S. Virchow Infection and the Immune Responses Produced in Poultry

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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ABBREVIATION LIST

Chapter 1

UK = United Kingdom

NTS = Non Typhoidal *Salmonella*

EU = European Union

FSA = Food Standards Agency

VLA = Veterinary Laboratories Agency

NCP = National Control Program

GIT = Gastrointestinal Tract

DPI = Days Post Infection

DPSI = Days Post Secondary Infection

SPI = Salmonella Pathogenicity Island

T3SS = Type Three Secretion System

WT = Wild Type

CKC = Chick Kidney Cells

SCV = Salmonella Containing Vacuoles

LGP = Lysosomal Membrane Glycoprotein

ORF = Open Reading Frame

SCI = Salmonella enterica Centisome 7 Genomic Island

NK Cell = Natural Killer Cell

IL-2 Receptor = IL-2R

HPI = Hours Post Infection

ROS = Reactive Oxygen Species

APC = Antigen Presenting Cell

FDC = Follicular Dendritic Cell

IDC = Interdigitating Dendritic Cell

WBC = White Blood Cell

LPS = Lipopolysaccharide

TCR = T Cell Receptor

Th Cell = T Helper Cell

IL = Interleukin

IFN = Interferon

GM-CSF = Granulocyte Macrophage Colony Stimulating Factor

TGF = Transforming Growth Factor

CpG-ODN = CpG Oligodeoxynucleotides

MDR = Multiple Drug Resistant

Chapter 2

RFLP = Restriction Fragment Length Polymorphism

IS-RFLP = Insertional Sequences Restriction Fragment Length Polymorphism

PFGE = Pulsed-Field Gel Electrophoresis

AFLP = Amplified Fragment Length Polymorphism

RAPD-PCR = Random Amplified Polymorphic DNA PCR

Rep-PCR = Repetitive Element PCR

VNTR = Variable Number of Tandem Repeat

MLVA = Multiple Locus VNTR Analysis

MLST = Multi Locus Sequence Typing

SNP = Single Nucleotide Polymorphism

ST = Sequence Type

PT = Phage Type

ECM = Extra Cellular Matrix

STM = Signature Tagged Mutagenesis

LB = Luria-Bertani Broth

TAE = Tris-Acetate Buffer

UV = Ultra-Violet Light

PEG = Polyethylene Glycol

TBE = Tris Borate Buffer

UPGMA = Unweighted Pair Group Method with Arithmetic Mean

SLV = Single Locus Variant

iNOS = Inducible Nitric Oxide Synthase

SOCS = Suppressor of Cytokine Signalling

Chapter 3

PRR = Pathogen Recognition Receptor

TLR = Toll-like receptor

NO = Nitric Oxide

RNS = Reactive Nitrogen Species

FCS = Fetal Calf Serum

MOI = Multiplicity of Infection

PBS = Phosphate Buffered Saline

MC29 = Myelocytomatosis Virus

Chapter 4

GALT = Gut-Associated Lymphoid Tissue

SPF = Specific Pathogen Free

BGA = Brilliant Green Agar

Chapter 5

WPI = Weeks Post Infection

MHC = Major Histocompatibility Complex

HSPs = Heat Shock Proteins

Chapter 6

PAMPs = Pathogen Associated Molecular Patterns

TIR = Toll IL-1 Receptor

EST = Expressed Sequence Tags

Tsps = Tail Spike Proteins

WPPI = Weeks Post Primary Infection

BCIP = 5-Bromo-4-Chloro-3-Indolyl Phosphate

NBT = Nitroblue Tetrazolium

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Salmonella Virchow infection and the immune response produced by poultry

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ABSTRACT

Relatively little is known about *Salmonella* Virchow as a pathogen. Its prevalence varies from country to country, however it is constantly associated with invasive disease, particularly in children and the immuno-compromised. The main sources of *S.* Virchow are humans and poultry. The aims of this study were to determine the genetic relatedness of *S.* Virchow isolates from difference sources in England, to characterise its infection biology using *in vitro* and *in vivo* based models, to establish the immune response produced by poultry in response to infection with the serovar and to begin to determine the level of protection and cross-protection that could be achieved against the serovar and *S.* Typhimurium.

The genetic relatedness of the *S.* Virchow isolates was determined using molecular typing techniques including MLST and PFGE. The isolates were screened for the presence of 12 virulence genes that have been associated with adhesion, invasion and persistence. Human and avian cell lines and *in vivo* poultry infection experiments were used to characterise *S.* Virchow's invasiveness, persistence and ability to elicit an immune response, compared to a well characterised *S.* Typhimurium isolate. Immune responses were evaluated by immunohistochemistry, RT-PCR and ELISA, to establish aspects of the innate, cellular and humoral response, as well as cytokine and chemokine expression. An *in vivo* poultry infection experiment was performed to gain an indication of the level of protection and cross-protection offered by primary infection with *S.* Virchow against secondary infection. Bacteriology, ELISA and western blot methods were used analyse this.

Overall, *S.* Virchow appears to be a relatively clonal serovar, regardless of the source and the results indicate this is widespread and not solely in the UK. All of the isolates possessed the 12 virulence genes, which could contribute to its virulence in some hosts. *S.* Virchow was particularly persistent and inflammatory in the human Caco2 cells, which is consistent with the increased virulence previously reported in humans. The *in vitro* HD11 assay and the *in vivo* poultry infection experiments were consistent in showing *S.* Virchow colonises the chicken intestine to high levels, causes transient systemic infection and stimulates a moderate inflammatory response, very similar to *S.* Typhimurium infection. *S.* Virchow infection stimulated all aspects of the chicken immune system, characteristic of a broad-range serovar. Initial results from the *in vivo* protection experiment showed primary infection with *S.* Virchow does offer some protection against systemic invasion, although adequate protection against caecal colonisation was not found. However, 2 proteins were identified that strongly reacted and cross-reacted with sera from infected chickens, providing optimism that a vaccine to protect against *S.* Virchow colonisation could be developed with further research.

Chapter 1
Introduction

1.1 INTRODUCTION

Salmonella is a Gram-negative, facultative anaerobic, bacterial pathogen that has a significant impact on human and livestock health worldwide (Grimont, 2000, DuPont, 2007, Mead, 2004). The *Salmonella* genus belongs to the Enterobacteriaceae family and is split into 2 species *Salmonella bongori* and *Salmonella enterica* (Tindall et al., 2005, Reeves et al., 1989). *Salmonella enterica* is divided into 6 subspecies by their biochemical properties. The subspecies can then be further divided by the Kauffman-White scheme into serovars, based on their lipopolysaccharide (O) and flagella protein (H) antigens (Tindall et al., 2005, Le Minor, 1988). Currently, there are over 2600 *Salmonella* serovars identified (EFSA, 2012).

Salmonella enterica subspecies enterica (I) mainly causes disease in humans and warm-blooded vertebrates and contains many serovars that are zoonotic i.e. can be transmitted from animals to humans and vice versa. The majority of zoonotic *Salmonella* serovars can infect a broad-range of hosts and typically cause limited gastroenteritis (Kaiser et al., 2000). Broad-range serovars have also been shown to cause severe systemic infection depending on the infecting serovar, age of the host and host genetics (Morgan et al., 2004, Wigley et al., 2006, Kaiser et al., 2000). In contrast, a small number of *S.* enterica serovars are highly host-restricted. Typically such serovars result in severe systemic infection in a single or narrow range of hosts (Shivaprasad, 2000).

1.2 Prevalence of *S. enterica* in the UK

Since the late 1990s *Salmonella* infection in humans in the United Kingdom (UK) has generally declined each year (Cogan and Humphrey, 2003). In 2010 there were 9685 laboratory confirmed cases of salmonellosis in the UK, which was nearly an 8% decline from reported cases in 2009 (DEFRA, 2010). However, it is estimated that for every laboratory confirmed case of human salmonellosis reported there are approximately 4.7 unreported cases in the community (Tam et al., 2012). In 2010, *Salmonella* Enteritidis (*S.* Enteritidis) and *S.* Typhimurium remained the 2 most common serovars

causing human salmonellosis, with *S.* Enteritidis accounting for 30% of cases (DEFRA, 2010).

The number of reported incidents of *Salmonella* in chickens in the UK rapidly increased during the late 1980s and early 1990s and has generally declined since 1997, which is thought to be due to the introduction of a vaccine against *S.* Enteritidis (Anonymous, 2001, Cogan and Humphrey, 2003). The number of reported incidents increased by 27% from 196 in 2007 to 249 in 2008; however, this is thought to be related to increased surveillance testing in commercial chicken flocks, rather than increased prevalence of *Salmonella* (DEFRA, 2008c). Recent reports indicate the prevalence of *Salmonella* serovars in poultry in the UK continues to decrease (DEFRA, 2010).

1.2.1 Sources of human salmonellosis

The main source of human salmonellosis is through consumption of contaminated food, particularly poultry meat and eggs (Little et al., 2007, Lublin and Sela, 2008, Currie et al., 2005, Chittick et al., 2006, Braden, 2006, Mead, 2004). Other sources of infection include beef, dairy products, pork and fish/shellfish (Anonymous, 2006, Braden, 2006). Food products such as salad vegetables, spices, herbs, chocolate and peanut butter have also been implicated in outbreaks of *Salmonella* (DuPont, 2007, DEFRA, 2008c, Anonymous, 2009, Werber et al., 2005, DEFRA, 2010). Handling reptiles in zoological gardens or keeping pet reptiles is also a potential risk for human salmonellosis, with transmission occurring through direct contact with the reptile or its surrounding environment (Pedersen et al., 2009).

The infectious dose for human salmonellosis is estimated to be between 10^6 – 10^8 CFU, although it has been suggested that this dose is significantly lower in many cases due to differences in the physiological state of the bacteria, temperature or pH (Mastroeni, 2006). Consistent with this, low numbers of bacteria are usually recovered from fatty foods implicated in *Salmonella* outbreaks, including chocolate and cheese, suggesting a low infectious dose caused disease (Werber et al., 2005). Clinical symptoms for infection include diarrhoea, abdominal pain, fever, headaches, vomiting and

joint pain (Santos et al., 2011). Additionally, *S. enterica* serovars can cause invasive disease in humans, resulting in bacteraemia (Jones et al., 2008). Prevalence of individual *Salmonella* serovars varies depending on the country (Langridge et al., 2009). The United States and Canada have a uniquely high prevalence of *S.* Heidelberg (Zhao et al., 2008). Zhao et al (2008) reported a 21.6% prevalence of *S.* Heidelberg between 2002 and 2006 in retail meat in America, with the main source being poultry meat (96.6%) (Zhao et al., 2008). In under-developed countries, such as sub-Saharan Africa, non-typhoidal *Salmonella* (NTS) is more associated with invasive disease and is therefore one of the most common causes of paediatric bacteraemia, resulting in high levels of morbidity and mortality (Ikumapayi et al., 2007).

1.2.2 Sources of *Salmonella* infection in poultry

Animal feed including feed ingredients and compound feed are thought to be a source of *Salmonella* infection in poultry; therefore, are also a potential risk for human salmonellosis (Papadopoulou et al., 2009). Animal feed can easily become contaminated and cross-contaminated from one site to another by infected rodents or other wildlife, such as gulls (Nesse et al., 2005). In addition, feed ingredients can become contaminated in feed mills, leading to contamination of animal feed (Papadopoulou et al., 2009). However, there are Codes of Practice monitoring this issue and animal feed contamination rates are low. In 2010, 0.9% of animal feedstuff and feedstuff ingredients were contaminated with *Salmonella* in the UK and only 4.7% of the *Salmonella* serovars isolated were considered of public health significance (DEFRA, 2010). Other sources of *Salmonella* infection in poultry include carry-over of infection from one flock to another due to inadequate cleaning and disinfecting of houses, cross-contamination between houses and reservoirs of infection on farms, such as rodents and flies (Carrique-Mas et al., 2008, Davies and Breslin, 2001).

1.2.3 Prevalence of *Salmonella* in broilers

Between October 2005 and September 2006, an European Union (EU)-wide survey was carried out to establish the baseline prevalence of *Salmonella* in

broiler flock holdings to determine a baseline of infection rates (Snow et al., 2008). In the UK, 382 holdings were sampled. The prevalence of *Salmonella* was found to be 10.7%. The most common serovars isolated were *S.* Ohio, followed by *S.* Kedougou (Snow et al., 2008). *S.* Enteritidis was not isolated during this survey and *S.* Typhimurium was only found on a single holding (Snow et al., 2008).

The Food Standards Agency (FSA) carried out a survey between May 2007 and September 2008 of *Campylobacter* and *Salmonella* contamination of fresh chicken meat at the point of sale in the UK (FSA, 2009). The weighted prevalence of *Salmonella* in fresh chicken meat was 6.6%, with *S.* Kentucky being the most common serovar isolated (FSA, 2009). The prevalence of *S.* Enteritidis and *S.* Typhimurium on fresh chicken meat was low, which mirrored the picture on broiler farms (Snow et al., 2008). Factors such as country of purchase, country of origin, chilled or frozen chicken meat and whole or portioned chicken meat were found to have an impact on *Salmonella* contamination, with country of purchase causing a more significant impact (FSA, 2009).

1.2.4 Prevalence of *Salmonella* in layers

An EU survey of *Salmonella* infections on commercial laying farms in the UK was carried out between October 2004 and September 2005 (Snow et al., 2007). 436 holdings were selected at random throughout the UK for sampling, with 11.7% of them found to be positive for *Salmonella* (Snow et al., 2007). *S.* Enteritidis, followed by *S.* Typhimurium, were the most common serovars isolated (Snow et al., 2007). However, it has been estimated that the prevalence of *Salmonella* on egg-laying holdings in the UK is higher than reported (Arnold et al., 2010). Using the Bayesian methods to analyse data from the EU survey it was estimated that the prevalence of *Salmonella* on egg-laying farms is 18%, rather than 11.7% (Arnold et al., 2010). The lower estimation was suggested to be due to the survey not taking into account the lack of sensitivity of voluntary surveillance and due to only one flock per holding being sampled (Arnold et al., 2010).

The Veterinary Laboratories Agency (VLA) carried out a follow up study on 23 holdings identified as *Salmonella* positive in the EU survey (Carrique-Mas et al., 2008). The VLA survey aimed to identify the proportion of houses in which there was a carry-over of *Salmonella* from one flock to the next and to determine the efficacy of the cleaning and disinfecting procedures on the empty houses, once infected flocks had been removed (Carrique-Mas et al., 2008). Results identified 11/13 cage houses and 4/7 free-range houses as *Salmonella* positive, with the majority of flocks being infected with the same serovar, as detailed in the EU survey (Carrique-Mas et al., 2008). Even though a direct relationship between the lack of effectiveness of cleaning and disinfecting procedures with carry-over to new flocks could not be proven in this study, it was clear that satisfactory cleaning and disinfecting procedures were not achieved in the majority of houses in this study. Reasons for *Salmonella* contamination on egg-laying farms were concluded to be due to poor cleaning and disinfecting procedures and the presence of a reservoir of infection, such as flies or rodents on these farms (Carrique-Mas et al., 2008).

1.2.5 Impact of *Salmonella* infection

Although cases of human salmonellosis in the UK are generally declining each year, *Salmonella* infection still remains a great burden clinically, economically and on morbidity and mortality. A study investigating the cost of *S.* Typhimurium and *S.* Enteritidis infections in England to the economy, estimated it to be more then £6.5 million in 2008, based on the number of reported cases (Santos et al., 2011). The highest cost of *Salmonella* infection was to the NHS, although cost to GP practices, GP home visits, patients (transport etc) and unpaid time off work were also taken into account (Santos et al., 2011).

1.3 Control measures for *Salmonella* infection

Prevention of S*almonella* infection in poultry is important because that will control entry of it into the food chain and thereby reduce the occurrence of human salmonellosis. A number of disease prevention strategies to reduce

spread of infection in poultry have been identified and *Salmonella* prevalence in poultry is monitored closely (Snow et al., 2007, DuPont, 2007).

1.3.1. *Salmonella* National Control Programme (NCP) in the UK

The NCP for *Salmonella* in breeding flocks was implemented in 2008 and aimed to soon have no more than 1% of breeding flocks testing positive for those *Salmonella* serovars most significant to public health (DEFRA, 2007a). The serovars chosen were the 5 most frequently isolated from humans and included *S.* Enteritidis, *S.* Typhimurium, *S.* Virchow, *S.* Hadar and *S.* Infantis. The NCP required that all registered breeding flocks should have specific samples taken from them on a regular basis. The samples would then be submitted to a laboratory, authorised by the Competent Authority, for the detection of the *Salmonella* serovars significant to public health (DEFRA, 2007a). If *S.* Enteritidis or *S.* Typhimurium were detected in a breeding flock, it would be slaughtered to prevent the serovars from entering the food chain. In 2010, 0.82% of flocks sampled tested positive for *Salmonella* (Anonymous, 2010).

Since the introduction of the NCP in breeding flocks, NCPs have also been introduced for layers (DEFRA, 2007b), broilers (DEFRA, 2008a) and turkeys (DEFRA, 2008b). The NCP for layers aimed to have a 10% reduction in *Salmonella* serovars significant to public health by the following year, with an initial baseline of 8% for *S.* Enteritidis and *S.* Typhimurium (DEFRA, 2007b). The NCP for broilers had the same initial aim as the NCP for breeders, with no more than 1% of flocks testing positive for *Salmonella* serovars significant to public health (DEFRA, 2008a). A baseline survey was carried out on turkey breeding and fattening farms between 2006 to 2007 (Snow et al., 2011). *Salmonella* prevalence was found to be 20.1% in breeding turkeys and 37.7% in fattening turkeys (Snow et al., 2011). *S.* Kottbus was the most frequently isolated serovar in both breeding and fattening turkeys (Snow et al., 2011). The NCP for breeding and fattening turkeys was implemented in 2010 and includes all 250 flocks in the UK (DEFRA, 2008b).

A baseline survey has also been carried out in breeding and slaughter pigs, to provide information for a NCP for pigs, which will be implemented in the future (EFSA, 2009, EFSA, 2008b, EFSA, 2011, EFSA, 2008a). The most common serovars isolated from breeding and slaughter pigs were *S.* Typhimurium and *S.* Derby (EFSA, 2009, EFSA, 2008b). A significant positive association was made between *Salmonella*-positive holdings of breeding pigs and *Salmonella*-positive slaughter pigs (EFSA, 2011). The breeding pigs baseline survey also revealed a positive correlation between increased size of pens and increased chance of being *Salmonella*-positive, although positivity varied from country to country (EFSA, 2011). The slaughter pigs baseline survey showed an association between *Salmonella*-positive lymph nodes and *Salmonella* surface contamination of pig carcasses (EFSA, 2008a).

1.3.2. Vaccination against *Salmonella* infection in poultry

Horizontal transmission of *Salmonella* infection in intensively reared poultry farms and vertical transmission to eggs is difficult to control. Vaccination is regarded as the optimum method of controlling and preventing spread of the disease (Barrow, 2007). Several vaccines have been developed and are available for commercial use. Although current vaccines have not succeeded in completely eliminating *Salmonella* infections in poultry, they have significantly reduced the prevalence of *S.* Enteritidis (Cogan and Humphrey, 2003).

Initially, vaccines used to prevent *Salmonella* infection constituted killed or inactivated bacteria. Nobilis Salenvac T® is a commercially available, inactivated, iron-restricted vaccine that immunizes poultry against *S.* Enteritidis and *S.* Typhimurium. Research on the efficacy of this vaccine has shown it greatly reduces caecal shedding of the challenge strain, compared to shedding in unvaccinated chickens (Clifton-Hadley et al., 2002). However, no difference in systemic colonisation was found between the vaccinated and unvaccinated chickens, suggesting not all aspects of the immune response are activated by the vaccine (Clifton-Hadley et al., 2002).

Live vaccines are commercially available and have been shown to be greater stimulators of the chicken immune system. The AviPro® Megan® Egg live vaccine is available for commercial use in the USA, New Zealand and Dominican Republic. This vaccine has been shown to significantly protect hens and their eggs against *S.* Enteritidis colonisation (Hassan and Curtiss, 1997). The main vaccines currently used in the UK are the Lohmann AviPro TAD *Salmonella* vac E® and TAD *Salmonella* vac T® live vaccines, which are delivered via drinking water (http://www.bnotharel.com/apage/523. php). A combination of these two vaccines has been shown to greatly reduce reproductive tract colonisation and internal egg contamination (Gantois et al., 2006). Live attenuated vaccines have been shown to stimulate the cellular as well as the humoral immune response, unlike killed or subunit vaccines. However, live attenuated vaccines are potentially unsafe due to the possibility of them becoming virulent (Barrow, 2007). To develop effective vaccines an understanding of the poultry immune response against *Salmonella* infection is vital (Desin et al., 2011, Beal et al., 2006b).

1.3.3 Consumer awareness of the importance of food safety

To reduce the incidence of foodborne illnesses, consumers need to be aware of the risk factors that would result in them becoming ill and also methods to avoid illness. Using a panel of 40 nationally known experts on food safety, the behaviours most associated with reducing the risk of contracting foodborne pathogens, including *Salmonella,* have been ranked (Hillers et al., 2003). In total, eight behaviours were ranked as being important for the control of *Salmonella* infection, which was more than for most pathogens. The top ranked behaviours included avoiding food associated with *Salmonella* (raw eggs and chicken) and preventing cross-contamination by cleaning knifes, cutting boards and surfaces (Hillers et al., 2003). Other behaviours included the use of a thermometer to cook foods adequately and washing hands with warm soapy water before and after handling raw poultry meat (Hillers et al., 2003). Other studies have shown that educating consumers about disinfection procedures when handling food would be of great clinical and economic benefit (Duff et al., 2003).

1.3.4 Genetic resistance to *Salmonella* infection in poultry

Current control and prevention methods for *Salmonella* infection in chickens have drawbacks including cost and public health issues. Breeding *Salmonella* resistant chickens has its advantages, as it is a low risk strategy to control colonization by the bacteria (Wigley, 2004). Several genetic loci have been linked to systemic resistance or susceptibility in chickens including, *NRAMP1, MHC class I* and *SAL1* (Wigley, 2004).

Inheritance of the whole *SAL1* region has been shown to be associated with resistance to salmonellosis in poultry (Mariani et al., 2001). Further studies on the *SAL1* locus against *Salmonella* infection have shown that resistance is not expressed at the level of the gut but systemically by the mononuclear phagocyte system (Wigley et al., 2002a). *Salmonella*-susceptible chicken lines had higher systemic bacterial counts and for a longer period of time, when compared with resistant ones (Wigley et al., 2002a). They also exhibited bacteraemia and a higher level of pathology, whereas resistant birds showed no bacteraemia and infection was limited to the spleen and liver, where it was then cleared (Wigley et al., 2002a). It has also been shown that macrophages from *Salmonella* susceptible chickens phagocytose far less *Salmonella* then *Salmonella*-resistant lines (Wigley et al., 2006).

