu=0.5$ ), (SH) *S. Hadar* ( $\mu=0.6$ ) and (SI) *S. Infantis* ( $\mu=0.6$ ). Values are expressed as means  $\pm$  standard errors of three independent experiments for sensitive strains (Sens) and as means for the Nal<sup>r</sup> strains.

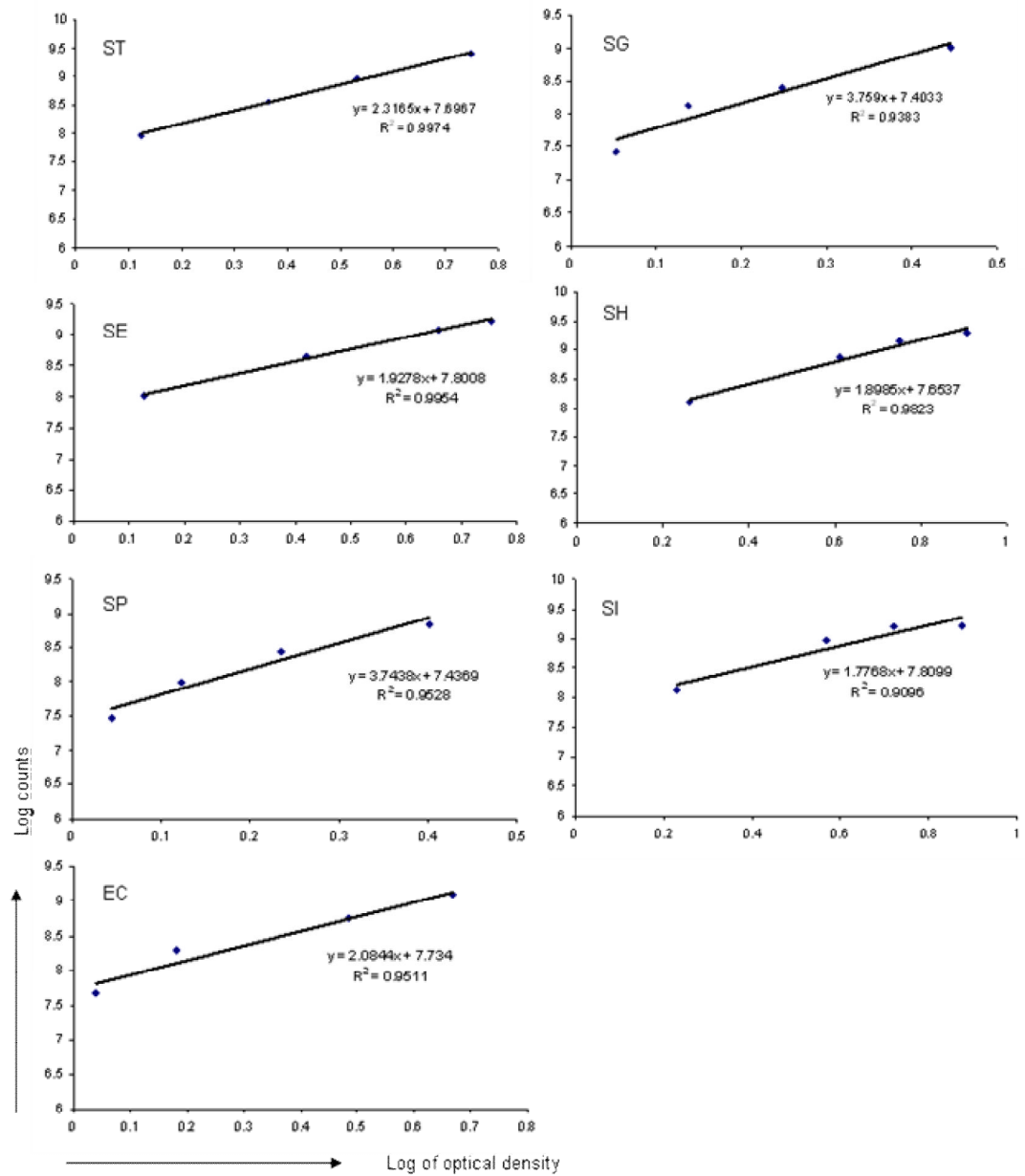


**Figure 7.2:** Growth curves of different *Salmonella* serovars based on the OD 600 nm. (ST) *S. Typhimurium* ( $\mu=0.6$ ), (SE) *S. Enteritidis* ( $\mu=0.6$ ), (SP) *S. Pullorum* ( $\mu=0.5$ ), (SG) *S. Gallinarum* ( $\mu=0.5$ ), (SH) *S. Hadar* ( $\mu=0.6$ ) and (SI) *S. Infantis* ( $\mu=0.6$ ). Values are expressed as means  $\pm$  standard errors of three independent experiments for sensitive strains (Sens) and as means for the  $\text{Nal}^r$  strains.



**Figure 7.3:** Calibration graphs between the bacterial counts and the optical density of different mutant strains of *Salmonella* serotypes which were used for estimation of the infection doses.

(SE/invA) *S. Enteritidis invA*, (SE/ssaR) *S. Enteritidis ssaR*, (SE/fliJ) *S. Enteritidis fliJ*, (SP/sipB) *S. Pullorum sipB*, (SP/spaS) *S. Pullorum spaS*, (SP/ssaU) *S. Pullorum ssaU* and (SG/flgL) *S. Gallinarum Flg<sup>+</sup>*.



**Figure 7.4:** Calibration graphs between the bacterial counts and the optical density of different *Salmonella* serotypes and *E. coli* K-12 which were used for estimation of the infection doses.

(ST) *S. Typhimurium*, (SE) *S. Enteritidis*, (SP) *S. Pullorum*, (SG) *S. Gallinarum*, (SH) *S. Hadar*, (SI) *S. Infantis* and (EC) *E. coli*-K12.

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**7.2 Red blood cells lysis buffer**

8.29g  $\text{NH}_4\text{Cl}$  (0.15 M) (Ammonium chloride)

1g  $\text{KHCO}_3$  (1.0 mM) (Potassium hydrogen carbonate)

37.2 mg  $\text{Na}_2\text{EDTA}$  (0.1 mM) (Disodium EDTA).

pH to 7.2-7.4

Make up to 1 litre and filter sterilise (0.22  $\mu\text{m}$  filter), store at room temperature.

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