Genetic resistance to *Salmonella* colonisation has also been shown at the level of the gut (Wigley, 2004). In-bred chickens, which are either resistant or susceptible to *Salmonella* colonisation, have differences in the rate of intestinal flow, the level to which the gut is colonised and the length of time the gut is colonised (Barrow et al., 2004). Resistance to gut colonisation by *Salmonella* is not genetically linked to the *MHC, NRAMP1* or *SAL1* loci (Wigley, 2004). It is also not thought to be linked to the adaptive immune response, as differences in IgG and IgA produced against *Salmonella* infection were not found between resistant and susceptible chicken lines (Barrow et al., 2004). Resistance to gut colonisation may be conferred by the innate immune response, as genetic resistance has been linked to differential heterophil function from resistant and susceptible chicken lines (Swaggerty et al., 2003). Heterophils isolated from resistant chicken lines showed significantly greater phagocytosis, degranulation and oxidative burst against

S. Enteritidis, compared to those from susceptible lines (Swaggerty et al., 2003).

1.3.5 Probiotic bacteria

Establishment of adult-type intestinal microflora provides resistance against colonisation by invading pathogens. This “competitive exclusion” concept is now exploited by administering newly hatched chickens with saline solutions containing the alimentary tract contents of an adult bird, to establish adult-type microflora (Mead, 2000). The basis of the competitive exclusion effect is mainly bacteriostatic, rather than bactericidal. Intestinal gut microflora are thought to inhibit pathogens such as *Salmonella* by acting as a physical barrier and filling all the environmental niches, reducing the caecal pH, producing volatile fatty acids and competing for nutrients (Mead, 2000, Dunkley et al., 2009). Probiotic bacteria have also been shown to have immuno-modulatory properties in their host. Administration of probiotics to chickens results in enhanced heterophil degranulation and oxidative burst activity after 24 hours (Farnell et al., 2006). Enhanced phagocytosis of *S.* Enteritidis in the gut and enhanced IgM and IgG titres have also been found after probiotic administration (Koenen et al., 2004). However, immuno-modulatory effects of probiotics are sometimes inconsistent and are suggested to vary due to the age of the host, host genetics, dose of probiotic bacteria and type of probiotic bacteria administered (Farnell et al., 2006, Koenen et al., 2004).

1.4 *S. enterica* colonisation in poultry

S. Typhimurium is one of the most well studied zoonotic *Salmonella* serovars in poultry (Beal et al., 2006b, Morgan et al., 2004, Withanage et al., 2004, Barrow et al., 1987, Smith and Tucker, 1975). In 1 day old chicks, *S.* Typhimurium colonises the gastrointestinal tract (GIT) and causes rapid severe systemic infection, leading to high morbidity and mortality rates (Barrow et al., 1987). However, in older chicks *S.* Typhimurium is limited to the GIT and the chickens usually exhibit no clinical symptoms and low mortality rates (Barrow et al., 1987, Morgan et al., 2004).

1.4.1 Caecal colonisation

Salmonella mainly infects poultry via the faecal-oral route and colonises the GIT rapidly. Following oral infection, *S.* Typhimurium can be detected in the caecal contents of chicks by 1 day post infection (DPI), at high levels (Withanage et al., 2004, Withanage et al., 2005b). The amount of *S.* Typhimurium in the caecal contents has been shown to peak at 6-7 DPI and then begins to decline (Withanage et al., 2005b, Beal et al., 2004). Low concentrations of *S.* Typhimurium can still be detected in the caecal contents around 28 DPI and chickens can still be positive for *Salmonella* by cloacal swab after enrichment past this time point (Withanage et al., 2005b, Beal et al., 2004). During secondary infection of chickens, lower concentrations of *S.* Typhimurium are detected in the caecal contents (Beal et al., 2004). Infected chickens demonstrate an enhanced clearance of *S.* Typhimurium and no viable counts can sometimes be detected in the caecal contents as early as 14 days post secondary infection (DPSI) (Beal et al., 2004, Withanage et al., 2005b).

1.4.2 Systemic infection

Following invasion of the gut, *Salmonella* is thought to be taken up by cells of the mononuclear phagocyte system, including macrophages and dendritic cells and transported via the lymphatic system to systemic sites (Chappell et al., 2009). Several studies have shown that *Salmonella* can survive within avian macrophages and replicate inside them, suggesting that they are the key for systemic invasion by *Salmonella* (Henderson et al., 1999, Wigley et al., 2002b). *Salmonella* has been shown to colonise systemic sites such as the liver, spleen, ovaries and bone marrow in poultry (Beal et al., 2004). Following *S.* Typhimurium infection of 1 week old chicks, bacteria can be detected in the liver by 7 DPI. However, this can be as early as 1 DPI in 1 day old chicks (Withanage et al., 2004, Withanage et al., 2005b). Following secondary infection of chickens with *S.* Typhimurium, chickens show a high level of protection against systemic infection, with no bacteria detected at systemic sites (Withanage et al., 2005b).

1.4.3 Pathology and clinical symptoms

Following primary infection with *S.* Typhimurium, 1 day old chicks display gross pathology including diarrhoea, bloody caecal contents, hepatosplenomegaly and early signs of anorexia (Withanage et al., 2004). As well as diarrhoea and anorexia, clinical symptoms include vent staining, disinclination to drink, lethargy, emaciation and eventually death (Barrow et al., 1987). In older chicks, less severe pathological findings are often observed at post mortem including serous typhlitis caused by inflammation of the caeca, hyperaemia and oedema of the caecal lamina propria and caecal tonsils, swelling and necrosis of single epithelial cells and dilated crypts with necrotic debris (Desmidt et al., 1998, Henderson et al., 1999, Withanage et al., 2005b). Although pathological findings can be observed in older chicks, they usually have no clinical symptoms of disease. This is a problem with animals such as broiler chickens as it allows them to shed *Salmonella* in their faeces asymptomatically, leading to horizontal transfer of infection, therefore increasing the frequency of serovars such as *S.* Typhimurium entering the food chain (Marin and Lainez, 2009, Kim et al., 2007).

1.4.4 *S.* Pullorum

S. Pullorum is highly host-adapted to poultry and rarely causes disease outside of this host, in which it causes severe systemic disease and septicaemia (Shivaprasad, 2000). In contrast to broad-range serovars, host-restricted serovars are not limited to the GIT. Upon intestinal invasion, *S.* Pullorum only colonises the GIT to low concentrations and instead targets lymphatic tissue, such as the Bursa of Fabricius, and causes rapid systemic infection (Henderson et al., 1999). The differences in the pathogenesis of disease caused by broad-range and host-restricted serovars are thought to be due to differences in early stage pathogenesis (Chappell et al., 2009).

Once the acute systemic infection has subsided *S.* Pullorum can develop into a persistent infection and survive in low numbers, in splenic macrophages, for months (Wigley et al., 2001). Intra-macrophage survival has been shown to be required for *S.* Pullorum persistence in poultry (Wigley et al., 2001). It has been reported that when a chicken reaches sexual maturity and starts

egg laying (commercial laying hens typically start around 17 or 18 weeks of age) the number of *Salmonella* in the spleen increases dramatically and spreads to the reproductive tract, leading to vertical transmission to the eggs (Wigley et al., 2005). As the immune system in chickens is suppressed at point-of-lay it has been suggested that this may play a role in the increased number of *Salmonella* and the spread of infection at this time point (Wigley et al., 2001, Wigley et al., 2005).

1.5 *Salmonella enterica* pathogenesis

Virulence genes have been identified in the *Salmonella* genome, which have been shown to be important for intestinal colonisation and systemic invasion. These virulence genes are often found in clusters that are termed *Salmonella* pathogenicity islands (SPI) and are thought to have been acquired through horizontal transfer (Parkhill et al., 2001). SPIs are important for the evolution of the bacteria as a pathogen (Parkhill et al., 2001). As well as containing virulence genes, SPIs have other characteristics including a lower GC content compared to the rest of the genome and an association with genes encoding tRNA genes (Hensel, 2004, Mills et al., 1995). Some of the SPIs also contain type three secretion systems (T3SS).

1.5.1 *Salmonella* pathogenicity island 1

SPI-1 spans a 40kb region of the 59-60 minute region of the *Salmonella* genome (Mills et al., 1995, Hensel, 2004). It contains a number of genes essential for invasion, including *prgH* and is absent from the non-invasive *E. coli* (Behlau and Miller, 1993, Mills et al., 1995). As closely related *E. coli* does not contain SPI-1, it has been suggested that SPI-1 was obtained by horizontal transfer from another organism and it is thought to have significantly advanced *Salmonella* as a pathogen (Mills et al., 1995). SPI-1 encodes a T3SS and also several proteins secreted by the T3SS (Galan, 1996, Galan and Zhou, 2000). The T3SS forms a needle-like structure and translocates effector proteins into the host cell in a contact-dependent manner, through interactions with the host cell membrane (Galan, 1996, Galan and Zhou, 2000, Ehrbar and Hardt, 2005, Schraidt et al., 2010, Haraga

et al., 2008). Environmental factors such as growth phase and oxygen tension are also thought to act as cues for T3SS formation and invasion into host cells (Lee and Falkow, 1990). The effector proteins translocated into the host cell stimulate cytoskeleton rearrangement and membrane ruffling, which allows bacterial uptake into the cell by macropinocytosis (Galan and Zhou, 2000, Hardt et al., 1998b, Haraga et al., 2008).

Several effector proteins translocated by the SPI-1 T3SS and involved in bacterial invasion into host cells have been identified. Hardt et al (1998) identified a protein called SopE, encoded on a cryptic bacteriophage in *S.* Typhimurium and demonstrated that it is transported by the SPI-1 T3SS (Hardt et al., 1998b). Mutants of *sopE* have a decreased level of cell invasion compared with wild type (WT) strains (Hardt et al., 1998b, Mirold et al., 2001). Differences in the morphology of membrane ruffles have been observed with *sopE* mutants, suggesting the SopE protein plays a role in aiding bacterial entry into cells by membrane ruffling (Hardt et al., 1998b). Another effector protein translocated into the host cell by the SPI-1 T3SS is SopB, which has been shown to have phosphatase activity (Norris et al., 1998). It is hypothesised that the SopB protein leads to the accumulation of inositol 1, 4, 5, 6-phosphate. This accumulation inhibits PtdIns 3, 4, 5 phosphate from closing Cl^- ion channels, therefore leading to an increase in Cl^- ions and resulting in diarrhoea (Norris et al., 1998). Mutations in *sopB* have been shown to reduce the magnitude of secretory and inflammatory responses caused by *Salmonella* invasion by significant amounts compared to WT strains (Wood et al., 1998). In the case of a triple mutation of *sopB, sopE* and *sopE2 Salmonella* has been shown to be >100-fold less invasive then WT strains (Mirold et al., 2001).

Some research has focused on the effect of attenuating SPI-1 on the pathogenicity of *Salmonella in vitro* in cell lines and *in vivo* in poultry. A range of SPI-1 mutant strains of *Salmonella* including *S.* Gallinarum, *S.* Pullorum, *S.* Typhimurium and *S.* Enteritidis have been studied. SPI-1 mutant strains were found to be significantly less invasive in non-phagocytic chick kidney cells (CKC) compared to WT strains (Jones et al., 2001, Wigley et al.,

2002b). SPI-1 mutant strains have also been found to be significantly less invasive in human epithelial cells (Caco2) compared to WT strains (Desin et al., 2009). *In vivo* infection experiments with SPI-1 mutant strains have shown reduced intestinal colonisation and invasion of systemic sites, as well as lower mortality rates, compared to WT strains (Jones et al., 2007, Jones et al., 2001, Wigley et al., 2002b, Desin et al., 2009). Overall the studies show that SPI-1 is important for invasion, as SPI-1 mutant colony counts are significantly reduced *in vitro* and *in vivo*. However, the absence of SPI-1 encoded functions only attenuates *Salmonella* invasion, suggesting SPI-1 is important but not essential for pathogenicity.

1.5.2 *Salmonella* pathogenicity island 2

SPI-2 is a 40 kb region of the *S. enterica* genome (Hensel et al., 1997). It is not present in *S. bongori* and it is thought to have been acquired after *S. enterica* diverged from *S. bongori* (Hensel et al., 1997). SPI-2 is characteristic of pathogenicity islands, having a lower GC content (41.4%) then the core genome and being inserted next to the $tRNA^{Val}$ gene (Hensel et al., 1997, Hensel, 2004). Another T3SS is encoded by SPI-2 (Shea et al., 1996). The SPI-2 T3SS is thought to have been acquired at a separate time to the SPI-1 T3SS and it has been shown to be important for virulence following epithelial invasion, unlike SPI-1 T3SS (Shea et al., 1996, Jones et al., 2001).

Salmonella replicates and survives within host cells in *Salmonella* containing vacuoles (scv). After 4-6 hours of *Salmonella* replication, long filamentous lysosomal membrane glycoprotein (lgp) structures can be observed, extending from the scv and connecting them (Garcia-del Portillo et al., 1993). The formation of lgp structures depends on viable-intracellular bacteria, an acidic pH and possibly host machinery to form and maintain the filamentous structures (Garcia-del Portillo et al., 1993). A virulence gene encoded on SPI-2 termed *sifA* has been identified as being required for the formation of these lgp structures and has been shown to be important for virulence in mice (Stein et al., 1996). It has been suggested that SifA is translocated to the vacuolar membrane by the SPI-2 T3SS and is required to maintain the

integrity of the scv (Beuzon et al., 2000). Another SPI-2 effector protein called spiC is directly transported into the host cell cytosol by the SPI-2 T3SS and interferes with intracellular trafficking (Uchiya et al., 1999). The role of spiC protein involves directly preventing the fusion of scv with phagosomes containing endosomes and lysosomes (Uchiya et al., 1999).

Some studies have found that SPI-2 mutants fail to persist in HD11 chicken macrophage-like cells, suggesting that SPI-2 is essential for intra-macrophage survival (Jones et al., 2001). *Salmonella* has been shown to survive in macrophages by causing an up-regulation of IL-10 via a protein kinase A signalling pathway, in an SPI-2 dependant manner (Uchiya et al., 2004). IL-10 has an inhibitory effect on macrophages and therefore creates a favourable environment for *Salmonella* to survive (Uchiya et al., 2004). However, more recently it has been found SPI-2 mutants can survive in HD11 cells and SPI-2 has been suggested to be more important for systemic distribution (Wisner et al., 2011b). SPI-2 mutant strains show no reduction of invasiveness in chick epithelial cell lines or *in vivo* in chickens (Wigley et al., 2002b, Jones et al., 2001). However, infection of chickens with SPI-2 mutants showed they were fully attenuated for virulence and absent from systemic sites (Jones et al., 2001, Wigley et al., 2002b, Jones et al., 2007). Infection of chickens with a *S.* Gallinarum SPI-2 mutant resulted in no morbidity and mortality, compared to 50% mortality observed in the group infected with a WT strain (Jones et al., 2001). These results suggest SPI-2 is essential for *S.* Gallinarum virulence and its ability to cause systemic fowl typhoid (Jones et al., 2001). In addition, no morbidity and mortality was observed during infection of 1 week old chicks with SPI-2 attenuated strains of *S.* Pullorum (Wigley et al., 2002b). Overall, the studies show SPI-2 is essential virulence and persistence in chickens; however, more research is needed to determine the role of SPI-2 in systemic survival.

1.5.3 *Salmonella* pathogenicity islands 3-6

Since the identification of SPI-1 and SPI-2, further clusters of virulence genes on the *Salmonella* genome have been identified and are all characteristic of SPIs. SPI-3 is a 17-kb pathogenicity island containing 10 open reading

frames (ORF) and 6 transcriptional units (Blanc-Potard et al., 1999). It has a GC content of 47.5% and is inserted next to the tRNA SelC locus (Blanc-Potard and Groisman, 1997). SPI-3 contains an *mgtCB* operon which encodes essential virulence genes (Blanc-Potard and Groisman, 1997, Hensel, 2004). The *mgtCB* operon, particularly the *mgtC* gene has been shown to be required for virulence in mice and is essential for *Salmonella* replication within macrophages (Blanc-Potard and Groisman, 1997). It has also been shown that expression of *mgtCB* is essential for growth in Mg^{2+} limiting environments (Blanc-Potard and Groisman, 1997).

SPI-4 is a 25kb region of the *Salmonella* genome located at 92 min on the chromosomal map (Wong et al., 1998). Publication of the whole genome sequence of *S.* Typhimurium LT2 organised the SPI-4 operon into 6 ORFs (McClelland et al., 2001), which have since been renamed *siiA*-*siiF* (Morgan et al., 2004). A type I secretion system has been shown to be encoded on SPI-4, as well as a 595 kDa secreted protein encoded by *siiE* (Morgan et al., 2007). HilA is encoded on SPI-1 and is thought to regulate either the transcription or secretion of the SiiE protein (Morgan et al., 2007). *In vivo* infection experiments have shown SPI-4 has a role in enteric colonisation of *Salmonella* in mice and calves (Morgan et al., 2004, Pullinger et al., 2007).

SPI-5 is inserted adjacent to the tRNA *serT* gene (Wood et al., 1998). It has been shown to encode 5 novel virulence genes named *pipA, pipB, pipC, pipD* and *orfX,* as well as the SPI-1 T3SS translocated SopB protein (Wood et al., 1998). The magnitude of inflammatory and secretory responses elicited by *S.* Dublin have been shown to significantly decrease if *pipA, pipB, pipD* or *sopB* genes are attenuated, implicating SPI-5 in enteric pathogenesis (Wood et al., 1998).

SPI-6 is a 59-kb region of the *Salmonella* genome, inserted next to the tRNA *aspV* gene (Townsend et al., 2001). SPI-6 has also been termed *Salmonella enterica* centisome 7 genomic island (SCI) (Folkesson et al., 2002). SPI-6 encodes the *saf* and *tcf* fimbrial operons (Townsend et al., 2001, Parkhill et al., 2001). It also encodes putative proteins with genetic similarities to

virulence genes found in other Gram-negative bacteria. The SCI proteins have been shown to localize to cytoplasmic and periplasmic components and the inner and outer membranes, suggesting they have a role in secretion or organelle biosynthesis (Folkesson et al., 2002). In addition, SCI mutants are impaired when trying to invade eukaryotic host cells, suggesting the SCI proteins are important for invasion (Folkesson et al., 2002).

1.5.4 *Salmonella* pathogenicity islands 7-10

SPI 7-10 have been mainly identified in the *S.* Typhi genome, a serovar restricted to humans. Differences in the *S.* Typhi genome compared to other *Salmonella* serovars include the presence of these additional SPIs and a large number of pseudogenes. It has been suggested these differences may contribute towards the host specificity of *S*. Typhi (Parkhill et al., 2001, Hansen-Wester and Hensel, 2002).

SPI-7 or “major pathogenicity island” is a 147kb region of the *S.* Typhi genome, inserted adjacent to the tRNA *pheU* gene (Hansen-Wester and Hensel, 2002, Hensel, 2004). This region has been shown to encode a number of virulence genes including the ViaB proteins, which are necessary for synthesis and export of the Vi capsular antigen (Hashimoto et al., 1993, Hansen-Wester and Hensel, 2002). It also encodes the *pilL-pilV* gene cluster encoding the type IVB pilus, which has been shown to be involved in the entry of *S.* Typhi into human intestinal cells (Hansen-Wester and Hensel, 2002, Zhang et al., 2000). SPI-7 also encodes another virulence protein sopE, which is encoded within a temperate prophage in *S.* Typhimurium (Hardt et al., 1998b, Mirold et al., 2001).

Based on the genome sequence of *S.* Typhi, SPI-8, SPI-9 and SPI-10 have been suggested, as they have characteristics of pathogenicity islands (Parkhill et al., 2001). SPI-8 has been shown to encode resistance to bacteriocins, SPI-9 encodes a type I secretion apparatus like SPI-4 and SPI-10 has been shown to encode *sefA-R* chaperone-usher fimbrial operon, as well as phage 46 (Parkhill et al., 2001, Townsend et al., 2001).

1.6 Immune response against *Salmonella*

The severity of *Salmonella* infection and the strength of the immune response produced against it are dependent on the infecting serovar, host genetics and host age (Morgan et al., 2004). High mortality rates are found in 1 day old chicks infected with *Salmonella* (Barrow et al., 1987). However, older chicks have no clinical symptoms and low mortality rates, which is thought to be due to a developed immune response (Beal and Smith, 2007, Withanage et al., 2005b). In addition, *Salmonella* serovars infecting a broad-range of hosts cause a different immune response to *Salmonella* serovars that are host restricted, which is thought to be due to differences in early stage pathogenesis (Chappell et al., 2009, Kaiser et al., 2000).

1.6.1 Innate immune response in poultry

The innate immune system is the first line of defence against pathogens entering the host. It includes the complement system and cells responsible for pathogen recognition, phagocytosis, immune modulation and activation of the adaptive immune response (Juul-Madsen, 2008). Physical barriers also prevent pathogen entry into the host and include the skin, mucosal surfaces and the normal commensal flora present on different surfaces (Juul-Madsen, 2008, Mead, 2000). Following infection several acute phase proteins increase significantly in the blood and include proteins such as C-reactive protein, mannan-binding lectin and fibrinogen (Juul-Madsen, 2008).

The complement system is a large component of the innate immune system and is found in chickens as well as in mammals (Kogut et al., 2003, Juul-Madsen, 2008). The proteins of the complement system circulate in the serum in an inactive form and become sequentially activated, in a cascade type manner, upon stimulation by a pathogen (Juul-Madsen, 2008). The complement system has 3 pathways of activation including the classical, lectin and alternative pathways, which all result in opsonisation of the pathogen, leading to enhanced phagocytosis, induction of inflammatory responses and enhancement of B and T cell responses (Juul-Madsen, 2008, Carroll, 2004).

Cells of the innate immune response have been well characterised in mammals and include natural killer (NK) cells, neutrophils, macrophages and dendritic cells. In mammals, NK cells have been shown to have cytotoxic activity towards pathogens (Cooper et al., 2001). NK cells also have an immuno-regulatory role, through secretion of cytokines (Cooper et al., 2001). Several characteristics of chicken TCR0 cells have lead to the conclusion that they are the avian equivalent of mammalian NK cells (Gobel et al., 2001, Gobel et al., 1994). These include intracellular expression of CD3, as well as the expression of several surface molecules such as CD8α chains, a binding site for chicken IgG and the IL-2 receptor (IL-2R) (Gobel et al., 1994). TCR0 cells also lack expression of CD4 and other antigens characteristic of T and B cells and exhibit spontaneous cytotoxic activity towards LSCC-RP9 cells, which are NK susceptible cells (Gobel et al., 1994). Sub-populations of NK cells have recently been defined in chickens including a $CD3^{-}CD8\alpha^{+}$ population and a $CD3^{-}CD8\alpha^{dim}$ population (Jansen et al., 2010).

Heterophils are avian polymorphonuclear cells (equivalent of mammalian neutrophils) that have been shown to have potent antimicrobial activity against bacterial species, including *Salmonella* (van Dijk et al., 2009). Heterophils exert their antimicrobial activity through oxidative burst and degranulation (Wu and Kaiser, 2011). Heterophils release antimicrobial peptides including cathelicidin-2 through degranulation, which has been shown to inhibit *S.* Typhimurium growth (van Dijk et al., 2009). Heterophils up-regulate multiple cytokines and chemokines upon *Salmonella* infection, showing they also have an immuno-regulatory role (Kogut et al., 2005, Kogut et al., 2003). The number of heterophils in the peripheral blood initially decreases in response to *S.* Typhimurium infection, but significantly increases again by 20 hours post infection (HPI) (Meade et al., 2009). This initial reduction is thought to indicate an influx of heterophils into the caecum, which agrees with other studies (Henderson et al., 1999, Desmidt et al., 1998, Withanage et al., 2004, Withanage et al., 2005b). Systemic colonisation by *S.* enterica serovars stimulates heterophil infiltration into organs, such as the spleen and liver (Withanage et al., 2004).

Macrophages are always present in the circulation, acting as scavengers and collecting cellular debris; however, they also have an important role in pathogen recognition and kill pathogens by phagocytosis (Kaspers, 2008). Phagocytosis of *S.* Typhimurium by avian macrophage cell lines is significantly enhanced by IFN-y (Okamura et al., 2005). Once macrophages have phagocytosed a pathogen they will kill it by releasing reactive oxygen species (ROS) (Withanage et al., 2005a). Macrophage cell lines from chickens resistant to *Salmonella* infection have been shown to clear bacteria within 24 hours, which was associated with a strong oxidative burst (Wigley et al., 2002a). These findings were in contrast to the weak response seen by macrophages from *Salmonella*-susceptible chickens; therefore macrophages are thought to be important for resistance to *Salmonella* infection (Wigley et al., 2002a). Macrophages are also a major antigen presenting cell (APC), therefore they are crucial for the adaptive immune response, as well as the innate immune response (Wu and Kaiser, 2011).

Dendritic cells are antigen presenting cells and have been shown to play an important role in pathogen recognition and activation of the adaptive immune response in mammals (Cooper et al., 2004). Two dendritic cell populations have been characterised, called follicular (FDCs) and interdigitating cells (IDCs) and have also been found in avian species (Del Cacho et al., 2009). FDCs have a star-shaped morphology, express molecular markers such as IgG, IgM, ICAM-1 and VCAM-1 and can induce B cell proliferation (Del Cacho et al., 2009). FDCs are present in the germinal centres of caecal tonsils, Peyer's patches, Harderian gland and spleen (Wu and Kaiser, 2011). IDCs are distinct from FDCs as they are elongated cells rather than star-shaped and they express different surface markers, which include CD45, MHC class I and selectin (Del Cacho et al., 2009).

Avian thrombocytes are the equivalent of mammalian platelets and are the most abundant white blood cell (WBC) in avian blood (Wu and Kaiser, 2011). Thrombocytes have phagocytic functions and have been shown to phagocytose *Salmonella* as well as other bacteria, although to a lesser extent than heterophils (Wigley et al., 1999). Oxidative burst activity has been

shown against a range of Gram-negative bacteria, suggesting thrombocytes have antimicrobial properties (Wigley et al., 1999). Upon stimulation with bacterial lipopolysaccharide (LPS), thrombocytes have increased expression of IL-1β, lL-6, IL-12 and cyclooxygenase-2, suggesting they are important effectors of the innate immune response during bacterial infection (Wu and Kaiser, 2011, Ferdous et al., 2008).

1.6.2 Adaptive immune response in poultry

S. Typhimurium infection of chickens induces a strong adaptive immune response. Studies have consistently shown that primary infection of chickens with *Salmonella* is followed by a rise in antigen-specific serum antibodies, which follow a classic pattern of a rise in specific IgM, followed by a rise in specific IgG and IgA, which all peak around 13 DPI (Beal et al., 2004, Withanage et al., 2005b). IgM then declines to levels similar to uninfected birds (Beal et al., 2004). IgG temporarily declines around 28 DPI and then sharply increases and remains elevated for at least 69 days (Beal et al., 2004). IgA remains constantly elevated for at least 69 DPI (Beal et al., 2004). Although the humoral response has been shown to strongly respond against *Salmonella* invasion, some studies have shown that B cells and antibodies are not essential for clearance of *Salmonella* infection (Desmidt et al., 1998). Furthermore, some studies have shown clearance of *Salmonella* is not affected by the absence of B cells (Beal et al., 2006a). It has been suggested that B cells and antibodies do play a role in *Salmonella* clearance, but other mechanisms exist to clear infection (Desmidt et al., 1998).

Avian T-cell structure and function is very similar to that of mammalian T-cells. T-cells can be divided into 2 main lineages based on the structure of their T-cell receptor (TCR). The 2 main lineages include, one comprising an α light chain and β heavy chain (αβ TCR) and the other lineage comprising a γ light chain and δ heavy chain (γδ TCR) (Berndt et al., 2006, Viertlbock, 2008). Three avian T-cell subpopulations can be detected in the peripheral blood including $TCR1^+$ (γδ TCR), $TCR2^+$ (αβ vβ1 TCR) and $TCR3^+$ (αβ vβ2 TCR) populations (Berndt et al., 2006). The T-cell lineages can be further subdivided by CD4 and CD8 surface molecules into a $CD4^+$ helper T-cell

population and a $CD8^+$ cytotoxic T-cell population, although it has been shown some T-cell populations can express both CD4 and CD8 (Viertlbock, 2008). Berndt et al (2006) showed that when chickens are infected with *S.* Enteritidis the amount of $TCR1^+$ $CD8\alpha^+$ T-cells significantly increases, suggesting a role in the primary immune response against *Salmonella,* as well as the possibility of forming memory T-cells (Berndt et al., 2006).

In mammals, T helper cells (Th) can be divided into Th1 and Th2 cells based on their function (Viertlbock, 2008). Th1 cells drive cell-mediated and inflammatory responses, whereas Th2 cells drive responses against helminthic worms and allergies (Kaspers, 2008). Both responses involve different sets of cytokines and chemokines. Th1 responses in chickens have been well documented but not much is known about Th2 responses (Avery et al., 2004).

Upon *S.* Typhimurium infection of chickens, T cell proliferation has been detected, particularly in *Salmonella* resistant chicken lines and in older chickens, suggesting clearance of enteric *Salmonella* is T-cell dependant (Beal et al., 2005). Rapid T-cell proliferation occurs shortly after primary infection with *S.* Typhimurium (Withanage et al., 2005b, Beal et al., 2004). The number of T cells declines at systemic sites around 20 DPI, coinciding with gut clearance of *Salmonella,* which has been suggested to indicate T-cell trafficking to the intestine (Beal, Powers et al. 2004; Withanage, Wigley et al. 2005). After this decline, antigen specific T-cell proliferation occurs again and T-cells remain elevated for at least 60 DPI (Beal, Powers et al. 2004).

Changes in IFN-γ mRNA levels in the spleen, coincides with changes in the number of T-cells (Beal et al., 2004). Following primary infection, IFN-γ levels peak in the spleen at 13 DPI and after declining peak again at 27 DPI (Beal et al., 2004). Additionally, T-cells have been shown to increase significantly in number between 7 and 14 DPI in the liver (Withanage et al., 2005b). Coinciding with the increase of T-cells in the liver, IFN- γ mRNA levels significantly increase up to 200-fold greater than control levels (Withanage et

al., 2005b). These findings support the idea of IFN- γ mediated T-cell responses to *Salmonella* infection.

1.6.3 Cytokine and chemokine production in poultry

Cytokines are defined as regulatory peptides, with molecular weights typically less than 30 kDa, that act as extracellular signals between cells of the immune system (Eckmann and Kagnoff, 2001). Cytokines can be produced by nearly every cell type and have pleiotrophic effects involving regulating and eliciting immune responses (Kaiser, 2008). Many of the identified mammalian cytokines are present in avian species, although fewer cytokines have been identified in birds compared to mammals (Kaiser et al., 2005). Avian cytokines typically have 25-35% amino acid identity to mammalian orthologues (Weining et al., 1998, Schneider et al., 2000, Degen et al., 2004). Currently, genes for 35 cytokines have been identified in the chicken genome including, 23 interleukins (IL), 8 type I interferons (IFN), IFN-γ, granulocyte macrophage colony-stimulating factor (GM-CSF), and 2 transforming growth factors (TGFs) (Kaiser et al., 2005). Chemokines have more restricted effects on the immune response compared to cytokines, being responsible for regulating circulation of immune cells and recruiting them to sites of inflammation (Kaiser, 2008). Chemokines can be divided into four groups based on the spacing of the first two cysteines at the amino termini including XC, CC, CXC and CX3C (Kaiser, 2008, Martins-Green, 2001, Hughes et al., 2007). A study on the chicken genome has identified 24 chemokines (Kaiser et al., 2005, Kaiser, 2008).

In mammals, cytokines and chemokines can be polarized functionally into type 1 or type 2 immune pathways. These responses are regulated by Th1 and Th2 cells. The chicken immune response differs from the mammalian immune response, as it lacks components of the Th2 response, including eosinophils and IgE (Kaiser et al., 2005). Additionally, a chicken orthologue for the Th2 cytokine IL-5 has been identified as a pseudogene (Avery et al., 2004). This suggests chickens may have a reduced requirement for Th2 cytokines, compared to mammals; however, a cluster of Th2 cytokines has been identified in chickens, encoding for IL-3, IL-4, IL-13 and GM-CSF (Avery

et al., 2004). Chicken IL-4 and IL-13 expression in lymphoidal tissue has been shown to be similar to that in mammals and co-stimulates the proliferation of B cells with CD154 (CD40) (Avery et al., 2004). However, IL-3 is expressed at high levels in lymphoidal and non-lymphoidal tissue compared to mammalian species, suggesting it is differentially regulated in chickens (Avery et al., 2004).

Th1 cytokine responses in avian species have been characterised in more detail then Th2 responses. Early production of IL-12 and IL-18 drive Th1 inflammatory responses and production of IFN-γ (Eckmann and Kagnoff, 2001). IL-12 is a heterodimer, consisting of two subunits, p35 and p40 (Degen et al., 2004). Several lymphoidal cell lines including, HD11, CU91 (T cell derived) and DT-40 (B cell derived) produce IL-12 following stimulation with ligands such as LPS, CpG-ODN and chCD40 (Degen et al., 2004). Chicken IL-12 has been shown to stimulate significant proliferation of spleen cells and production of IFN-γ by T-cells (Degen et al., 2004). ChIL-18 exhibits about 30% sequence similarity to mammalian IL-18 (Schneider et al., 2000). ChIL-18 is important for systemic resistance to *Salmonella* infection (Raupach et al., 2006). Macrophages from resistant chickens produce significantly greater IL-18 than those from susceptible ones (Wigley et al., 2006). This suggests that *Salmonella*-resistant macrophages are more efficient at stimulating IFN-γ production and initiating the adaptive immune response (Wigley et al., 2006). Biologically activate IL-18 can stimulate primary chick spleen cells to produce a significantly increased amount of IFN-γ (Schneider et al., 2000).

A third lineage of Th cells has been identified, called Th17, which produces IL-17 under the influence of IL-23 (Kaiser, 2008). There are 6 members of the IL-17 family in humans, of which 4 of these have been identified in the chicken genome (Kaiser et al., 2005). IL-17 is thought to contribute to inflammation in the gut, following *S.* Enteritidis infection of chickens (Crhanova et al., 2011).

1.7 *Salmonella* Virchow

Since the 1990s, *S.* Virchow has increased in prevalence in the UK and is often reported as the third most frequent serovar isolated from human salmonellosis cases (Ispahani and Slack, 2000, Matheson et al., 2010). *S*. Virchow infection is most commonly associated with gastroenteritis in humans, but has been frequently associated with bacteraemia, especially in the immuno-compromised and in children (Matheson et al., 2010, Ispahani and Slack, 2000, Gulcan et al., 2012). In rare cases, *S.* Virchow has also been shown to cause severe complications with other illnesses, such as deep vein thrombosis and has been shown to cause invasive disease in otherwise healthy adults (Schifferdecker et al., 2009, Eckerle et al., 2010). The main source of *S.* Virchow infection in humans is thought to be poultry, however sporadic cases of *S.* Virchow from different sources have been reported (Bennett et al., 2003, Callaway et al., 2011, Sato et al., 2000). Five serovars including *S.* Virchow have been given priority in prevalence studies on farms by the EU, for control of entry into the food chain, due to their significant risk to public health (Arnold et al., 2010, Snow et al., 2008, Snow et al., 2007).

S. Virchow has a uniquely high prevalence and association with invasive disease in some countries. In Israel, *S.* Virchow is 1 of 3 serovars most commonly isolated from human patients and accounted for 15% of all stool and 22% of all blood isolates between 1997 and 2002 (Weinberger et al., 2006). Studies in Israel have found *S.* Virchow to be very invasive in children and the elderly and to have a high resistance to antibiotics (Weinberger and Keller, 2005, Weinberger et al., 2006, Weinberger et al., 2004). *S.* Virchow is also frequently isolated from humans in Switzerland, being ranked between the 4th and 8th most frequently isolated serovar between 2004 and 2009 (Bonalli et al., 2011). In other countries, including the United States, *S.* Virchow infection in humans is uncommon compared to other serovars, although it has still been found to be an invasive serovar (Jones et al., 2008).

Clinical evidence from a combined dataset of 10 countries indicates that there is a high association between *S.* Virchow and invasive disease, although invasiveness of *Salmonella* serovars varies from country to country (Langridge et al., 2009, Jones et al., 2008). The invasive index for *S.* Virchow, based on the ratio of the number of blood culture positives divided by total isolations of each serovar, is 4.4, more than double that of *S*. Enteritidis (1.8) and *S*. Typhimurium (1.6) that numerically cause the greatest burden of non-typhoidal salmonellosis (Langridge et al., 2009).

Increasing antimicrobial resistance of *S.* Virchow has been reported in previous studies. A study in France and Belgium found some *S.* Virchow isolates express the $bla_{\text{CTX-M-2}}$ gene, which is located on a large conjugative plasmid (Bertrand et al., 2006). This gene confers resistance to ciprofloxacin, which is a fluoroquinolone used to treat invasive salmonellosis in immuno-compromised patients (Bertrand et al., 2006). A high incidence of resistance to nalidixic acid, a quinolone used to treat invasive salmonellosis, has also been reported in *S.* Virchow, with 90% of strains isolated in Israel between 1997 and 2004 conferring resistance (Solnik-Isaac et al., 2007). A study comparing antimicrobial resistance genes in *S.* Virchow, *S.* Enteritidis and *S.* Hadar strains, isolated from humans and food-producing animals in England and Wales found *S.* Virchow strains, isolated from humans, had a greater number of different resistance genes compared to the other 2 serovars (Hopkins et al., 2007). A wider study testing non-typhoidal *Salmonella* strains, isolated from 10 different European countries, for antimicrobial susceptibility showed that 73% of *S.* Virchow isolates were resistant to at least one antimicrobial and had an increased resistance to all of the antimicrobials tested, particularly nalidixic acid (Meakins et al., 2008). Multiple drug resistant (MDR) clonally related *S.* Virchow isolates, from humans, have been detected that express *Salmonella* genomic island I (variant SGI I-J3), which is largely responsible for the diversity of MDR *S.* Virchow isolates (Chu et al., 2012). A widespread increase in antimicrobial resistance of *S.* Virchow is a major concern to public health, as the serovar is associated with invasive disease and antimicrobials are used to treat invasive salmonellosis.

1.8 Aims of this thesis

S. Virchow is a continuing and growing public health problem in the UK and worldwide, being associated with invasive disease in humans and showing high antimicrobial resistance to therapeutic drugs. Even though *S.* Virchow is the third most common serovar isolated from humans in the UK and a highly prevalent serovar in other countries, not much is known about its genetic structure and pathogenic behaviour. Additionally, an understanding of the chicken's immune response to *S.* Virchow would be most valuable and provide important opportunities to investigate the potential of developing a vaccine against this serovar.

The aims of this thesis have been to characterise the genetic relationship of *S.* Virchow strains isolated in England, from human and avian sources, using molecular typing techniques; to determine the infection biology of *S.* Virchow *in vitro* using human and avian cell lines and *in vivo* in poultry; to establish the immune response produced by poultry against infection; begin to determine the potential for a vaccine against the serovar by investigating homologous and heterologous protection offered by primary infection with *S.* Virchow in poultry.

Chapter 2
Molecular characterisation of *S.* Virchow

2.1 Introduction

The 12 *S.* Virchow strains used throughout this thesis were isolated from different sources, at different times, in England. The stains were compared using molecular typing techniques and PCR virulotyping before any *in vitro* and *in vivo* invasion studies, to determine the genetic relatedness of the isolates. Molecular typing methods are often used to compare isolates of the same serovar and of different serovars genetically, to show similarities and differences between strains and also to determine the primary source of bacterial contamination in foodborne outbreaks. Several molecular typing techniques are available to use and are chosen depending on the time taken to return the results, the discriminatory ability, the reproducibility of the results, how interpretable the results are and the resources available (Foley et al., 2009, Maiden et al., 1998).

Restriction-, amplification- and sequencing-based methods are available for molecular typing (Foley et al., 2009). Restriction-based methods are based on digestion of the genomic DNA and analysis of the fragments produced. Restriction-based methods include plasmid analysis, restriction fragment length polymorphism (RFLP) analysis, ribotyping, insertional sequences (IS)-RFLP and pulsed-field gel electrophoresis (PFGE). Amplification-based methods involve PCR of selected target genes and include amplification profiling, amplified fragment length polymorphisms (AFLP), random amplified polymorphic DNA PCR (RAPD-PCR), repetitive element PCR (Rep-PCR), variable number of tandem repeat (VNTR) and multiple locus VNTR analysis (MLVA). Sequencing-based methods identify DNA sequence polymorphisms at specific loci and include multi-locus sequence typing (MLST) and single nucleotide polymorphism (SNP) analysis.

PFGE and MLST are often used to discriminate between *S.* enterica isolates. PFGE compares restriction profiles of genomic DNA that has been digested by a specific enzyme into smaller fragments and separated by electrophoresis. PFGE is currently considered the “gold standard” of molecular typing methods for bacterial foodborne pathogens (Foley et al.,

2009, Cimons, 2000). Criteria to aid the interpretation of the genetic relatedness of bacterial isolates compared by PFGE have been published (Barrett et al., 2006, Tenover et al., 1995). PFGE has high discriminatory power, is reproducible and compares the whole genome rather than selected regions; however, it is often labour intensive and requires 2-4 days to obtain results (Foley et al., 2009, Ammari et al., 2009). MLST targets house-keeping genes within the genome and compares specific nucleotide base changes to determine the genetic relatedness of different strains. MLST is highly reproducible, but sometimes is not adequate enough to distinguish between closely related isolates, due to the low rate of genetic variability in house-keeping genes (Foley et al., 2009, Maiden et al., 1998).

Several previous studies have compared the genetic relatedness of *S.* enterica isolates to each other using PFGE and MLST and compared the discriminatory ability of PFGE and MLST. Comparison of 81 *S.* Newport isolates by PFGE identified 43 patterns and grouped them into 3 major clusters, whereas MLST defined 12 sequence types (ST), with ST 45 encompassing 61.7% of the isolates (Harbottle et al., 2006). Antimicrobial susceptibility typing identified 48% of the *S.* Newport isolates as MDR – AmpC, with 97% of these isolates characterised as either ST 45 or ST 116 by MLST (Harbottle et al., 2006). Additionally, eBURST analysis of the MLST STs separated the genetically related and the distantly related STs from each other, showing the use of MLST as a tool to compare evolutionary changes (Harbottle et al., 2006). Although PFGE may not provide as much information as MLST, the high discriminatory power of it is more useful when determining the primary source during epidemiological outbreaks (Harbottle et al., 2006). Although MLST has proven to be a useful molecular typing method, previous studies have shown care needs to be taken when choosing the house-keeping genes to target. Fakhr et al (2005) compared 85 *S.* Typhimurium isolates from cattle by MLST using a different set of house-keeping genes to those now available on the database online and found no differences between the isolates (Fakhr et al., 2005). In comparison, PFGE identified 50 distinct patterns that grouped into 3 main clusters, showing the advantage of

screening the whole genome rather than targeting selected genes, particularly during epidemiological outbreak studies (Fakhr et al., 2005).

In addition to PFGE and MLST, AFLP, rep-PCR and VNTR and MLVA have been used for genotypic characterisation of *Salmonella*. AFLP analysis involves digesting the whole genome with a restriction enzyme and then ligating short adaptor sequences complementary to the ends of the restriction fragments for targets during PCR (Foley et al., 2009). Following PCR, the fragments are separated by electrophoresis (Foley et al., 2009). AFLP has similar discriminatory power to PFGE and is less laborious (Torpdahl et al., 2005). However, the reproducibility of AFLP is poor as the procedure and analysis is subject to person-to-person variation, making comparisons between different laboratories very difficult (Torpdahl et al., 2005). Rep-PCR amplifies sequences that flank repeat elements and the amplified sequences are then separated by electrophoresis (Foley et al., 2009). Rep-PCR has been shown to be a highly discriminatory for *Salmonella* (Ben-Darif et al., 2010). Advances in sequencing of bacterial genomes have allowed the development of VNTR and MLVA analysis. Many areas of bacterial genomes contain directly repeated DNA motifs and the number of these can be highly variable (Foley et al., 2009). VNTR analyses the number of repeated copies at specific loci and MLVA targets multiple VNTR loci to distinguish between different bacterial strains (Foley et al., 2009). MLVA is highly discriminatory and rapid; however the protocol for one serovar does not often work for others (Kang et al., 2011). Therefore, it has been suggested MLVA in combination with PFGE may be useful for epidemiological outbreak investigations (Kang et al., 2011).

Comparison of bacterial genomes to determine the presence or absence of virulence genes is often done because they have been shown to be important for invasion and survival of the bacteria in its host. Transcriptional changes in identified virulence genes have been shown during *Salmonella* invasion of the host's gut. *S.* Enteritidis phage type (PT) 4 infection of newly-hatched chicks showed that of the 4380 genes represented on a microarray, 714 were up-regulated and 753 were down-regulated by bacteria isolated

from the gut lumen, in comparison to ones grown *in vitro* in broth (Dhawi et al., 2011). The up-regulated genes included those required for energy generation, carbohydrate metabolism, protein turnover, transport (chaperones) and stress responses, including those for oxidative stress (Dhawi et al., 2011). The down-regulated genes included those required for amino acid metabolism, translation, replication and cell wall biogenesis (Dhawi et al., 2011). Gene expression changes indicated a wide range of carbon sources were metabolised in the gut and a range of electron acceptors were used (Dhawi et al., 2011). A similar experiment infecting newly hatched chicks with *S.* Typhimurium F98 was conducted to determine the change in virulence and metabolism genes in the gut compared to the gene expression *in vitro* in broth (Harvey et al., 2011). Major changes in *S.* Typhimurium F98 gene expression were found, due to adaptation to the caecal environment. An up-regulation of genes required for amino acid and carbohydrate metabolism, co-enzymes and lipid transport was found, as well as a down-regulation of genes required for cell cycle regulation, translation and DNA replication (Harvey et al., 2011).

Factors such as limited nutrients, low oxygen, temperature and osmolarity affect the change in gene expression of *Salmonella* in the host's gut; however changes in virulence genes have also been found to allow adhesion to components of the gut extra-cellular matrix (ECM) (Berndt et al., 2009). Incubation of *S.* Enteritidis with ECM proteins resulted in an increased expression of the flagella subunit protein gene *fliC*, which is important for host cell invasion (Berndt et al., 2009). *S.* Infantis was included in this study as a serovar with low levels of invasiveness and did not exhibit an increase in *fliC* expression when exposed to ECM proteins (Berndt et al., 2009). In another study, *S.* Enteritidis isolates that were poorly invasive in Caco2 cells and *in vivo* in mice were found to have impaired motility and impaired secretion of flagella-associated proteins including FlgK, FljB and Flg or SPI-1 TTSS proteins SipA and SipD (Shah et al., 2011).

The requirement for virulence genes for host intestinal colonisation and systemic spread has been shown to be conserved between species and also

to sometimes be host-specific. Through signature tagged mutagenesis (STM), 119 mutants of *S.* Typhimurium ST4/74 were identified as having impaired intestinal colonisation in pigs (Carnell et al., 2007). Of the 119 mutants, 54 were located on SPIs showing their importance for intestinal colonisation of pigs (Carnell et al., 2007). The *safABCD* operon is located on SPI-6 and encodes *Salmonella* specific putative atypical fimbriae (Folkesson et al., 2002, Parkhill et al., 2001). A mutation in *safA* has been shown to impair *S.* Typhimurium ST4/74 in intestinal colonisation of pigs but not in calves and chickens (Carnell et al., 2007, Morgan et al., 2004). Mutations in the *siiA-siiF* genes located on SPI-4 also impair intestinal colonisation in a host-dependant manner. *S.* Typhimurium ST4/74 *siiE* or *siiF* mutants have shown reduced invasion of bovine enterocytes and reduced numbers in intestinal colonisation of cattle (Morgan et al., 2007). However, SPI-4 mutants are not impaired in pigs (Carnell et al., 2007) or chickens (Morgan et al., 2004). A study to determine the effects of mutations in virulence genes on *S.* Dublin intestinal colonisation and systemic spread in cattle has shown SPI-4 mutants to be impaired in colonising the ileal mucosa and lymph nodes but not systemic sites, suggesting SPI-4 plays a role in enteric but not systemic disease (Pullinger et al., 2007).

The importance of virulence genes in colonisation and systemic invasion of *Salmonella* in chickens has been shown through *in vitro* cell-based models and *in vivo* infection experiments. SPI-1 mutant strains of a range of *Salmonella* serovars including *S.* Typhimurium, *S.* Gallinarum and *S.* Pullorum have been shown to have impaired invasion of CKC cells and reduced intestinal colonisation *in vivo* in chickens compared to WT strains (Jones et al., 2001, Wigley et al., 2002b, Jones et al., 2007). SPI-2 has been shown to be important for systemic infection in chickens, although there are conflicting studies concerning its role. *In vivo* infection experiments using *S.* Gallinarum and *S.* Pullorum SPI-2 mutants have shown SPI-2 is required for virulence and systemic persistence of these serovars in chickens (Wigley et al., 2002b, Jones et al., 2001). *S.* Typhimurium SPI-2 mutants also show significantly reduced systemic disease in chickens compared to the WT strain (Jones et al., 2007). *In vitro* invasion assays using HD11 chicken

macrophage-like cells found a *S.* Gallinarum SPI-2 mutant failed to survive in the cells and therefore concluded SPI-2 is essential for intra-macrophage survival (Jones et al., 2001). More recently, SPI-2 mutants of *S.* Typhimurium and *S.* Enteritidis were found to survive equally as well in HD11 cells compared to the WT strain and it has been suggested SPI-2 is important for systemic distribution, rather than intra-macrophage survival (Wisner et al., 2011b).

Some virulence factors have been included as potential vaccine candidates due to their absence resulting in attenuation of *Salmonella* infection. *S.* Enteritidis challenge of chickens vaccinated with either SPI-1 or SPI-2 defective mutants showed a reduction in caecal colonisation of chickens vaccinated with SPI-1 mutants and no systemic spread in ones vaccinated with either SPI-1 or SPI-2 compared to unvaccinated chickens (Matulova et al., 2012). Chickens vaccinated with SPI-2 structural or effector proteins have also been found to elicit a strong humoral response following *S.* Enteritidis challenge (Wisner et al., 2011a).

The aim of this study was to compare the *S.* Virchow isolates by molecular typing methods to determine their genetic relatedness. MLST was chosen to assign the isolates ST numbers and to determine their phylogenetic relationship. PFGE was done in combination with MLST to determine the genetic relatedness of the isolates, as it has been shown to sometimes have more discriminatory power. Additionally, the *S.* Virchow isolates were screened by PCR for 12 genes associated with virulence to get an indication of the array of virulence factors they possessed in comparison to each other and to a representative *S.* Typhimurium isolate.

2.2 Materials and Methods

2.2.1 *Salmonella* isolates

Throughout this thesis, 12 *S.* Virchow isolates were used to characterise the serovar (Table 2.1). Of these isolates, 6 were received from Dr Chris Parry, Medical Microbiology, University of Liverpool and were isolated from human gastroenteritis patients on Merseyside, between 2005 and 2008.The other 6 isolates were provided by Professor Roberto La Ragione from the Veterinary Laboratories Agency, Weybridge (now AHVLA) and consisted of 4 isolated from live chickens, 1 isolated from a turkey and 1 environmental isolate from a poultry house. *S.* Typhimurium F98 was included in all of the experiments as a comparison, as it is a well characterised strain in chickens (Barrow et al., 1987, Smith and Tucker, 1975). *S.* Typhimurium 238 (DT 40) and *S.* Typhimurium 244 (DT 56) were also included in some of the experiments as comparisons and are highly associated with garden bird mortality (Pennycott et al., 2006, Hughes et al., 2008, Hughes et al., 2010).

Table 2.1: *S.* Virchow isolates

ID	Source
S. Virchow 51	Human
S. Virchow 52	Human
S. Virchow 53	Human
S. Virchow 54	Human
S. Virchow 55	Human
S. Virchow 56	Human
S. Virchow 57	Environmental
S. Virchow 58	Chicken
S. Virchow 59	Chicken
S. Virchow 60	Chicken
S. Virchow 61	Turkey
S. Virchow 62	Chicken

2.2.2 MLST

All 12 *S.* Virchow isolates were grown in 10 ml Luria-Bertani (LB) broth in an orbital shaking incubator for 18 hours, at 37°C and 150 rpm. Genomic DNA was prepared from the broths using a Wizard® Genomic DNA Purification kit following the manufacturer's instructions (Promega, Southampton, UK). Seven house-keeping genes were targeted by PCR and were *thrA, purE, sucA, hisD, aroC, hemD* and *dnaN*. The primers used were obtained from the *Salmonella* MLST database online (http://mlst.ucc.ie/mlst/dbs/Senterica) and are detailed in Table 2.2. The reaction mixture was the same for each gene and consisted of 10X buffer, 2.5 mmol/l $MgCl_2$, 250 µmol/l each of dATP, dCTP, dGTP and dTTP, 25 pmol/l each of the forward and reverse primers, 1 unit of Taq polymerase (Invitrogen, Paisley, UK) and 2 µl DNA template, made up to a final reaction volume of 50 µl with molecular grade water. PCR thermocycler conditions included an initial denaturation step of 94°C for 10 minutes, followed by 34 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, with a final step of 72°C for 5 minutes (Ikumapayi et al., 2007). PCR products were run on a 2% agarose gel in Tris-acetate buffer (TAE) at 120 volts for 40 minutes. The gel was visualised under ultra-violet (UV) light.

PCR products were purified using a polyethylene glycol (PEG) precipitation method. PCR products were transferred to 96 well plates and 60 µl 20% (w/v) PEG_{8000}, 2.5M NaCl was added to each well. The plates were incubated overnight at 4°C and then centrifuged at 2750 rcf for 60 minutes at 4°C. To remove the PEG, plates were centrifuged inverted at 500 rpm for 60 seconds. The DNA pellets in the wells were washed twice with 150 µl of ice-cold ethanol by adding the ethanol to the wells and centrifuging the plates at 2750 rcf for 10 minutes. The ethanol was removed from the wells by centrifuging the plates at 500 rpm for 60 seconds. Following the washes, the plates were air dried on the bench for 10 minutes. DNA pellets were re-suspended in 50 µl sterile water by vortexing and then centrifuging briefly.

Forward and reverse nucleotide sequences of the PCR products were determined using the primers listed in Table 2.3 and Big Dye ready reaction

mix (Applied BioSystems). The reaction mixture consisted of 0.50 µl Big Dye, 3.74 µl 5X buffer, 4µl each of the forward and reverse primers and 3µl of the PCR product, made up to a total volume of 20 µl with molecular grade water. Thermocycler conditions included 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 2 minutes.

An ethanol precipitation method was used for the sequencing reaction clean-up. Firstly, 52 µl of 100% ethanol and 3M sodium acetate in a ratio of 25:1 was added to each well and the plates were vortexed and centrifuged briefly at 500 rpm. The plates were then incubated at room temperature for 45 minutes and centrifuged at 2750 rcf for 1 hour at 4°C. To remove the ethanol and sodium acetate, the plates were centrifuged inverted, at 500 rpm for 1 minute. The pellets were washed with 150 µl ice-cold 70% ethanol, by centrifuging the plates at 2750 rcf for 10 minutes. The ethanol was removed by centrifuging the plates inverted, at 500 rpm for 1 minute and then the plates were left to air dry at room temperature for 10 minutes. Following this, 10 µl of HiDi (formamide) was added to each well, the plates were vortexed and then centrifuged briefly. The DNA was heat-denatured by heating the plates at 94°C for 10 minutes and then the plates were loaded onto an ABI Prism 3130x/ Genetic Analyzer sequencer (Applied BioSystems).

ChromasPro version 1.42 and Mega 4.1 software were used to analyse the chromatograms generated by the sequencer. Sequences were submitted to the *Salmonella* MLST database online (http://mlst.ucc.ie/mlst/dbs/Senterica) and STs were assigned to each isolate. For further analysis eBURST version 3 diagrams were constructed online (http://eburst.mlst.net) using the *S.* Virchow isolates in this study and *S.* Virchow isolates submitted to the online database, following the online instructions.

Table 2.2: *Salmonella* MLST amplification primers

Target Gene		Primer Sequence (5'-3')	Product Length (bp)
ThrA	F	GTCACGGTGATCGATCCGGT	852
	R	CACGATATTGATATTAGCCCG	
purE	F	ATGTCTTCCCGCAATAATCC	510
	R	CGAGAACGCAAACTTGCTTC	
sucA	F	AGCACCGAAGAGAAACGCTG	643
	R	GGTTGTTGATAACGATACGTAC	
HisD	F	GAAACGTTCCATTCCGCGCAGAC	894
	R	CTGAACGGTCATCCGTTTCTG	
aroC	F	CCTGGCACCTCGCGCTATAC	826
	R	CCACACACGGATCGTGGCG	
hemD	F	GAAGCGTTAGTGAGCCGTCTGCG	666
	R	ATCAGCGACCTTAATATCTTGCCA	
dnaN	F	ATGAAATTTACCGTTGAACGTGA	833
	R	AATTTCTCATTCGAGAGGATTGC	

2.2.3 PFGE

All 12 *S.* Virchow isolates, *S.* Typhimurium F98 and *S.* Typhimurium 238 were grown on nutrient agar plates overnight at 37°C. Bacterial colonies were put in bijoux bottles containing 2 ml suspension buffer and the optical densities were measured, until an OD_{610} value of 1.35 was obtained for each isolate. PFGE methodology was based on the Standardized PulseNet Rapid *Escherichia coli* PFGE method with slight modifications (Anonymous, 2004, Hughes et al., 2008, Ribot et al., 2006). Once the OD value had been obtained 200 µl of the cell suspension, 10 µl (20 mg/ml) proteinase K and 200 µl molten agarose (1% PFGE agarose dissolved in 1x TE buffer and 1% Sodium Dodecyl Sulfate) were mixed together by pipetting briefly. The mixtures were transferred to duplicate plug moulds and left to set for 20 minutes at 4°C. The plugs were transferred to 3 ml cell lysis buffer (15 µl of 20 mg/ml proteinase K, 50mM Tris, 50 mM EDTA and 1% N-lauryl sarcosine) and incubated in an orbital shaking incubator for 2 hours at 54°C and 150

rpm. The lysis buffer was removed and the plugs were incubated with pre-heated sterile distilled water for 15 minutes at 54°C and 150 rpm. The plugs were washed twice with sterile distilled water and four times with 1x TE buffer and then one set of plugs were incubated in 500 µl 0.1x TE buffer for 20 minutes at 37°C and 150 rpm and the other set of plugs were stored in 2ml 1x TE buffer at 4°C. The 0.1x TE buffer was removed and replaced with 200 µl *XbaI* restriction buffer (Promega) and incubated for 15 minutes at 37°C and 150 rpm. The restriction buffer was removed and replaced with 200 µl restriction buffer containing 50U *XbaI* (Promega) and the samples were incubated statically at 37°C for 2 hours.

Fragments of the digested samples were separated out on a 1% agarose gel (PFGE agarose, BioRad Laboratories) in 1x Tris-Borate buffer (TBE) at 210 volts and 14°C for 19 hours. Pulse times were ramped at 2.2-54.2 seconds and a re-orientation angle of 120° was applied. Bacteriophage λ DNA concatemers were used as a molecular marker. The gel was stained for 20 minutes in 1% ethidium bromide and the gel was visualised under UV light. BioNumerics version 4 software was used for image analysis. A position tolerance of 2% was used and a dendrogram was constructed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA).

Table 2.3: *Salmonella* MLST sequencing primers

Target Gene		Primer Sequence (5'-3')
thrA	F	ATCCCGGCCGATCACATGAT
	R	CTCCAGCAGCCCCTCTTTCAG
purE	F	CGCATTATTCCGGCGCGTGT
	R	CGCGGATCGGGATTTTCCAG
sucA	F	AGCACCGAAGAGAAACGCTG
	R	GGTTGTTGATAACGATACGTAC
hisD	F	GTCGGTCTGTATATTCCCGG
	R	GGTAATCGCATCCACCAAATC
aroC	F	GGCACCAGTATTGGCCTGCT
	R	CATATGCGCCACAATGTGTTG
hemD	F	GTGGCCTGGAGTTTTCCACT
	R	GACCAATAGCCGACAGCGTAG
dnaN	F	CCGATTCTCGGTAACCTGCT
	R	CCATCCACCAGCTTCGAGGT

2.2.4 PCR Virulotyping

All 12 *S.* Virchow isolates and *S.* Typhimurium F98 were grown on nutrient agar plates overnight at 37°C. Crude boil lysate DNA samples were prepared by adding 2-3 bacterial colonies to 0.5 ml sterile distilled water and boiling the preparations at 100°C for 10 minutes. The isolates were screened for the presence of 12 genes associated with virulence, which were *prgH, sopB, sopE, invA, sitC, spiC, sifA, misL, orfL, pipD, iroN* and *pefA* (Skyberg et al., 2006, Hughes et al., 2008), using the primers in Table 2.4. Reaction mixtures were the same for each gene and included 1.25 units Taq polymerase, 75 mmol/l Tris-HCl (pH 8.8 at 25°C), 20 mmol/l $(NH_4)_2SO_4$, 2.5mmol/l $MgCl_2$, 0.01% (v/v) Tween-20, 0.2 mmol/l each of dATP, dCTP, dGTP and dTTP, 4 mmol/l each of the forward and reverse primers and 1 µl of DNA in a total volume of 25 µl (Hughes et al., 2008). PCR thermocycler conditions included an initial denaturation step of 94°C for 3 minutes, followed by 30 cycles of 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute, with a final step of 72°C for 5 minutes (Hughes et al., 2008). PCR products were run on a 2%

agarose gel in TAE buffer for 30 minutes at 120 volts. The gel was visualised under UV light. Amplicon size was determined by comparison with Φ X174 Hae III digest DNA marker (ABgene, Epsom, UK).

Table 2.4: PCR virulotyping primers

Virulence Gene		Primer Sequence (5'-3')
prgH	F	GCCCGAGCAGCCTGAGAAGTTAGAAA
	R	TGAAATGAGCGCCCCTTGAGCCAGTC
sopB	F	CGGACCGCCCAGCAACAAAACAAGAAGAAG
	R	TAGTGATGCCCGTTATGCGTCAGTGTATT
sopE	F	TCAGTTGGAATTGCTGTGGA
	R	TCCAAAAACAGGAAACCACAC
InvA	F	CTGGCGGTGGGTTTTGTTGTCTTCTCTATT
	R	AGTTTCTCCCCCTCTTCATGCGTTACCC
SitC	F	CAGTATATGCTCAACGCGATGTGGGTCTCC
	R	CGGGGCGAAAATAAAGGCTGTGATGAAC
SpiC	F	CCTGGATAATGACTATTGAT
	R	AGTTTATGGTGATTGCGTAT
SifA	F	TTTGCCGAACGCGCCCCCACACG
	R	GTTGCCTTTTCTTGCGCTTTCCACCCATCT
misL	F	GTCGGCGAATGCCGCGAATA
	R	GCGCTGTTAACGCTAATAGT
OrfL	F	GGAGTATCGATAAAGATGTT
	R	GCGCGTAACGTCAGAATCAA
pipD	F	CGGCGATTCATGACTTTGAT
	R	CGTTATCATTCGGATCGTAA
IroN	F	ACTGGCACGGCTCGCTGTCGCTCTAT
	R	CGCTTTACCGCCGTTCTGCCACTGC
pefA	F	GCGCCGCTCAGCCGAACCAG
	R	CAGCAGAAGCCCAGGAAACAGTG

2.3 Results

2.3.1 MLST

MLST was used to determine the genetic relatedness of the *S.* Virchow isolates. The genetic sequences of all 7 house-keeping genes for each isolate was submitted to the *Salmonella* MLST database online (http://mlst.ucc.ie/mlst/dbs/Senterica). Overall, 11 *S.* Virchow isolates were identified as ST 16. The environmental isolate differed from the others and was identified as a novel ST, being assigned the number ST 648. The environmental isolate differed by 3 bases in the *dnaN* gene and 2 bases in the *purE* gene compared to the other isolates. In addition to ST 16 being the most prevalent ST amongst the isolates in this study, it was the most common ST submitted to the MLST database online and was mainly isolated from humans (Figure 2.1). An eBURST diagram representing all of the *S. enterica* isolates submitted to the online database was constructed to show the relationship of *S.* Virchow to other serovars. *S.* Virchow clustered in its own group quite close to the *S.* Typhimurium cluster located in the centre of the diagram (Figure 2.2).

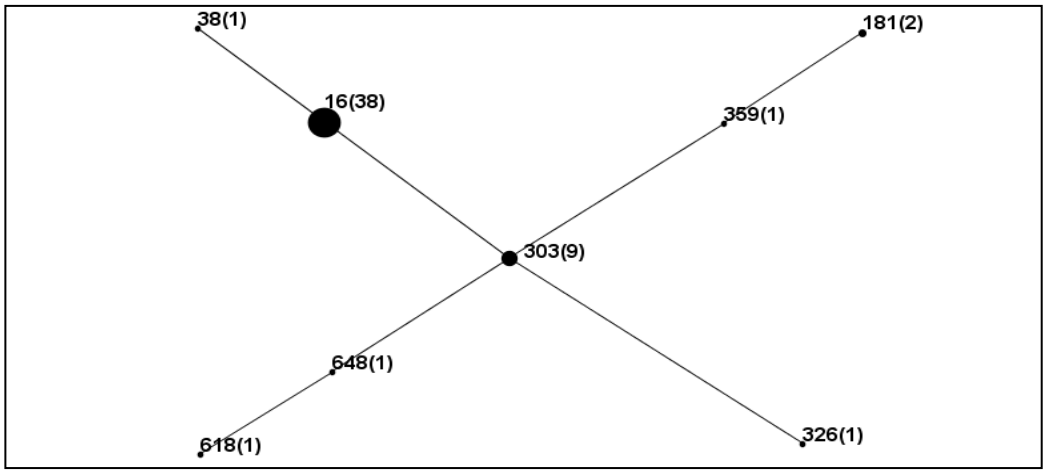

Figure 2.1: eBURST ver. 3 diagram of *S.* Virchow isolates from this study and those available in the *Salmonella* MLST database online. The numbers in brackets represent the number of isolates of that particular ST. The founder is ST 303, which suggests this ST is related to the largest number of other STs. Single locus variants (SLV) are connected by a line.

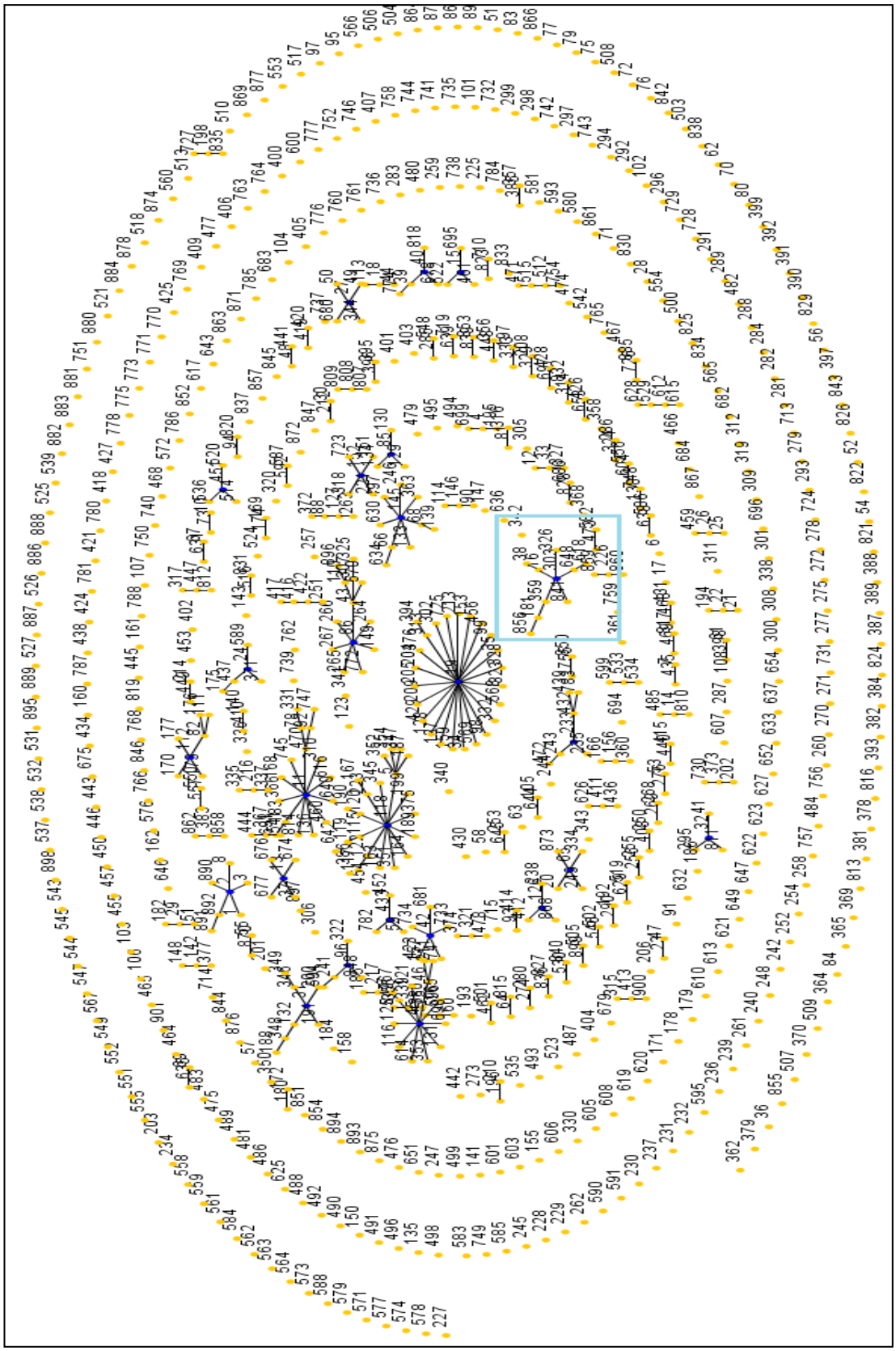

Figure 2.2: eBURST ver. 3 diagram of all the *S. enterica* isolates in the MLST database. Each cluster is generally representative on a particular serovar. The *S.* Virchow cluster is highlighted by a blue box.

2.3.2 PFGE

PFGE was used to further determine the genetic relatedness of the *S.* Virchow isolates. Dendrogram analysis of the *S.* Virchow isolates revealed different banding patterns or pulsotypes between them (Figure 2.3). The *S.* Virchow isolates were grouped into 2 genetically related clusters and *S.* Typhimurium F98 and *S.* Typhimurium 238 were grouped into a separate cluster (Figure 2.3).

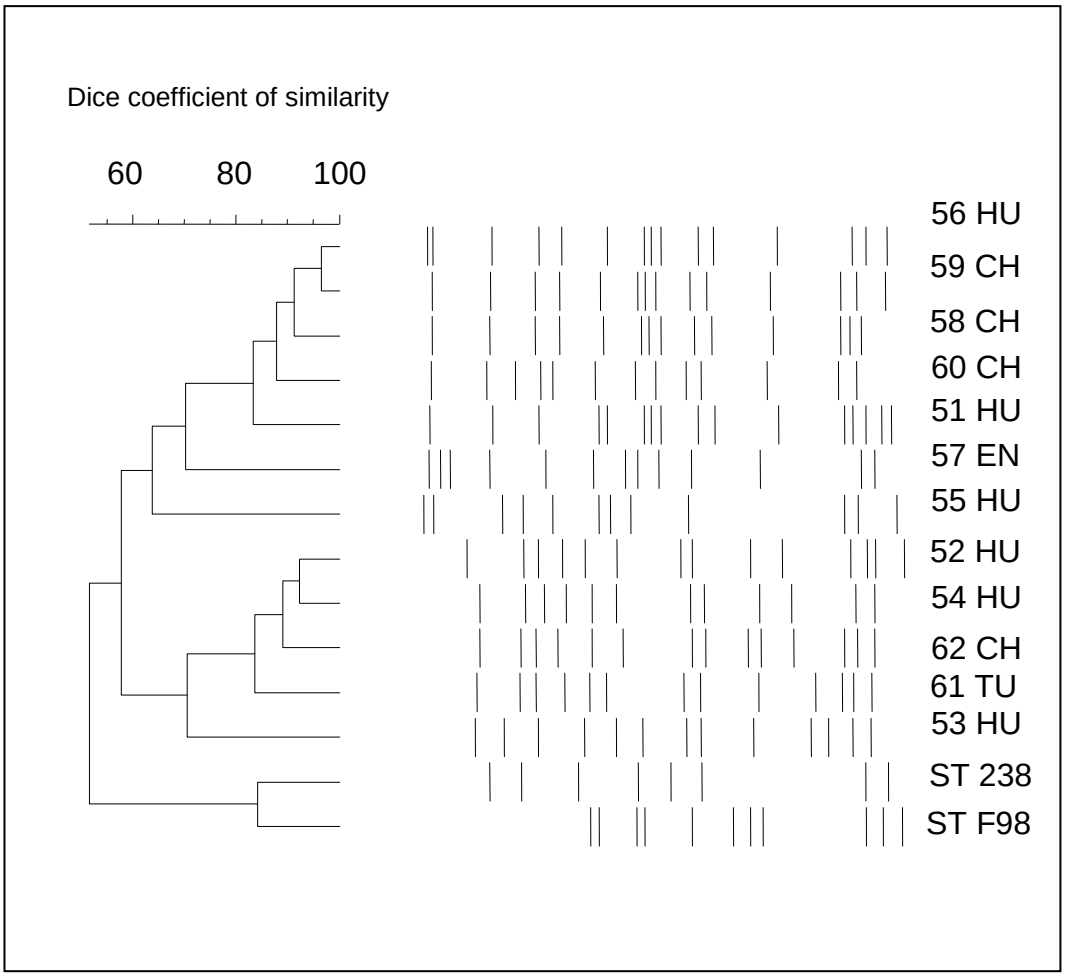

Figure 2.3: Results from a dendrogram using DICE (tolerance 1.0-1.0%) (Minimum height > 0.0%, minimum surface > 0.0%) (0-100%) coefficient for PFGE using *XbaI* digestion. Dendrogram of the relatedness between *S.* Virchow isolates to each other and to *S.* Typhimurium based on PFGE. The dendrogram was constructed using BioNumerics software by Unweighted Pair Group Method with Arithmetic Mean.

2.3.3 PCR Virulotyping

The *S.* Virchow isolates were positive for all 12 genes, which were *prgH, sopB, sopE, invA, sitC, spiC, sifA, misL, orfL, pipD, iroN* and *pefA*. *S.* Typhimurium F98 was positive for 10 of the virulence genes and lacked *pefA* (Figure 2.4) and *sopE* (Figure 2.5).

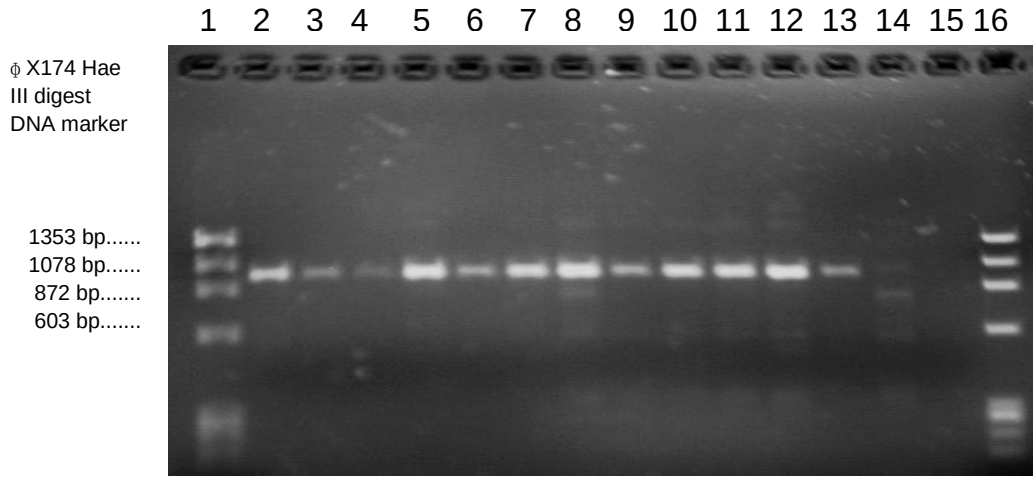

Figure 2.4: Gel image of isolates positive for *pefA*. Lane 1 = ϕ X174 Hae III digest DNA marker, Lane 2-13 = *S.* Virchow 51-62 isolates, Lane 14 = *S.* Typhimurium F98, Lane 15 = negative control and Lane 16 = ϕ X174 Hae III digest DNA marker.

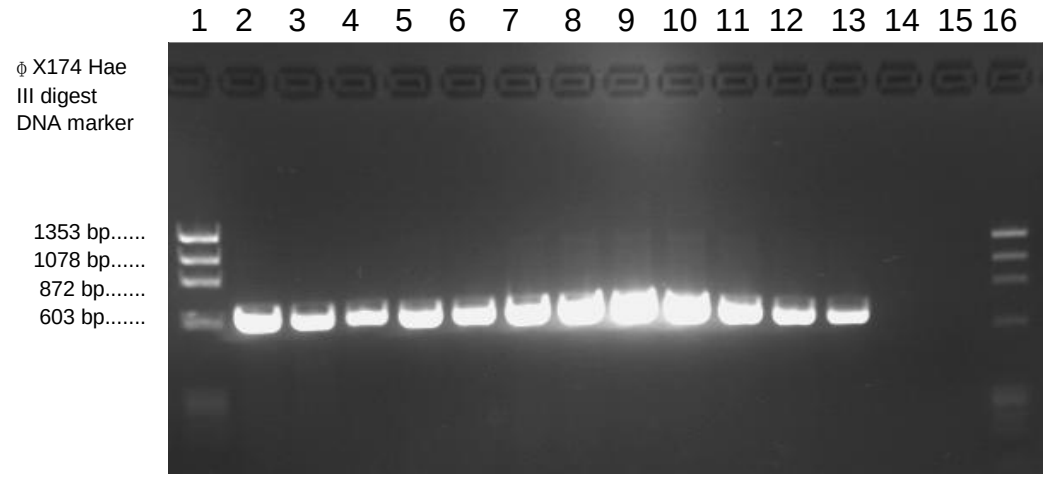

Figure 2.5: Gel image of isolates positive for *sopE*. Lane 1 = ϕ X174 Hae III digest DNA marker, Lane 2-13 = *S.* Virchow 51-62 isolates, Lane 14 = *S.* Typhimurium F98, Lane 15 = negative control and Lane 16 = ϕ X174 Hae III digest DNA marker.

2.4 Discussion

Molecular typing of the *S.* Virchow isolates revealed that they are very similar genetically, although some differences were identified. MLST analysis identified 11/12 *S.* Virchow isolates as the same ST, ST 16. The environmental isolate was identified as a novel one and assigned the ST, ST 648. Results from this study and from *S.* Virchow isolates submitted to the *Salmonella* MLST online database indicate ST 16 is the predominant ST for *S.* Virchow. This is evident not only in the UK but countries such as Germany, France and Denmark, from where *S.* Virchow isolates have also been submitted. In total, 8 different STs have been assigned to *S.* Virchow isolates submitted to the online database. Construction of an eBURST diagram identified ST 303 as the founder ST, which is the ST that most of the others deviate from by one SLV and therefore, may represent how *S.* Virchow has diversified (Figure 2.1). Except for ST 303 and ST 16, the other STs only consist of 1 or 2 isolates in the MLST database, indicating they are not as common. The eBURST diagram (Figure 2.1) showed the STs of *S.* Virchow do not vary much from the founder, as the connecting black lines are representative of SLVs, suggesting *S.* Virchow is a relatively clonal group.

Previous studies have highlighted the importance of MLST as an evolutionary tool. The majority of *S.* Typhimurium isolates infect a wide range of hosts, mainly causing limited gastroenteritis and are identified as ST 19. However, some *S.* Typhimurium isolates have varying STs and have shown distinct differences in their invasive phenotype. *S.* Typhimurium ST 313 has been found to be a predominant ST causing invasive disease in humans and is rarely found outside of Africa (Kingsley et al., 2009). Additionally, *S.* Typhimurium ST 568 has been identified as a predominant ST in passerine birds, causing invasive disease and high rates of mortality, but it is rarely isolated in other hosts, suggesting it is host-adapted (Hughes et al., 2010). Although MLST may occasionally lack discriminatory ability for *Salmonella* strains of the same serovar, it has proven a great tool for showing evolutionary relationships of serovars and does separate strains into clusters of the same serovar. Therefore, it has recently been suggested that MLST

should be used to replace serotyping, by allowing the identification of genetically related strains of *Salmonella enterica* rather than typing based on 'O' and 'H' antigen serology (Achtman et al., 2012).

PFGE grouped the *S.* Virchow isolates into 2 main clusters and the *S.* Typhimurium isolates into a separate cluster, with no distinction between isolates from human and avian species. Within the clusters, the *S.* Virchow isolates differed by an average of 3 bands, indicating that they are closely related (Barrett et al., 2006, Tenover et al., 1995). PFGE is based on the whole genome and therefore, sometimes has more discriminatory power than MLST. However, it does not show the evolutionary relationship of the isolates like MLST does (Foley et al., 2009). Therefore, MLST and PFGE were used in combination in this study, to determine the genetic relatedness of the *S.* Virchow isolates. Although PFGE analysis revealed more genetic variation between the *S.* Virchow isolates than MLST, both methods showed the isolates to be highly genetically related and indicated *S.* Virchow in the UK is relatively clonal.

PFGE has been used in previous studies to determine the genetic relatedness of *S.* Virchow, in countries where the prevalence is higher than others. In Israel, *S.* Virchow has a uniquely high prevalence in humans and is a dominant cause of NTS (Weinberger and Keller, 2005). During a study between 1997 and 2002 *S.* Virchow accounted for 15% of all stool and 22% of all blood isolates of *Salmonella enterica* in Israel (Weinberger et al., 2006). Similar to the findings in this study, Weinberger et al (2006) found PFGE grouped the *S.* Virchow isolates into 2 main clusters, which differed at most by 3 bands, indicating as in the UK *S.* Virchow is relatively clonal (Weinberger et al., 2006). Additional comparison of *S.* Virchow isolates in Israel between 1997 and 2004 showed an even closer relationship between the isolates, emphasising even more the clonality of *S.* Virchow (Solnik-Isaac et al., 2007). The study by Solnik-Isaac et al (2007) included human as well as poultry isolates and in agreement with this study showed isolates from both sources had closely related PFGE profiles (Solnik-Isaac et al., 2007). Switzerland is another country reporting increasing prevalence of *S.* Virchow

(Bonalli et al., 2011). Between 2004 and 2009 1.5% of all clinical isolates from humans were identified as *S.* Virchow, ranking it at between 4th and 8th most common serovar over the study period (Bonalli et al., 2011). PFGE separated the isolates into 4 clusters, with no common characteristics found within a particular cluster (Bonalli et al., 2011). Bonalli et al 2011 concluded from the PFGE that the *S.* Virchow strains isolated over the study period were closely genetically related and widespread.

PFGE is often used to determine the source of *Salmonella* outbreaks and cases unrelated to the outbreak, due to its high discriminatory ability. Between October 2001 and March 2002 an estimated excess of 439 cases of *S.* Oranienburg was reported in Germany (Werber et al., 2005). The outbreak was linked to a source of chocolate and was confirmed by the *Salmonella* isolated from the chocolate and humans having indistinguishable PFGE profiles (Werber et al., 2005). During 2001, an international outbreak of *S.* Stanley was linked back to a particular brand of Asian-style peanuts, also through indistinguishable PFGE profiles from the human cases and the peanuts (Kirk et al., 2004).

PCR virulotyping of the 12 *S.* Virchow isolates found that they were all positive for 12 genes associated with virulence, *prgH, sopB, sopE, invA, sitC, spiC, sifA, misL, orfL, pipD, iroN* and *pefA*. Similar sets of virulence genes have been used in previous studies, as they represent SPIs 1-5 and have all been associated with virulence of *Salmonella* (Skyberg et al., 2006, Hughes et al., 2008, Dione et al., 2011). The gene *prgH* is encoded in SPI-1and is a major component of the base structure of the SPI-1 T3SS (Kubori et al., 2000, Schraidt et al., 2010). *In vivo* infection of mice with *S.* Typhimurium *prgH* mutants has found the bacteria to be defective in transcytosis across the epithelial barrier, showing the importance of PrgH to the structure of the T3SS (Behlau and Miller, 1993).

SopB is an effector protein translocated by the SPI-1 T3SS, although it is encoded by SPI-5 (Wood et al., 1998). SopB is translocated into the host intestinal cells via a *sip*-dependant pathway and promotes fluid secretion and

an inflammatory response through phosphatase activity (Norris et al., 1998, Galyov et al., 1997). *S.* Dublin strains with mutations in *sopB* have been shown to have a decreased ability to induce fluid secretion and inflammation in calf intestine loops (Norris et al., 1998). SPI-5 also encodes the virulence genes *pipA, pipB, pipC, pipD* and *orfX* (Wood et al., 1998). Mutations in *pipD* as well as *pipA* and *pipB* significantly reduce the magnitude of the secretory and inflammatory responses of bovine ligated ileal loops in response to *S.* Dublin infection compared to the WT strain (Wood et al., 1998). Therefore, SPI-5 genes appear to be important for the enteropathogenicity of *Salmonella*, rather than systemic spread.

SopE protein is encoded on a cryptic bacteriophage and is an effector protein of the SPI-1 T3SS (Hardt et al., 1998b). *S.* Typhimurium strains with mutations in *sopE* are not as invasive into host cells as WT strains and host cells also exhibit differences in morphology (Hardt et al., 1998b). It has been suggested SopE has a role in host cell invasion through the induction of cytoskeleton rearrangements and membrane ruffling, by activating the host Rho GTPases, CDC42 and Rac-1 (Hardt et al., 1998b, Hardt et al., 1998a, Haraga et al., 2008, Friebel et al., 2001). As *sopE* is encoded on a cryptic bacteriophage it is not present in all *Salmonella* serovars (Dione et al., 2011). The frequency of *sopE* in the top 10 most prevalent serovars in England and Wales in 2001 revealed *sopE* was present in 47 out of 158 *S.* Typhimurium isolates and these were definitive phage types most associated with major epidemics (Hopkins and Threlfall, 2004). Therefore, outbreaks of certain strains of a particular serovar may be due to enhanced virulence, provided by the presence of genes such as *sopE*. The presence of *sopE* was found in a large proportion of *S.* Virchow isolates, with only antimicrobial-sensitive strains negative for *sopE* (Hopkins and Threlfall, 2004). The presence of *sopE* in *Salmonella* isolates from human and animal sources in India has also been investigated (Rahman et al., 2004). The presence of *sopE* was found to be conserved amongst only a few serovars including *S.* Virchow, *S.* Enteritidis and *S.* Gallinarum (Rahman et al., 2004). A protein named SopE2 has been identified that is highly homologous to SopE and unlike the *sopE* gene, is conserved in pathogenic strains of *Salmonella* (Bakshi et al., 2000).

SopE2 like SopE is a guanine nucleotide exchange factor, although they activate different Rho GTPase pathways (Friebel et al., 2001). Following *S.* Typhimurium infection of T84 epithelial cells, SopE2 stimulates an increase in IL-8 production (Huang et al., 2004). SopE2 and flagellin have been shown to interact to further increase IL-8 production upon invasion (Huang et al., 2004). SopE2 has also been shown to up-regulate inducible nitric oxide synthase (iNOS) in a SipB-, SipC- and SipD-dependant manner upon *S.* Typhimurium invasion of murine macrophage cells (Cherayil et al., 2000). These results combined indicate SopE2 has a role in the induction of inflammation and shows the importance of SopE2 for bacterial pathogenesis. A *sopB, sopE* and *sopE2* triple mutant of *S.* Typhimurium has been shown to be incapable of membrane ruffling and has >100-fold reduced invasion *in vitro* in COS7 epithelial cells (Mirold et al., 2001).

SitC is encoded in SPI-1, along with 3 additional ORFs termed *sitA, sitB* and *sitD* (Zhou et al., 1999). The *sitABCD* operon has high sequence homology to the *yfe* ABC iron transport system of *Yersinia pestis* and is repressed under iron-rich growth conditions in a *fur*-dependent manner, suggesting that it encodes a putative iron transport system (Zhou et al., 1999). The *sitABCD* operon is expressed following intestinal invasion, during systemic invasion of mice by *S.* Typhimurium (Janakiraman and Slauch, 2000). *S.* Typhimurium *sit* null mutants are significantly attenuated in mice, indicating SitABCD plays an important role in iron acquisition in mice (Janakiraman and Slauch, 2000). Iron is important for bacterial growth, but environments within the host often have low concentrations of this metal, therefore iron acquisition mechanisms are important. Previous studies have suggested the existence of redundant iron uptake systems in *S. enterica* and other systems have been identified. The *iroN* gene is encoded in the *iroA* locus of *S. enterica* and is conserved throughout the species, although it is absent from *S. bongori* (Baumler et al., 1998). *IroN* has been shown to encode a 78 kDa outer membrane protein that has high homology to enterochelin receptors, suggesting it also has a role in iron acquisition (Baumler et al., 1998). IroN has been shown to transport similar substrates to other outer membrane receptor proteins, such

as enterobactin and also to mediate uptake of selected substrates such as corynebactin (Rabsch et al., 1999).

SpiC protein is encoded on SPI-2 and is transported into the host cell via the SPI-2 TTSS (Uchiya et al., 1999). *S.* Typhimurium infection of J774 macrophage cells has been shown to require a functional *spiC* gene for inhibition of the fusion of *Salmonella*-containing phagosomes with lysosomes and inhibition of trafficking of vesicles (Uchiya et al., 1999). Furthermore, a *S.* Typhimurium *spiC* mutant was attenuated for virulence in J774 macrophage cells, indicating inhibition of cellular trafficking is important for *Salmonella* pathogenesis (Uchiya et al., 1999). SpiC has also been shown to up-regulate protein kinase A, leading to CREB phosphorylation and an increase in IL-10, which creates a favourable environment for *Salmonella* to survive within macrophages (Uchiya et al., 2004). In addition, SpiC protein regulates the transcription of FliC protein, which is a component of the flagella filaments (Uchiya and Nikai, 2008). Up-regulation of *fliC* activates MAPK pathways in *Salmonella*-infected macrophages, which stimulates an up-regulation of suppressor of cytokine signalling (SOCS)-3 (Uchiya and Nikai, 2008). SOCS-3 inhibits cytokine signalling by the macrophage cells, therefore reducing the immune response against the bacteria (Uchiya and Nikai, 2008). Overall, findings indicate SpiC is important for the survival and persistence of *Salmonella* in macrophages.

SifA protein is encoded on SPI-2 and transported into the host cell by the SPI-2 T3SS (Beuzon et al., 2000). SifA is required to maintain the integrity of the scv within the host cell and does this by forming lgp structures (Beuzon et al., 2000, Garcia-del Portillo et al., 1993, Stein et al., 1996). SifA has been shown to be important for virulence in mice (Stein et al., 1996) and has also been suggested to have a role in systemic persistence in calves (Pullinger et al., 2007).

The MisL protein is encoded on SPI-3 and has shown similarities to the immunoglobulin A1 protease family of autotransported proteins, which are found only in pathogenic bacteria (Blanc-Potard et al., 1999). *S.* Typhimurium

misL mutants are highly attenuated in the intestinal colonisation of chicks, indicating that it is required for virulence in this host (Morgan et al., 2004). Further research to determine the role of MisL protein has shown it binds to intestinal ECM proteins including fibronectin and collagen IV (Dorsey et al., 2005). MisL therefore appears to be an extracellular matrix adhesion involved in attachment to the intestinal cell wall and intestinal colonisation (Dorsey et al., 2005).

Identification and characterisation of SPI-4 found putative proteins that have significant homology to toxin secretion proteins, suggesting SPI-4 encodes a Type I secretion system involved in toxin secretion (Wong et al., 1998). A large secreted protein called SiiE has since been characterised (Morgan et al., 2004). The OrfL protein, which had been previously characterised, also mapped to this region and has been shown to be needed for intra-macrophage survival in mice (Wong et al., 1998, Baumler et al., 1994). *In vivo* challenge of mice with *S.* Typhimurium SPI-4 mutants showed them to be attenuated and implicated SPI-4 in intestinal colonisation (Morgan et al., 2004).

The PefA protein is encoded on a large 90 kb virulence plasmid, in a 7 kb region along with 4 other proteins including PefC, Orf5, Orf6 and Orf8 (Friedrich et al., 1993). The *pefA* locus was found to have some sequence homology to various fimbrial/pilin shaft subunits found in *E. coli* (Friedrich et al., 1993). Furthermore, a transposon insertion in *pefA* abolishing its expression eliminated the production of fimbriae (Friedrich et al., 1993). Formation of surface filamentous structures by the *pef* genes and up-regulation of the genes following *in vivo* infection suggest *pefA* has a role in attachment to the surface of the small intestine (Rotger and Casadesus, 1999).

The previous studies outlined above have shown the 12 virulence genes screened for in this study are important for adhesion, invasion and persistence of *S. enterica* in a range of hosts. Mutations in these genes result in attenuated virulence of the bacteria. The presence of these virulence

genes, particularly *sopE* and *pefA*, has been shown to be varied in different *Salmonella* serovars and may partly explain the differences in virulence observed between different serovars (Hughes et al., 2008, Gassama-Sow et al., 2006, Skyberg et al., 2006, Dione et al., 2011). In this study all of the *S.* Virchow isolates were positive for the 12 virulence genes, suggesting that *S.* Virchow can cause intestinal and systemic disease.

Overall, the experiments in this study have consistently shown that *S.* Virchow isolates in England are genetically very similar, regardless of their source. Although the sample size was relatively small, additional isolates from the MLST database were included and confirmed further that *S.* Virchow is a relatively clonal serovar. Presence of all of the virulence genes examined, including *sopE,* indicate that *S.* Virchow has high levels of potential virulence, particularly as *sopE* is usually found in isolates of *S*. Typhimurium associated with epidemics in humans and animals (Hopkins and Threlfall, 2004).

Chapter 3
In vitro studies of pathogen-host interactions

3.1 Introduction

In vitro experiments using cell lines are regularly used in research to determine specific characteristics of a pathogen in a particular host and to identify the nature of the host-pathogen interaction. Characteristics of interest include the pathogen's mechanisms for invading the host cell, how invasive the pathogen is in the host cell and if the pathogen can persist in the host cell. Host cell immune responses are also of importance for research aimed at vaccine development. Information gained from *in vitro* experiments can provide a baseline from which hypotheses for *in vivo* experiments can be generated (Babu et al., 2006).

To understand a pathogen's mechanisms for invading host cells, much research has focused on models that mimic the effect of conditions in the host on the pathogen's growth. For example, invasiveness of *Salmonella* into Madin-Darby canine kidney cells was affected by the concentration of oxygen. Specifically, low concentrations of oxygen induce *Salmonella* adherence and invasiveness, indicating oxygen limitation could be an environmental cue that triggers expression of genes required for invasiveness within the intestinal lumen of the host (Lee and Falkow, 1990).

In vitro cell invasion and persistence assays are important for indicating how virulent a pathogen can be in a host and for determining the mechanisms of pathogenesis. STM is a method that allows the investigation of the function of a gene of interest, by inactivating the gene through a mutation. For example, using strains of *S.* Typhimurium modified by STM to infect calf and chicken intestinal cells, mutants have been identified that can no longer infect these cells, therefore indicating possible roles for certain genes involved in pathogenesis (Morgan et al., 2004).

An insight into host immune responses can also be gained through *in vitro* studies. Broad-range *Salmonella* serovars cause limited gastroenteritis in their hosts, whereas host-restricted serovars cause rapid systemic infection (Shivaprasad, 2000). The different mechanisms broad-range and host-

restricted serovars have for infecting their host has been shown to result in different immune responses (Henderson et al., 1999). Broad-range serovars cause an acute inflammatory response, which is not seen with host-restricted serovars (Kaiser et al., 2000). *In vitro* and *in vivo* cell studies have been used to identify and quantify these different immune responses and may have important implications for future vaccine development (Kaiser et al., 2000).

In vitro studies are especially important for determining pathogen-host interactions and immune responses in human hosts, where *in vivo* studies are inevitably very limited and rarely conducted (Salazar-Gonzalez et al., 2004). Several cell lines are regularly used as human intestinal epithelial cell representatives including HT29 (enterocyte-like differentiation), T84 cells (secretary differentiation) and Caco2 (enterocyte-like differentiation) cells (Nandakumar et al., 2009, Vo et al., 2007, Shah et al., 2011). Interactions between these cell lines and enteropathogenic bacteria such as *Salmonella, Campylobacter* and *E. coli* are a focus for research (Nandakumar et al., 2009, Vo et al., 2007, Shah et al., 2011).

Several studies have documented immune responses by human intestinal epithelial cells against invasive and non-invasive enterobacteria. Upon *Salmonella* infection into human T84 colonic epithelial cells, IL-8 was secreted within 90 minutes to high concentrations (Eckmann et al., 1993). High concentrations of IL-8 against *Salmonella* infection have been shown using other human intestinal epithelial cell lines, such as Caco2 and HT29 cells (Vo et al., 2007, Witthoft et al., 1998). IL-8 is a pro-inflammatory cytokine suggested to be the initial signal for an acute inflammatory response upon bacterial invasion of mucosal surfaces (Eckmann et al., 1993). It has been suggested that IL-8 is produced when bacteria invade cells, as non-invasive bacteria, such as *E. coli*, do not induce such a response (Eckmann et al., 1993). In contrast, other research has shown non-invasive bacteria do stimulate human intestinal epithelial cells to produce IL-8 and suggests LPS is the predominant signal to stimulate cells for this activity (Nandakumar et al., 2009).

Chicken cell lines are also regularly used for *in vitro* research into host-pathogen interactions. Chicken cell lines currently used in research include HD11 (macrophage-like), MQ-NCSU (macrophage), CKC (kidney) and CEF (embryonic fibroblast) (Hughes et al., 2008, Withanage et al., 2005a, Kaiser et al., 2000). While there are currently very limited chicken intestinal epithelial cell lines available for use for studying immune responses against infection, chicken macrophage cell lines are used regularly. They are of a particular value as macrophages function as part of the innate immune response as well as providing an environment where intracellular pathogens, such as *Salmonella*, can survive and replicate (Uchiya et al., 2004, Juul-Madsen, 2008). Survival in macrophages is thought to be important for *Salmonella* to cause systemic infection and persistent infection in poultry (Wigley et al., 2005, Wigley et al., 2002b).

Invasion assays using macrophage cell lines can be useful for modelling mechanisms by which these cells can become activated by a pathogen and for determining the immune response produced by them. Macrophages recognise pathogens through pathogen recognition receptors (PRRs) and become activated (Juul-Madsen, 2008). Toll like receptors (TLRs) are PRRs and are important for recognising generic patterns on pathogenic organisms and initiating a rapid immune response (Juul-Madsen, 2008, Barton and Medzhitov, 2003). Chicken TLR5 has been found to be expressed on different immune cells, including macrophages (Iqbal et al., 2005a). Using HD11 chicken macrophage cells, which bear TLR5 constitutively, it has been shown TLR5 recognises bacterial flagellin expressed by *S.* Typhimurium and initiates an inflammatory response (Iqbal et al., 2005b, Chadfield and Olsen, 2001, Hayashi et al., 2001).

Activated macrophages can engulf pathogens through phagocytosis and produce antimicrobial mediators such as ROS and nitric oxide (NO) (Kaspers, 2008). Cell invasion assays using RAW264.7 and J774 murine macrophage cell lines have shown that infection by *S.* Typhimurium and *S.* Dublin lead to an increase in NO via up-regulation of iNOS (Cherayil et al.,

2000). Up regulation of iNOS and NO upon *S.* Dublin invasion has also been shown using human colonic epithelial cells (Witthoft et al., 1998).

Cell invasion assays have lead to a greater understanding of the role of cytokines and chemokines during the immune response to *Salmonella*. The Th1 cytokine IFN-γ can prime macrophage cell lines to be more reactive against pathogens, resulting in significantly greater production of antimicrobial properties, such as ROS and NO, in the presence of microbial agonists, including LPS (He et al., 2011). Additionally, the chicken Th2 cytokine IL-4 exerts bi-directional regulatory affects on macrophage production of ROS and NO (He et al., 2011). Although an increase in ROS and NO is observed following stimulation of macrophage cell lines with bacterial agonists, infection of MQ-NCSU macrophage cells with different *Salmonella* serovars showed the bacteria were not completely cleared from the cells, indicating other mechanisms are needed for *Salmonella* clearance (Withanage et al., 2005a).

In vitro studies have shown broad-range and host-restricted *Salmonella* serovars can survive and replicate within macrophages, despite the antimicrobial properties of these cells (Henderson et al., 1999). Cell invasion assays have been used to determine the mechanisms *Salmonella spp.* use to survive within macrophages. SPI-2 encodes a T3SS, which is essential for intra-macrophage survival of *S.* Gallinarum within HD11 chicken macrophage cells; however it is not needed for survival within non-phagocytic cells, indicating a particular role for SPI-2 in intra-macrophage survival (Jones et al., 2001). Further research using *in vitro* cell-based models has shown that *S.* Typhimurium causes an indirect SPI-2 dependent up-regulation of IL-10 in macrophages (Uchiya et al., 2004). Macrophages are a main source of IL-10, which acts as a negative feedback mechanism to inhibit macrophage production of ROS and reactive nitrogen species (RNS) (Bogdan et al., 1991). It has been suggested that stimulation of IL-10 production by *Salmonellae* provides a favourable environment for them to survive in, within macrophages (Uchiya et al., 2004).

The aim of this *in vitro* study was to determine key features of *S.* Virchow infection biology and to obtain an indication of how inflammatory the serovar is during infection. Initially, Vero cells were used to determine the invasiveness of *S.* Virchow in epithelial tissue. Following on from this, Caco2 cells were used to model *S.* Virchow invasiveness and persistence in human intestinal epithelial tissue, as well as to show the inflammatory response in human tissue against *S.* Virchow. Finally, HD11 cells were used to determine *S.* Virchow's invasiveness and persistence in macrophages, to indicate *S.* Virchow's ability to cause systemic infection in chickens and to determine inflammatory responses produced by chickens against *S.* Virchow.

3.2 Materials and Methods

3.2.1 Mammalian and chicken Cell Culture

Vero cells (Barrow and Lovell, 1989) were grown at 37°C in Eagles minimum essential medium (Sigma) containing 2mM L-glutamine, 100 units / 0.1mg/ml penicillin / streptomycin, 1x MEM non-essential amino acid solution, 0.075% sodium bicarbonate solution and 10% fetal calf serum (FCS).

Caco2 (colorectal adenocarcinoma) cells were cultured at 37°C in Dulbecco's modified Eagles medium (Sigma) containing 2mM L-glutamine, 50 units / 0.05mg/ml penicillin / streptomycin and 10% FCS (Witthoft et al., 1998, Vo et al., 2007).

HD11 chicken macrophage-like cells (Beug et al., 1979) were cultured at 37°C and 5% CO_2 in RPMI media (Lonza) supplemented with 2mM L-glutamine, 100 units / 0.1mg/ml penicillin / streptomycin, 2.5% FCS, 2.5% chicken sera and 10% tryptose phosphate broth (Kaiser et al., 2000).

3.2.2 Bacterial Cultures

Twelve *S.* Virchow isolates (See Chapter 2, Table 2.1 for details), *S.* Typhimurium isolates F98, 238 and 244, LPS and a negative control were

included in the gentamicin protection assays. Bacterial cultures were grown overnight in 10ml LB broth at 37°C and 150 rpm in an orbital shaking incubator. Bacterial cultures were adjusted to $1x10^8$cfu/ml and added to the cells at a multiplicity of infection (moi) of 10 (Kaiser et al., 2000). LPS from *S.* Typhimurium (Sigma, UK) was reconstituted to 200µg/ml and 100µl per well was added to triplicate cell wells.

3.2.3 Gentamicin Protection Assay

To determine *S.* Virchow invasiveness into epithelial tissues, gentamicin protection assays were performed, initially using Vero cells. Vero cells were passaged into 24-well plates, 24 hours prior to the start of the assay, in antibiotic-free media. Each isolate was added separately to cell wells in triplicate (100µl per well) and the cells were incubated for 1 hour at 37°C. After a 1 hour incubation with the *Salmonella* isolates the media on the cells was replaced with media containing 100µg/ml of gentamicin sulphate and the cells were incubated for 1 hour at 37°C, to kill any extracellular bacteria (Jones et al., 2001). Following incubation, the cells were washed twice with 1× phosphate buffered saline (PBS) to remove any extracellular bacteria remaining and lysed with 1xPBS containing 0.5% Triton-X100. Serial dilutions of the supernatant were plated onto nutrient agar and incubated overnight to obtain viable bacterial counts. The assay was performed on three separate occasions and a mean bacterial count was taken for each isolate.

A gentamicin protection assay was also performed using Caco2 and HD11 cells to determine *S.* Virchow invasiveness in these cell lines. In addition, a parallel set of plates were set up during these assays. Instead of lysing the cells with 0.5% Triton-X100, they were incubated for a further 24HPI with 20µg/ml of gentamicin, to determine the persistence of *S.* Virchow in them (Jones et al., 2001). At 24HPI, the cells were incubated for 1 hour at 37°C in media containing 100µg/ml of gentamicin. After the 1 hour incubation, the cells were washed, lysed and serially diluted as described above, to obtain viable bacterial counts.

3.2.4 IL-8 ELISA

Human IL-8 CytoSet ELISA kits (Invitrogen, UK) were used to determine IL-8 production in the supernatant samples collected during the Caco2 invasion assays at 4, 8 and 24 HPI (Nandakumar et al., 2009, Vo et al., 2007). ELISAs were performed following the manufacturer's instructions. Absorbance was determined using a microplate reader at 450nm and the concentration of IL-8 in samples was determined using a standard curve, with a concentration range of 12.5pg/ml - 800pg/ml.

3.2.5 NO assay

To determine NO production by HD11 cells against *S.* Virchow invasion, a Griess reagent kit was used to measure nitrite, a stable metabolite of nitric oxide, in the cell supernatant (Barton and Medzhitov, 2003, Okamura et al., 2005). Measurements were taken at 4, 8 and 24 HPI following the manufacturer's instructions. Absorbance was determined using a microplate reader at 550nm and nitrite concentration was determined using a standard curve with a concentration range of 1-100µM.

3.2.6 Statistical analysis

Statistical analysis was performed using SPSS 16.0. Bacterial counts, concentration of IL-8 and concentration of nitrites were compared using one-way ANOVA. Significance between the values was taken if the *P* value was <0.05.

3.3 Results

3.3.1 Vero cell gentamicin protection assay

To determine the invasiveness of *S.* Virchow in epithelial cells, Vero cells were initially used. All 12 *S.* Virchow isolates showed similar levels of invasiveness, with the mean counts being $\log_{10}$ 5.42-6.43 cfu/ml (Figure 3.1). The mean colony count for *S.* Typhimurium F98 was $\log_{10}$ 4.04 cfu/ml, which was significantly lower than those for all 12 *S.* Virchow isolates (*P* = <0.001).

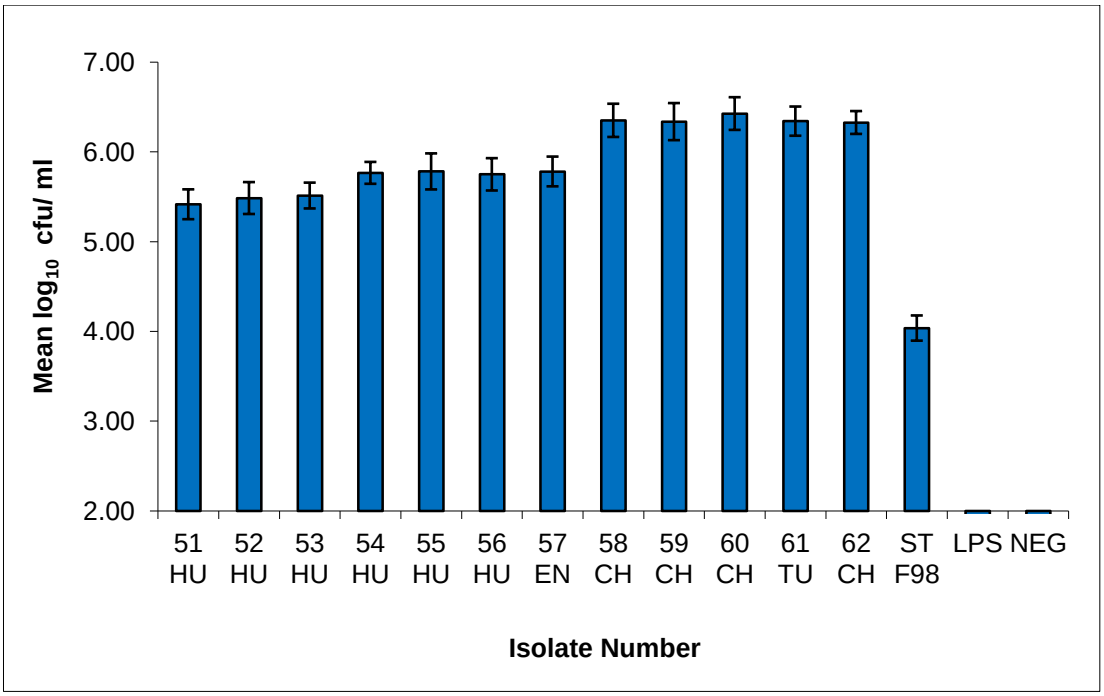

Figure 3.1: Mean $\log_{10}$ cfu/ml of *S.* Virchow and *S.* Typhimurium from Vero cells at 1 HPI. Error bars represent standard error of the mean.

3.3.2 Caco2 cell gentamicin protection assay

Viable counts of *Salmonella* were made 1 HPI to assess invasiveness and 24 HPI to determine persistence in Caco2 cells. At 1 HPI, the colony counts for the 12 *S.* Virchow isolates ranged from $\log_{10}$ 5.3 to $\log_{10}$ 7.0 cfu/ml (Figure 3.2). No significant difference was found between the highest and lowest colony count (*P* = >0.187). The 12 *S.* Virchow isolates had similar colony counts at 1 HPI compared to *S.* Typhimurium F98 and *S.* Typhimurium 244, with their counts being $\log_{10}$ 6.90 and $\log_{10}$ 6.70 cfu/ml, respectively.

At 24 HPI, *S.* Virchow colony counts ranged from $\log_{10}$ 6.2 to $\log_{10}$ 7.3 cfu/ml (Figure 3.2). *S.* Virchow colony counts mainly increased at 24 HPI compared to 1 HPI, with isolates 54, 55, 60, 61 and 62 significantly increasing in number (*P* = <0.001). *S.* Virchow colony counts were similar to *S.* Typhimurium F98 ($\log_{10}$ 6.80 cfu/ml) and *S.* Typhimurium 244 ($\log_{10}$ 7.90 cfu/ml) at 24 HPI, although *S.* Virchow isolates 58 and 60 had significantly greater bacterial recovery than *S.* Typhimurium F98 (*P* = <0.038).

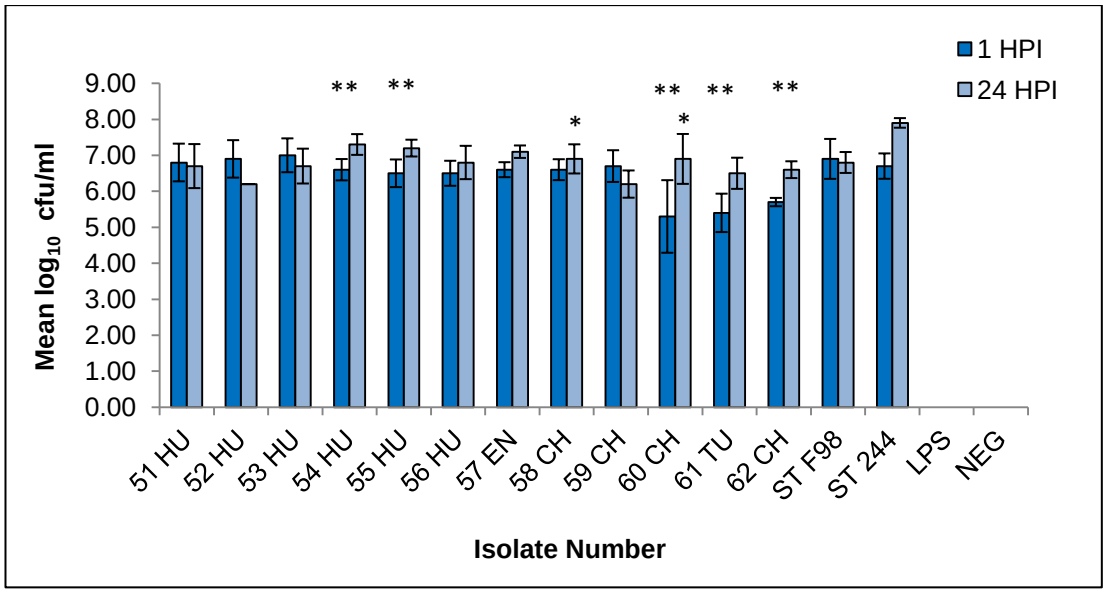

Figure 3.2: Mean $\log_{10}$ cfu/ml of *S.* Virchow and *S.* Typhimurium at 1 HPI and 24 HPI in Caco2 cells. Error bars represent standard error of the mean.
* = Isolate has significantly greater colony counts than *S.* Typhimurium F98,
** = Isolate shows significantly greater bacterial recovery at 24 HPI compared to 1 HPI.

3.3.3 IL-8 production by Caco2 cells

The IL-8 concentration in Caco2 cell supernatant was measured at 4, 8 and 24 HPI, for an indication of an acute inflammatory response produced by these cells in response to *S.* Virchow infection. IL-8 was detected in cell supernatant at 4 HPI against all 12 *S.* Virchow isolates (Figure 3.3). It was not detected in the supernatant of cells infected with *S.* Typhimurium F98 and *S*. Typhimurium 244 or against LPS antigen at this time point. IL-8 could be detected in increased concentrations at 8 HPI in the supernatant of cells infected with *S.* Virchow (Figure 3.3). At 8 HPI, a low concentration of IL-8 could be detected against *S.* Typhimurium F98 and *S.* Typhimurium 244 (Figure 3.3). Maximal concentrations of IL-8 could be detected at 24 HPI against the *S.* Virchow isolates (Figure 3.4). Increased concentrations of IL-8 were detected against *S.* Typhimurium F98 and *S.* Typhimurium 244 (Figure 3.4), although IL-8 concentrations were significantly greater against 9 *S.* Virchow isolates (P = <0.05) (Figure 3.4). IL-8 production could be detected against LPS antigen by 24 HPI (Figure 3.4).

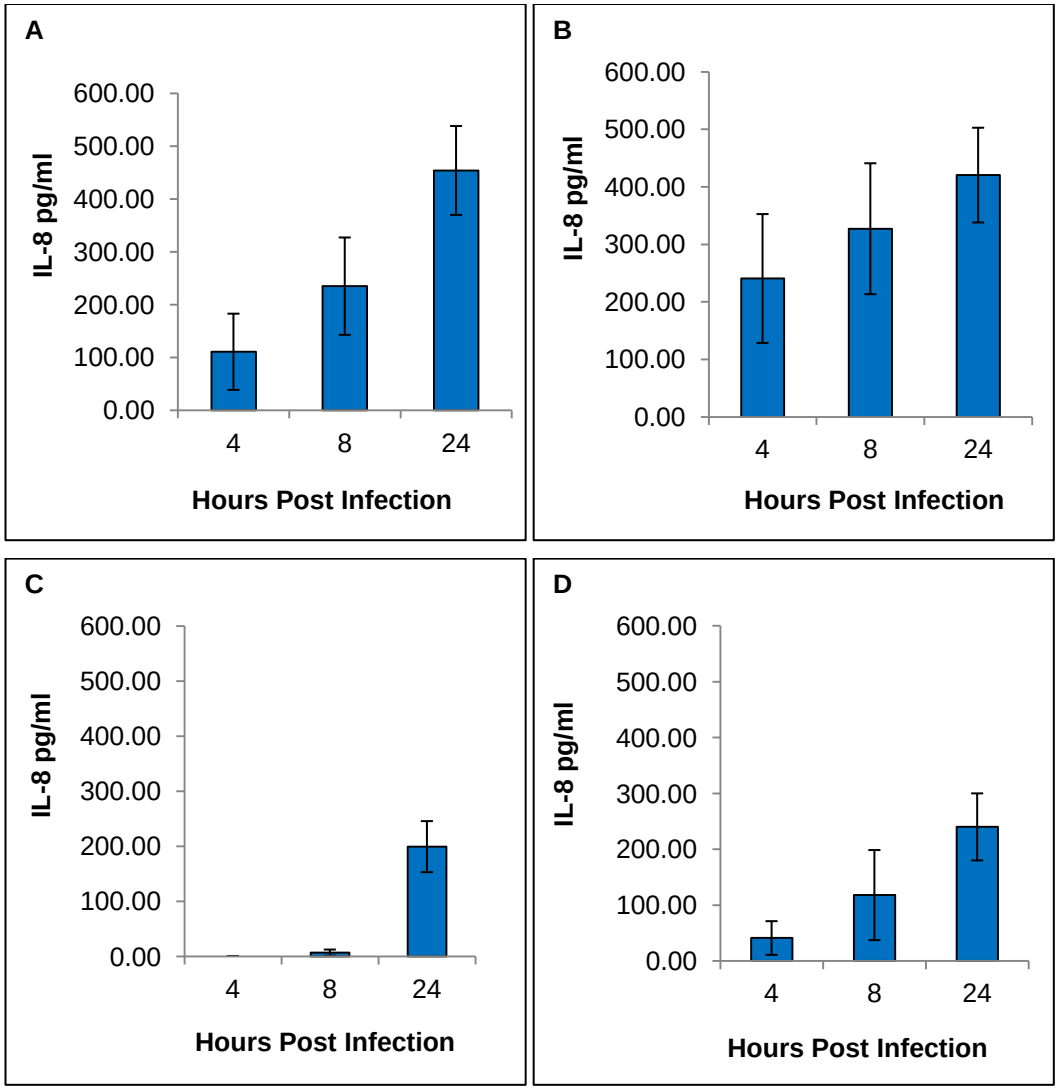

Figure 3.3: IL-8 production by Caco2 cells at 4, 8 and 24 HPI. A = *S.* Virchow 58, B = *S.* Virchow 59, C = *S.* Typhimurium F98, D = *S.* Typhimurium 244.

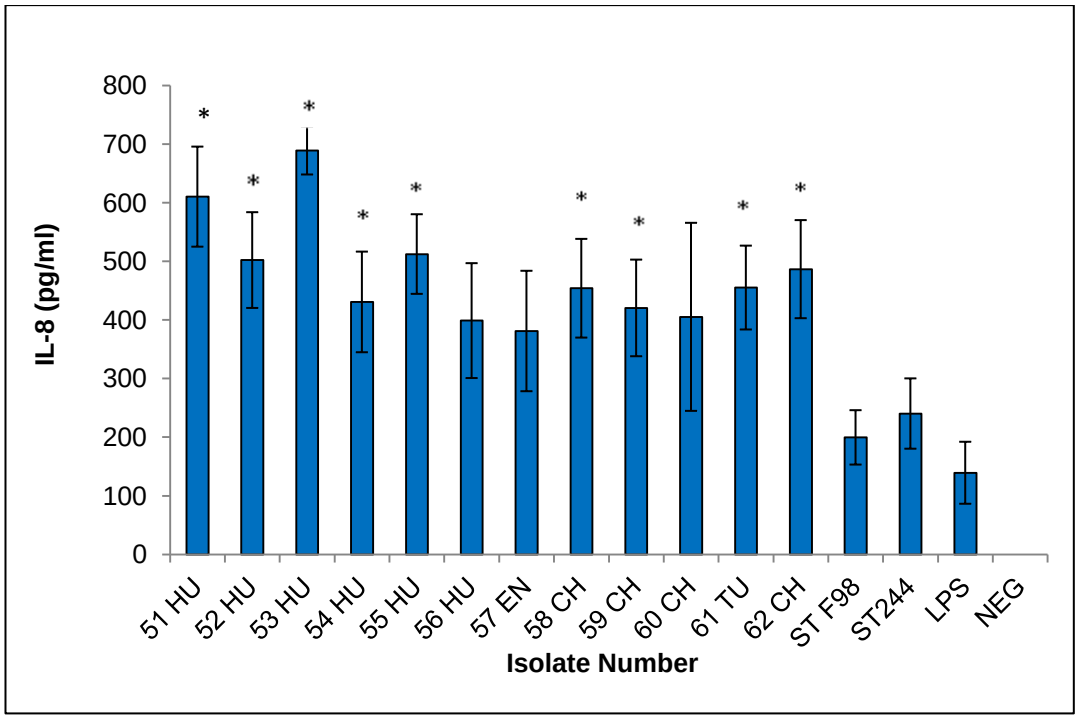

Figure 3.4: IL-8 production by Caco2 cells at 24 HPI in response to *S.* Virchow (51 HU-62 CH), *S.* Typhimurium F98, *S.* Typhimurium 244 and LPS. * = *S.* Virchow isolates that stimulated a significantly greater production of IL-8 compared to *S.* Typhimurium isolates F98 and 244.

3.3.4 HD11 gentamicin protection assay

Colony counts of *S.* Virchow were taken 1 HPI and 24 HPI to determine invasiveness and persistence of the serovar in HD11 chicken macrophage-like cells. At 1 HPI, colony counts for *S.* Virchow ranged from $\log_{10}$ 6.09 cfu/ml to $\log_{10}$ 6.48 cfu/ml (Figure 3.5). At 1 HPI, *S.* Typhimurium F98 had a mean colony count of $\log_{10}$ 6.20 cfu/ml, which was similar to the colony counts for the *S.* Virchow isolates. *S.* Typhimurium 238 and *S.* Typhimurium 244 were included in the assay and had colony counts of $\log_{10}$ 6.22 cfu/ml and $\log_{10}$ 6.31 cfu/ml, respectively. No significant differences in colony counts were found between the *S.* Virchow and *S.* Typhimurium isolates at 1 HPI (*P* = >0.181).

At 24 HPI, colony counts for *S.* Virchow had decreased slightly, ranging from $\log_{10}$ 4.57 cfu/ml to $\log_{10}$ 5.99 cfu/ml (Figure 3.5). A significant difference was

found between the isolates with the lowest (*S.* Virchow 60) and highest (*S.* Virchow 53) bacterial recovery (*P* = >0.001), showing variation in ability to persist until 24 HPI. A decrease in colony counts for the *S.* Typhimurium isolates at 24 HPI was observed. *S.* Typhimurium F98, *S.* Typhimurium 238 and *S.* Typhimurium 244 had mean colony counts of $\log_{10}$ 5.98 cfu/ml, $\log_{10}$ 5.55 cfu/ml and $\log_{10}$ 5.41 cfu/ml, respectively. Colony counts of *S.* Virchow were similar to colony counts of *S.* Typhimurium at 24 HPI; however, *S.* Virchow isolates 52, 54 and 60 had significantly lower counts (*P* = <0.028). Overall, between 1 HPI and 24 HPI all of the *S.* Virchow and *S.* Typhimurium isolates had reduced colony counts, being significant in 5 *S.* Virchow isolates, *S.* Typhimurium 238 and *S.* Typhimurium 244 (*P* = <0.031) (Figure 3.5).

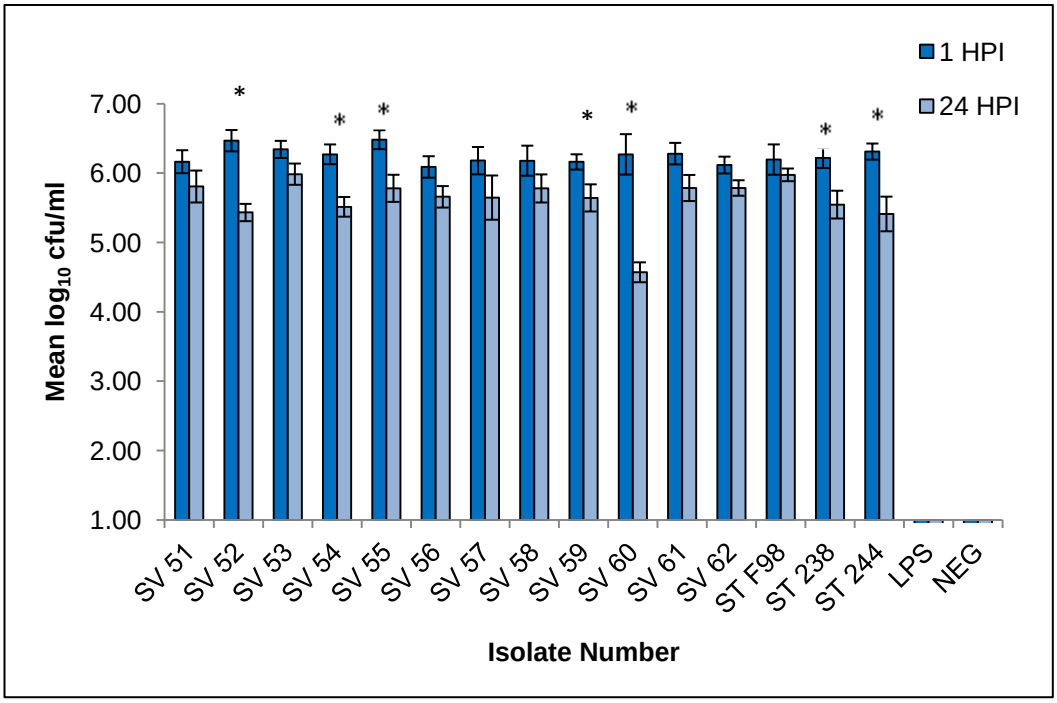

Figure 3.5: Mean $\log_{10}$ cfu/ml of *S.* Virchow and *S.* Typhimurium isolates at 1 HPI and 24 HPI in HD11 cells. Error bars represent standard error of the mean. * = Isolates with a significantly lower bacterial recovery at 24 HPI compared to 1 HPI.

3.3.5 Quantification of nitrites

A Griess reagent kit was used to measure nitrites in the supernatant of HD11 cells infected with *S.* Virchow at 4, 8 and 24 HPI. At 4 HPI, nitrites could be detected in the supernatant of cells infected with the *S.* Virchow isolates and *S.* Typhimurium isolates, as well as supernatant of cells incubated with LPS antigen (Figure 3.6). No significant difference was found between the concentrations of nitrites produced in response to *S.* Virchow compared to *S.* Typhimurium at this time point (P = >206).

The concentrations of nitrites in the cell supernatants increased at 8 HPI compared to 4 HPI, in response to all of the *S.* Virchow isolates (Figure 3.6). The concentration at 8 HPI was significantly greater against *S.* Virchow isolates 52, 53, 54, 55, 56, 58, 59 and 60, compared to 4 HPI (P = <0.013). The concentration of nitrites produced in response to the *S.* Typhimurium isolates decreased slightly at 8 HPI compared to 4 HPI; however, this decrease was not significant (P = >0.336). A significantly higher concentration of nitrites was produced in response to *S.* Virchow isolates 52, 53, 54, 55, 56, 58, 59 and 60 compared to *S.* Typhimurium F98 at 8 HPI (P = <0.006). At 24 HPI, the concentration of nitrites had increased in response to all of the *S.* Virchow and *S.* Typhimurium isolates, as well as against the LPS antigen (Figure 3.6 & 3.7). The concentration of nitrites at 24 HPI was significantly greater then at 8 HPI in response to all of the *S.* Virchow isolates and all of the *S.* Typhimurium isolates, as well as in response to the LPS (P = <0.05).

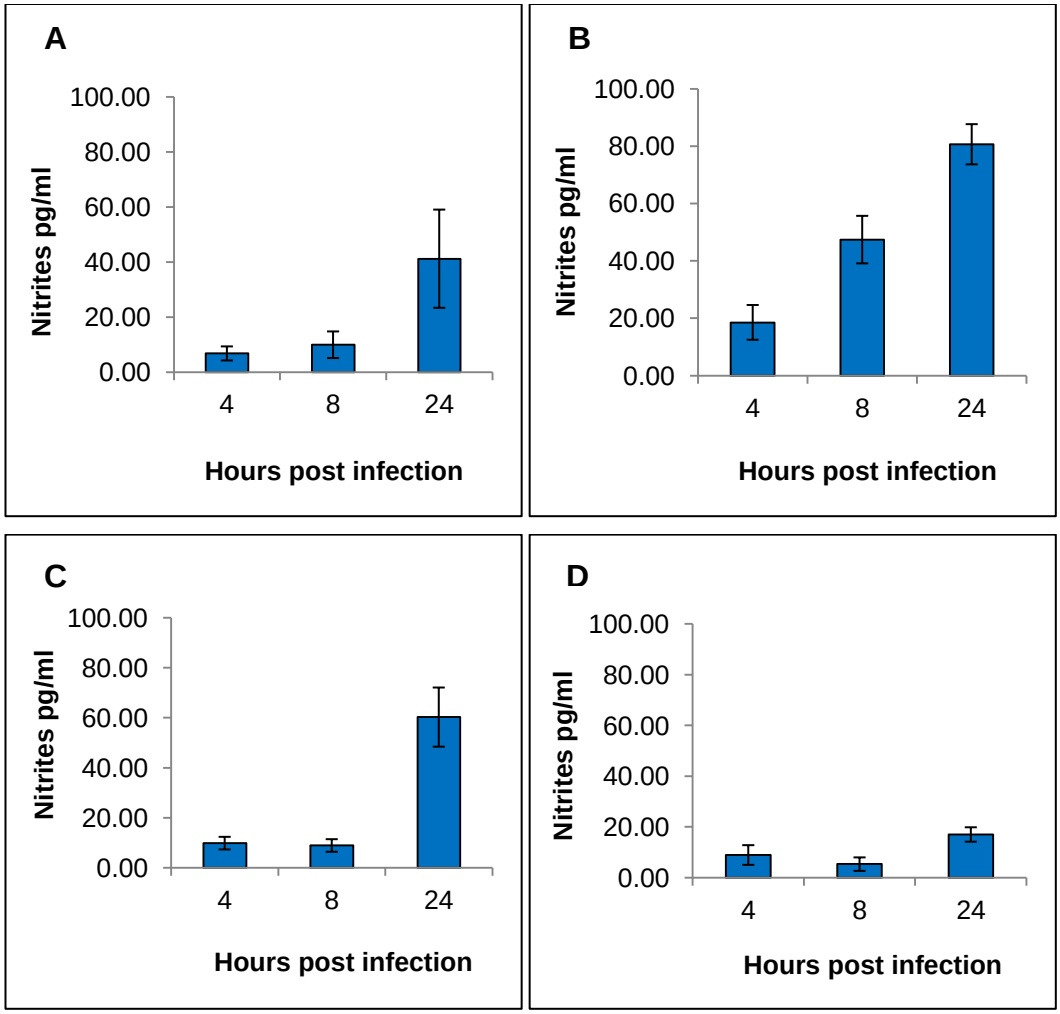

Figure 3.6: Concentration of nitrites in HD11 cell supernatant at 4, 8 and 24 HPI. A = *S.* Virchow 57, B = *S.* Virchow 58, C = *S.* Typhimurium F98, D = LPS antigen.

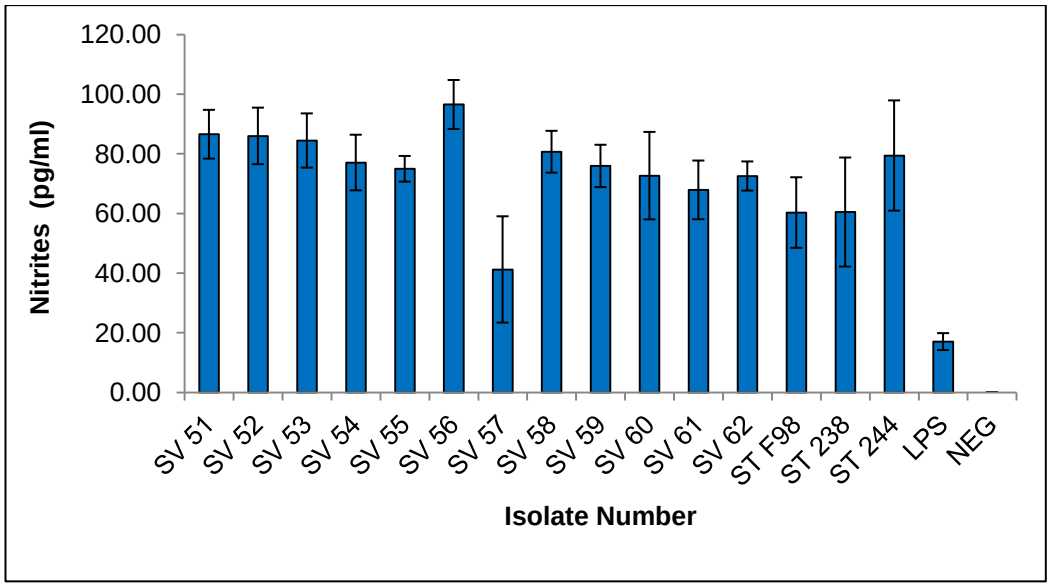

Figure 3.7: The effects of different S Virchow and S Typhimurium isolates on the concentration of nitrites in HD11 cell supernatant at 24 HPI. Error bar represents the standard error of the mean.

3.4 Discussion

This study has shown that *S.* Virchow is an invasive serovar in epithelial tissue, in particular human intestinal tissue, which is consistent with reports of *S.* Virchow causing invasive disease in humans. *S.* Virchow has shown the ability to persist and replicate over 24 hours in Caco2 cells and the results indicate it causes a large inflammatory response in these cells. *S.* Virchow also showed the ability to invade HD11 chicken macrophage-like cells, which stimulated an immune response, with increasing nitrite concentrations being detected in the supernatant over a 24 hour infection period.

The 12 *S*. Virchow isolates included in this study were all significantly more invasive into Vero cells then *S.* Typhimurium F98 (*P* = <0.001). *S.* Virchow was also similarly invasive compared to *S.* Typhimurium F98 and *S.* Typhimurium 244 in Caco2 cells, if not significantly more so (*P* = <0.002). No significant difference was found between the lowest and highest colony counts for the *S.* Virchow isolates (*P* =0.187), although they did differ slightly in their ability to invade Caco2 cells. Differences in invasion potential of *S.* Enteritidis isolates into Caco2 cells have been shown to be associated with

motility and ability to secrete flagellin proteins or SPI-1 T3SS proteins, but not their ability to form biofilms (Shah et al., 2011). *S.* Enteritidis isolates which were less invasive into Caco2 cells had impaired motility and impaired secretion of flagella-associated proteins or SPI-1 T3SS proteins (Shah et al., 2011). Although all of the *S.* Virchow isolates had a range of virulence genes encoded on SPI 1-5, it may have been useful to investigate motility and possibly biofilm formation of the isolates to account for differences in invasiveness into Caco2 cells.

Even though the *S.* Virchow colony counts varied slightly they were all high compared to the invasiveness of *S.* Enteritidis isolates into Caco2 cells (Shah et al., 2011). In this study, isolates were grouped based on invasiveness. The isolates considered as having high invasiveness had an average colony count of $\log_{10}$ 5.58 cfu/ml, with the highest count being $\log_{10}$ 5.91 cfu/ml. The *S.* Virchow colony counts were comparable to, if not higher than these counts, suggesting that *S.* Virchow is more invasive than *S.* Enteritidis into human intestinal tissue. This is consistent with clinical evidence implying that it is an invasive serovar in humans and more invasive than *S.* Enteritidis and *S.* Typhimurium (Langridge, 2008).

Over the 24 hour infection period, some *S.* Virchow isolates decreased in bacterial recovery; however, 8 out of the 12 isolates increased, with 5 showing a statistically significant increase (P = <0.001). Although there are some differences in the colony counts of the *S.* Virchow isolates, those at 24 HPI show *S.* Virchow can persist and replicate in Caco2 cells. Invasion and persistence in human intestinal epithelial tissue is essential for the serovar to invade cells of the mononuclear phagocyte system, such as macrophages and cause systemic infection (Eckmann and Kagnoff, 2001).

IL-8 production by Caco2 cells in response to *S.* Virchow was determined at 4, 8 and 24HPI, as an *in vitro* assessment of the inflammatory response produced by these cells against *S.* Virchow infection. IL-8 could be detected in Caco2 cell supernatant at 4HPI, which increased in concentration at 8 HPI and increased in concentration further at 24 HPI. In comparison, *S.*

Typhimurium F98 and *S.* Typhimurium 244 stimulated IL-8 production by Caco2 cells; however, the concentration of IL-8 produced was less than the concentration of IL-8 produced against *S.* Virchow. One hypothesis for the concentration of IL-8 produced in response to a strain of *Salmonella* is that the amount of IL-8 produced is dependent on how invasive the strain is (Vo et al., 2007). Therefore, the higher the intracellular colony counts, the higher the predicted concentration of IL-8 (Vo et al., 2007). My results did not support this hypothesis, showing no correlation between colony count at 1 HPI and IL-8 production. Furthermore, the *S.* Typhimurium isolates were similarly invasive into Caco2 cells compared to the *S.* Virchow isolates; however, elicited a weaker production of IL-8.

A *S.* Typhimurium LPS control was included in the assay to determine if the main stimulus for IL-8 production was invasion into the cell by the *Salmonella* or extracellular stimulation by recognition of LPS. During the assays, no *Salmonella* colony counts were detected from the LPS wells. IL-8 production against LPS could not be detected until 24 HPI. At this time point, the concentration of IL-8 produced by the Caco2 cells against 10 of the 12 *S.* Virchow isolates was still significantly greater than the IL-8 produced against the LPS (P = <0.033). The results suggest that invasion of *S.* Virchow into Caco2 cells is the main stimulus for IL-8 production by the cells and this is supported by other studies. Eckmann et al (1993) showed invasive bacteria such as *S.* Dublin stimulated T84 cells (colonic epithelial) and HeLa (cervical epithelial) cells to produce IL-8, whereas non-invasive bacteria such as *E.* Coli DH5α stimulated hardly any IL-8 production (Eckmann et al., 1993). Additionally, the T84 cells responded differently depending on the invading bacteria. Some strains invaded the T84 cells equally as well, however T84 cells only responded and produced IL-8 against some of them (Eckmann et al., 1993). These findings were comparable to the findings in this study, as *S.* Virchow and *S.* Typhimurium were similarly invasive into Caco2 cells; however, a greater IL-8 response was produced against *S.* Virchow. These results show certain cell types may respond differently to invasion depending on the invading strain of bacteria. Although these results suggest bacterial

invasion is the main stimulus for IL-8 production, other mechanisms may initiate IL-8 release from cells.

The results outlined in this study showed a low IL-8 production against LPS antigen; however, several previous studies have shown IL-8 production is stimulated by LPS interactions with host cell surface receptors. Interactions between LPS and TLR4 have been shown to activate host cells to produce of IL-8 and TNFα (Haraga et al., 2008). Other bacterial components have also been shown to interact with TLRs to stimulate production of IL-8, including bacterial flagellin and TLR5 (Chadfield and Olsen, 2001). A recent study using two human intestinal cell lines showed invasive and non-invasive bacteria can stimulate cells to produce IL-8, supporting studies that show interactions of bacterial surface components with host cell surface receptors, as well as invasion can activate cells to produce an immune response (Nandakumar et al., 2009). Additionally, non-invasive *Vibrio cholerae* stimulated high concentrations of IL-8 production by HT29 (enterocyte-like) cells; however, low concentrations of IL-8 production by T84 (crypt-like) cells, highlighting the differences in the immune response produced, depending on the cell type (Nandakumar et al., 2009).

Following *S.* Typhimurium invasion into cells, the SPI-1 T3SS effector protein sopE2 stimulates IL-8 production, by activating proteins that are part of the TLR signalling pathways (Huang et al., 2004, Kucharzik et al., 2005). In addition, presence of flagellin in combination with the effects of sopE2 had an enhanced effect on IL-8 production (Huang et al., 2004). These results support both hypotheses that invasion of bacteria into cells, and bacterial interactions with surface TLRs, stimulates IL-8 production and also that both mechanisms could act synergistically to cause a greater IL-8 response (Huang et al., 2004).

IL-8, irrespective of mechanism of production, is thought to be an initial signal for an acute inflammatory response and is a potent chemo-attractant of neutrophils in mammals (Tapping et al., 2000). Chicken IL-8 has been shown to have a similar function and is a strong chemo-attractant for

monocytes/macrophages and lymphocytes (Martins-Green, 2001). The rapid and high concentrations of IL-8 produced by Caco2 cells against *S.* Virchow in this study indicate *S.* Virchow is a highly inflammatory serovar in human intestinal epithelial tissue. The high inflammatory response produced by intestinal cells against *S.* Virchow is usually observed with broad range serovars that cause limited gastroenteritis. However, studies of clinical data have shown *S.* Virchow to be an invasive serovar in humans (Langridge, 2008). Overall, *S.* Virchow appears to be a serovar that would usually cause limited inflammatory gastroenteritis in humans. However, *S*. Virchow has a tendency to overcome host defences and cause invasive disease and septicaemia, particularly in more vulnerable people, such as children with less developed immune systems, the immuno-compromised and the elderly who have weaker immune systems (Ispahani and Slack, 2000, Matheson et al., 2010, Gulcan et al., 2012, Weinberger et al., 2004, Schifferdecker et al., 2009).

The HD11 cell line is an avian myelocytomatosis virus (MC29) transformed chicken macrophage-like cell line and has been widely used for *in vitro* studies to represent chicken macrophages (Beug et al., 1979). The *S.* Virchow isolates all showed very similar levels of invasiveness into HD11 cells, with colony counts ranging from $\log_{10}$ 6.09 cfu/ml to $\log_{10}$ 6.48 cfu/ml at 1 HPI. In addition, the colony counts were very similar when comparing *S.* Virchow to *S.* Typhimurium at 1 HPI (P = >0.181). At 24 HPI, all of the *S.* Virchow isolates had decreased in colony count, with counts ranging from $\log_{10}$ 4.57 cfu/ml to $\log_{10}$ 5.99 cfu/ml, showing the ability of macrophages to limit infection. Although the colony counts had decreased at 24 HPI, compared to 1 HPI, they still remained high showing *S.* Virchow can persist in HD11 cells. However, the length of time they can persist in these cells may be limited. Some *Salmonella* serovars including *S.* Typhimurium have been shown to survive in HD11 cells for at least 48 hours; however, the counts were significantly lower at this time point than compared to earlier time points (Setta et al., 2012a). Intra-macrophage survival has been shown to be essential for systemic infection, as it allows the bacteria to disseminate to tissues such as the spleen, via the macrophages circulating in the blood and

lymphatic system (Wigley et al., 2005, Wigley et al., 2002b, Henderson et al., 1999, Chappell et al., 2009). Therefore, the possible limited persistence of *S.* Virchow in HD11 cells is consistent with the poultry infection experiment (Chapter 4), showing *S.* Virchow causes transient systemic infection in poultry, being cleared from systemic sites by 26 DPI.

Nitrite concentration in HD11 cell supernatant was measured at 4, 8 and 24 HPI to determine macrophage responses to *S.* Virchow infection. The concentration of nitrites produced in response to *S.* Virchow was similar to that produced in response to *S.* Typhimurium and LPS at 4 HPI. However, at 8 HPI and 24 HPI, the concentrations of nitrite detected against *S.* Virchow were much higher than nitrite detected against *S.* Typhimurium and LPS. The importance of the production of NO and ROS by activated macrophages for *Salmonella* killing has been well documented and previous studies have shown a large increase in NO production by HD11 chicken macrophage cells by 24 HPI against *S.* Typhimurium and *S.* Enteritidis (Babu et al., 2006, Setta et al., 2012a).

Research has shown that oxidative burst activity is greater in chickens genetically resistant to systemic salmonellosis compared to chickens genetically susceptible to systemic salmonellosis, suggesting macrophage activity in chickens is important against *Salmonella* infection (Wigley et al., 2002a). Therefore, the greater response of HD11 cells to *S.* Virchow compared to *S.* Typhimurium may suggest chicken macrophages are more resistant against *S.* Virchow infection. Although, this greater response did not reduce *S.* Virchow colony counts any more than *S.* Typhimurium colony counts by 24 HPI. Similar results have been shown using MQ-NCSU chicken macrophage cells. Infection with *S.* Typhimurium, *S.* Enteritidis and *S.* Gallinarum resulted in increased production of intracellular nitrite; however, the bacteria were not eliminated, suggesting other mechanisms as well as nitrite production are needed to eliminate *Salmonella* from macrophages (Withanage et al., 2005a).

In contrast, other research has shown treatment of HD11 cells with live *S.* Enteritidis culture completely abolishes NO production (He et al., 2011). It has been suggested that the ability of *S.* Enteritidis to abolish NO production may be a mechanism to ensure intra-macrophage survival (He et al., 2011). Increasing concentrations of NO against *S.* Virchow and *S.* Typhimurium in this study suggest these serovars do not use the same mechanism as *S.* Enteritidis to down-regulate NO. Even though an increasing amount of nitrites were produced against *S.* Virchow and *S.* Typhimurium, the colony counts for the isolates only decrease slightly over a 24 hour infection period, suggesting they are initially resistant to killing by phagocytosis. The greater survival of *S.* Virchow and *S.* Typhimurium in macrophages and the larger production of nitrites may suggest these two serovars are more inflammatory in chickens then *S.* Enteritidis. Other research comparing differential macrophage responses to *S.* Typhimurium and *S.* Enteritidis has also showed *S.* Typhimurium results in an increased inflammatory response (Okamura et al., 2005).

The size and the type of responses seen by macrophage cells reported in previous studies have varied according to the infecting *Salmonella* serovar and the responding cell type. Colony counts of *S.* Enteritidis after 1 HPI have been shown to be lower in J774A.1 mouse macrophage cells than HD11 cells, indicating J774.1 cells have a more dynamic bactericidal activity and HD11 cells are less effective (Babu et al., 2006). Using J774A.1 cells and HD11 cells it has also been shown no differences in oxidative burst occur against host-restricted and broad range *Salmonella* serovars, suggesting host-adaption is not linked to the serovars ability to avoid oxidative bursts (Chadfield and Olsen, 2001).

Cell lines are used regularly to determine mechanisms of pathogenesis and immune responses produced against a pathogen. Although useful information can be obtained from *in vitro* cell assays differences between results from different cell lines and between *in vitro* and *in vivo* results are often found and highlight the limitations of *in vitro* studies. Several studies have found that IFN-γ primes macrophages and therefore enhances the

production of ROS and NO against *Salmonella* (Okamura et al., 2005, He et al., 2011). In contrast, NO production is similar against *Salmonella* infection whether the macrophages are primed with IFN-γ or not (Babu et al., 2006). Differences in oxidative burst have also been described between primary macrophages and immortalised cell line macrophages (Chadfield and Olsen, 2001). Differences between *in vitro* findings show the limitations of cell lines and the need for *in vivo* models of infection.

This study has shown that *S.* Virchow is an invasive and persistent serovar in human intestinal epithelial cells, which is consistent with clinical data showing it has a tendency to cause systemic disease. High concentrations of IL-8 detected in the Caco2 cell supernatant following co-culture with *S.* Virchow indicate that it is a highly inflammatory serovar in human intestinal tissue. Although *S.* Virchow had similar colony counts compared to *S.* Typhimurium at 1 HPI and 24 HPI, concentrations of IL-8 produced in response to *S.* Virchow were significantly higher than against *S.* Typhimurium. The reason for this difference is unclear, although *S.* Typhimurium F98 lacks *sopE*, a homologue of *sopE2* shown to up regulate IL-8 production, which could be a contributing factor.

S. Virchow can invade and persist in HD11 chicken macrophage-like cells over a 24 hour period. NO could be detected in the cell supernatant as early as 4 HPI against *S.* Virchow and increased at 8 HPI and 24 HPI. The high concentrations of NO produced in response to *S.* Virchow indicate phagocytic activity by the macrophage cells and the highest concentrations detected at 24 HPI coincide with a decrease in bacterial colony counts. Although colony counts were lower at 24 HPI, this decrease was not significant and *S.* Virchow colony counts still remained high, suggesting *S.* Virchow is resistant to macrophage killing. *S.* Virchow colony counts were very similar to *S.* Typhimurium, indicating *S.* Virchow behaves in a similar way in chicken macrophages. This similar infection biology has been supported and confirmed in the *in vivo* poultry infection experiments described in Chapter 4.

Chapter 4
Poultry infection experiments

4.1 Introduction

Research conducting *in vivo* infection of chickens with *Salmonella* is often performed to establish the invasion, colonisation and persistence mechanisms of these bacteria. While *in vitro* assays are available for this, they have limitations and often do not provide a true representation of mechanisms *in vivo*. The main focus of research has been on broad-range serovars *S.* Typhimurium and *S.* Enteritidis and host-restricted serovars *S.* Pullorum and *S.* Gallinarum, as these serovars have caused the greatest burden to human health, poultry health and the economy (Santos et al., 2011, Shivaprasad, 2000, DuPont, 2007).

S. Typhimurium and *S.* Enteritidis are the 2 most common serovars causing human salmonellosis worldwide, although prevalence of serovars does vary from country to country (DEFRA, 2008c, DEFRA, 2010, EFSA, 2012). The consumption of contaminated poultry meat and eggs is the main vehicle of transmission of *Salmonella* infection in humans (Mead, 2004, Currie et al., 2005, Braden, 2006, Chittick et al., 2006, Little et al., 2007, Lublin and Sela, 2008, FSA, 2009). Chickens over 5 days old usually have no clinical symptoms of *Salmonella* infection, therefore enabling undetected spread of the disease in breeder farms and hatcheries, as well as horizontal transfer from contaminated faeces, during transport to the slaughterhouse or during slaughter (Kim et al., 2007, Marin and Lainez, 2009, Davies and Breslin, 2001, Corry et al., 2002). A better understanding of how *Salmonella* serovars commonly found in the human food chain infect and colonise chickens is essential for developing preventative methods against infection and subsequently reducing the pathogen's entry into the food chain (Dunkley et al., 2009).

In chickens older than 3-4 days, *S.* Typhimurium infection usually causes limited gastroenteritis (Morgan et al., 2004, Barrow et al., 1987). As part of this process, *S.* Typhimurium will colonise the intestinal lumen and target epithelial cells for invasion, using the SPI-1 T3SS, which injects effector proteins into the target cell in a contact-dependant manner to induce

membrane ruffling and cytoskeleton rearrangement, aiding entry into the cell (Ehrbar and Hardt, 2005, Hardt et al., 1998b). Colonisation of the intestinal tract and invasion into the epithelial cells by *S.* Typhimurium causes an acute inflammatory response in chickens, leading to an influx of heterophils and secretion of cytokines and chemokines (Meade et al., 2009, Withanage et al., 2004, Withanage et al., 2005a).

S. Enteritidis is highly associated with human salmonellosis via the consumption of contaminated eggs (Braden, 2006, Dunkley et al., 2009, Little et al., 2007). Vertical transmission of *S.* Enteritidis via the trans-ovarian route has been suggested for the mechanism of egg contamination (Okamura et al., 2001). Following intravenous infection of mature laying hens, *S.* Enteritidis colonised the reproductive tissue to significantly higher levels than the other *Salmonella* serovars, indicating that it has a higher affinity or tropism for reproductive tissues (Okamura et al., 2001). Furthermore, out of the six different serovars ,*S.* Enteritidis was the most frequent serovar detected in laid eggs (Okamura et al., 2001). A similar study that intravenously inoculated mature laying hens with a range of serovars including *S.* Enteritidis found it colonised the reproductive tract to significantly higher levels than the other serovars, except *S.* Typhimurium (Gantois et al., 2008). *S.* Typhimurium, *S.* Virchow, *S.* Hadar and *S.* Heidelberg all showed the ability to grow in egg albumen and penetrate the yolk sac at 25°C; therefore, the ability of *S.* Enteritidis to contaminate eggs was attributed to its preference for reproductive tissues and its enhanced ability to survive at 42°C, rather than its ability to grow in eggs (Gantois et al., 2008).

Previous studies have found that *S.* Enteritidis colonises the yolk more frequently than the albumen (Gast and Holt, 2000). However, other data from contaminated eggs from naturally infected hens has shown that there is a delay before yolk invasion and rapid growth of *Salmonella* because the vitelline membrane in fresh eggs inhibits yolk invasion by *Salmonella* (Humphrey and Whitehead, 1993). More recently, it has been shown *S.* Enteritidis can grow to significantly greater levels then the inoculum used in egg albumen, once it reaches a threshold of 250 cells per 25ml (Cogan et al.,

2001). Two possible reasons were suggested for this observation. At the threshold the combined enterochelin activity is able to out-compete ovotransferrin for iron (Cogan et al., 2001). Alternatively, the death of some cells in the albumen allows others to utilise them as a source of iron, or an energy source (Cogan et al., 2001). *S.* Heidelberg has also been shown to be frequently associated with human salmonellosis through consumption of contaminated eggs (Chittick et al., 2006).

In contrast to broad-range serovars, host-restricted serovars, *S.* Pullorum and *S.* Gallinarum, do not colonise the intestinal tract to high levels (Henderson et al., 1999). Host-restricted serovars have been shown to target gut-associated lymphocytic tissue (GALT), such as the bursa of Fabricius, rather than intestinal epithelial cells for invasion (Henderson et al., 1999, Shivaprasad, 2000). This leads to an acute systemic infection, which can become persistent with *S.* Pullorum surviving in low numbers in splenic macrophages for months (Wigley et al., 2001). During invasion of *S.* Pullorum into GALT, the inflammatory response that occurs with broad-range serovars is not seen (Chappell et al., 2009). Differences in the disease caused by broad-range and host-restricted *Salmonella* serovars have been suggested to be due to these differences in early-stage pathogenesis (Kaiser et al., 2000).

S. Pullorum and *S.* Gallinarum are avian-specific serovars and cause rapid severe systemic infection in poultry, leading to pullorum disease and fowl typhoid respectively (Shivaprasad, 2000). In countries where these serovars are not under control, substantial economic losses in the poultry industry can occur. Therefore, understanding the mechanisms *S.* Gallinarum and *S.* Pullorum use to invade poultry, which result in high morbidity and mortality may help prevent and control infection by these serovars and reduce economic losses, as well as improve poultry health.

Additionally to *S.* Enteritidis, *S.* Pullorum has been shown to infect eggs via vertical transmission and can cause persist infection, surviving in low numbers in splenic macrophages for months following experimental infection

of chickens (Wigley et al., 2001). When chickens reach sexual maturity and start egg laying, the number of *S.* Pullorum in the spleen increases dramatically and colonises the reproductive tract, leading to the contamination of eggs (Wigley et al., 2001). At point-of-lay the hen's immune system is temporarily suppressed and this is thought to play a role in the re-emergence and spread of bacteria at this time point (Wigley et al., 2001, Wigley et al., 2005).

S. Virchow is the third most common serovar resulting in human salmonellosis in the UK and is common in other countries (Matheson et al., 2010, Weinberger and Keller, 2005, Bonalli et al., 2011). Between 2009 and 2010, *S.* Virchow was the 9th most common serovar isolated from broiler meat in the EU, accounting for 1.3% of isolates (EFSA, 2012). Although *S.* Virchow is commonly isolated from chickens, its invasion mechanisms and pathogenic behaviour are poorly understood compared to *S.* Typhimurium and *S.* Enteritidis (Bertrand et al., 2006, Fashae et al., 2010, Marin and Lainez, 2009). One study has shown, during intravenous infection of poultry with *S.* Virchow and several other serovars, that *S.* Virchow colonises systemic sites such as the spleen to similar levels as *S.* Enteritidis and *S.* Typhimurium (Gantois et al., 2008). However, this study is somewhat limited given that faecal-oral infection is by far the main route of infection. Additionally, previous studies have investigated the ability of *S.* Virchow to survive in eggs, although it is uncommon in eggs. Findings have shown *S.* Virchow can survive in yolk, however it has a very limited ability to survive on egg shells and in albumen (Lublin and Sela, 2008, Gantois et al., 2008). This suggests the main vehicle of transmission of *S.* Virchow to humans is via poultry meat rather than eggs.

The aims of this study were to conduct *in vivo* poultry infection experiments, initially to determine if *S.* Virchow colonises the caeca and extra-intestinal sites, such as the spleen and to what levels it does so. Following on from this, the aims were to infect poultry for a longer period of time to observe the pattern of colonisation and to determine the effects *S.* Virchow has on the caeca and spleen by histological analysis.

4.2 Materials and Methods

4.2.1 Bacterial isolates and chickens

Experiment 1: Thirty two 1-day old specific pathogen free (SPF) Rhode Island Red chicks were obtained from the Institute of Animal Health, Compton, UK. They were housed separately in 6 groups of 5 or 6 chickens at a temperature of 30°C, which was reduced to 20°C at 3 weeks of age. The chickens were given *ad-libitum* access to a vegetable protein-based diet (SDS, Witham, Essex) and water. All experiments were conducted within local ethical guidelines and according to national legislation.

S. Virchow 55, 56, 59, 60 and *S.* Typhimurium F98 and 238 were included in this experiment (See Chapter 2, Table 2.1 for details). The isolates were grown in LB broth for 18 hours in an orbital shaking incubator at 37°C and 150rpm to late log phase.

Experiment 2: Forty five 1-day old SPF Rhode Island Red chicks were obtained from the Institute of Animal Health, Compton, UK. They were housed separately in 3 groups of 15 chickens at a temperature of 30°C, which was reduced to 20°C at 3 weeks of age. The chickens were given *ad-libitum* access to food and water, as described above. All experiments were conducted within local ethical guidelines and according to national legislation.

S. Virchow 60 and *S.* Typhimurium F98 were included in this experiment (See Chapter 2, Table 2.1 for details) and were grown overnight as described above.

4.2.2 Experiment 1 – Pilot poultry infection experiment

At 26 days of age, the chickens were infected orally with 10^8 cfu/ml of *Salmonella* culture in a 0.3 ml volume of LB broth. Each group was infected with a different serovar (*S.* Virchow 55, 56, 59, 60 and *S.* Typhimurium F98 and 238) and the groups were kept in separate rooms. The chickens were

checked twice a day for any signs of being unwell. At 3 DPI, the chickens were killed by cervical dislocation for bacteriological analysis.

4.2.3 Experiment 2 – Poultry infection experiment

At 7 days old, the chickens were infected orally with 10^8 cfu/ml of *Salmonella* culture in a 0.3 ml volume of LB broth. Group 1 was infected with *S.* Virchow 60, group 2 was infected with *S.* Typhimurium F98 and group 3 was left uninfected as a control. The chickens were checked twice a day for any signs of being unwell. At 5, 11 and 26 DPI 5 birds from each group were killed be cervical dislocation for bacteriological and histological analysis.

4.2.4 Post mortem and bacteriology

During both experiments, the caecal contents and spleen were taken aseptically at each post mortem. Spleen and caecal contents were added to 1 x PBS in a 1:10 dilution. Spleen samples were homogenised using a MicroStomacher 80 (Seward, UK) and the caecal contents were vortexed to form a suspension. Spleen and caecal content samples were serially diluted in 1 x PBS to 10^{-5} and 10^{-11} respectively and plated onto Brilliant Green agar (BGA) (Oxoid, UK). The plates were incubated at 37^{o}C for 18 hours and then the bacteria were enumerated.

During post mortem analysis for experiment 2, additional samples were collected and stored for histology and for the immunological experiments outlined in Chapter 5. Ileum and spleen samples were collected and stored in 4% paraformaldehyde at room temperature for histology. Ileum, spleen and caecal tonsil samples were embedded onto cork in O.C.T. compound (tissue-tek), snap frozen in liquid nitrogen and stored at -80°C for immunohistochemical analysis. Ileum, spleen and caecal tonsil samples were also stored in RNAlater (Sigma-Aldrich, UK) at -20°C for RT-PCR. Serum samples were collected from the heart using 21 mm needles. The serum was centrifuged at 13000 x *g* for 5 minutes. The supernatant was removed and stored at -20°C for ELISA and western blot.

4.2.5 Histological analysis

Ileum and spleen tissue samples from experiment 2 were stored in 4% paraformaldehyde for histological analysis. Tissue samples were placed in plastic cassettes and put on a tissue processor overnight, to dehydrate the tissue and embed it in paraffin wax. A microtome was used to cut 4 µm sections of the tissue samples, which were collected onto slides.

The sections were stained using haematoxylin and eosin. Samples were dewaxed in xylene for 5 minutes and then transferred to containers of descending grades of alcohol (100%, 96%, 86% and 70%) to distilled water. Sections were stained for 5 minutes using Mayer's Haemalum and then placed under running water for 6 minutes. Following this, sections were stained with Eosin for 2 minutes. Sections were dehydrated in 3 containers of 95% alcohol for 1 minute per container (repeated 3 times), 3 containers of absolute ethanol and 3 containers of xylene and then mounted in D.P.X.

4.3 Results

4.3.1 Experiment 1 – Pilot poultry infection experiment

Colony counts of *Salmonella* were taken at 3 DPI. All of the *S.* Virchow isolates colonised the gut to high levels, with caecal content counts ranging between $\log_{10}$ 5.5-7.4 cfu/g (Figure 4.1). No significant differences were found between caecal content colony counts for the *S.* Virchow infected groups (P = >0.233). The *S.* Virchow isolates had similar caecal content colony counts to *S.* Typhimurium F98 and *S.* Typhimurium 238, which had a counts of $\log_{10}$ 6.40 cfu/g and $\log_{10}$ 6.00 cfu/g respectively. No significant differences were found between the *S.* Virchow colony counts and the *S.* Typhimurium colony counts (P = >0.107).

S. Virchow isolates 55, 59 and 60 showed the ability to systemically colonise the chickens by 3 DPI, with spleen counts ranging between $\log_{10}$ 1.5-2.1 cfu/g (Figure 4.1). The 3 *S.* Virchow isolates showed similar levels of colonisation

in the spleen compared to *S.* Typhimurium F98 and *S.* Typhimurium 238. The spleen colony counts for *S.* Typhimurium F98 and *S.* Typhimurium 238 were $\log_{10}$ 2.00 cfu/g and $\log_{10}$ 1.80 cfu/g respectively. No significant differences were found between colony counts of the *S.* Virchow infected groups and the *S.* Typhimurium infected groups in the spleen (*P* = >0.148).

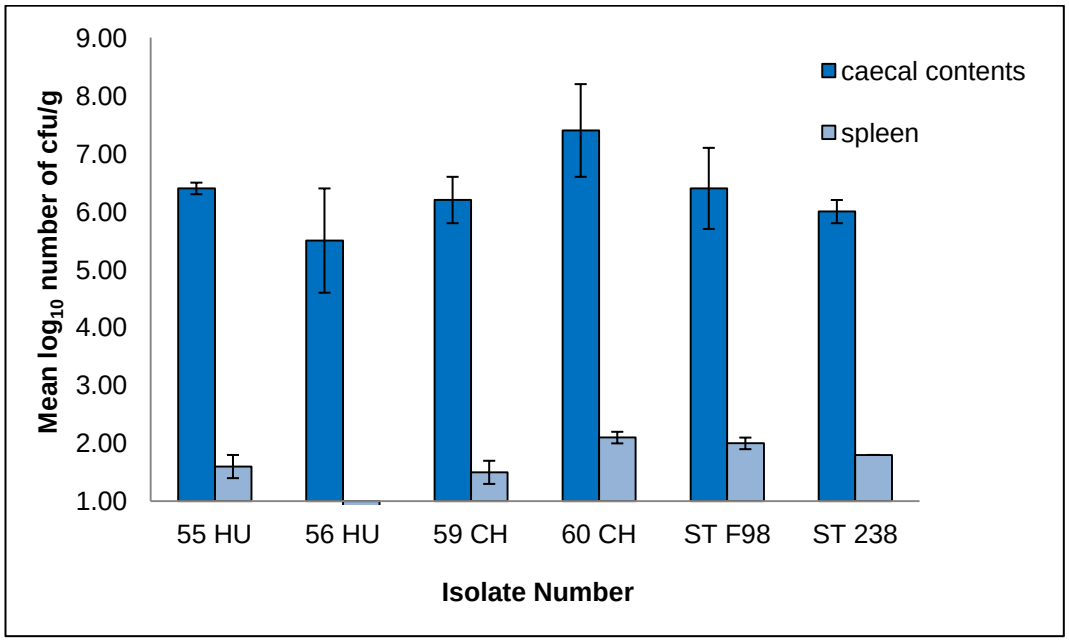

Figure 4.1: Experiment 1: Log_{10} colony counts for the caecal contents and spleen at 3 DPI. *S.* Virchow = 55 HU, 56 HU, 59 CH and 60 CH; ST F98 = *S.* Typhimurium F98; ST 238 = *S.* Typhimurium 238. Error bars represent the standard error of the mean, which was calculated from 5 birds per time point.

4.3.2 Experiment 2 – Poultry infection experiment

Colony counts of *Salmonella* were taken at 5, 11 and 26 DPI from the caecal contents and spleen. *S.* Virchow 60 was present in the caecal contents by 5 DPI at a concentration of $\log_{10}$ 8.0 cfu/g, which increased to $\log_{10}$ 9.0 cfu/g by 11 DPI and declined to $\log_{10}$ 7.0 cfu/g by 26 DPI (Figure 4.2). *S.* Virchow 60 showed a similar pattern of infection to *S.* Typhimurium F98, which was at a concentration of $\log_{10}$ 9.0 cfu/g at 5 DPI, $\log_{10}$ 12.0 cfu/g at 11 DPI and $\log_{10}$ 7.0 cfu/g at 26 DPI. The caecal content counts for *S.* Virchow showed no

significant differences to counts of *S.* Typhimurium F98 at each time point (*P* = >0.068).

S. Virchow 60 was isolated from the spleen at a concentration of $\log_{10}$ 3.0 cfu/g at 5 DPI and at 11 DPI; however, it was cleared from the spleen by 26 DPI (Figure 4.3). *S.* Typhimurium F98 increased from $\log_{10}$ 2.0 cfu/g at 5 DPI to $\log_{10}$ 4.0 cfu/g at 11 DPI; however it was also cleared from the spleen by 26 DPI and was not significantly higher in the spleen than *S.* Virchow at any time point (*P* = >0.136).

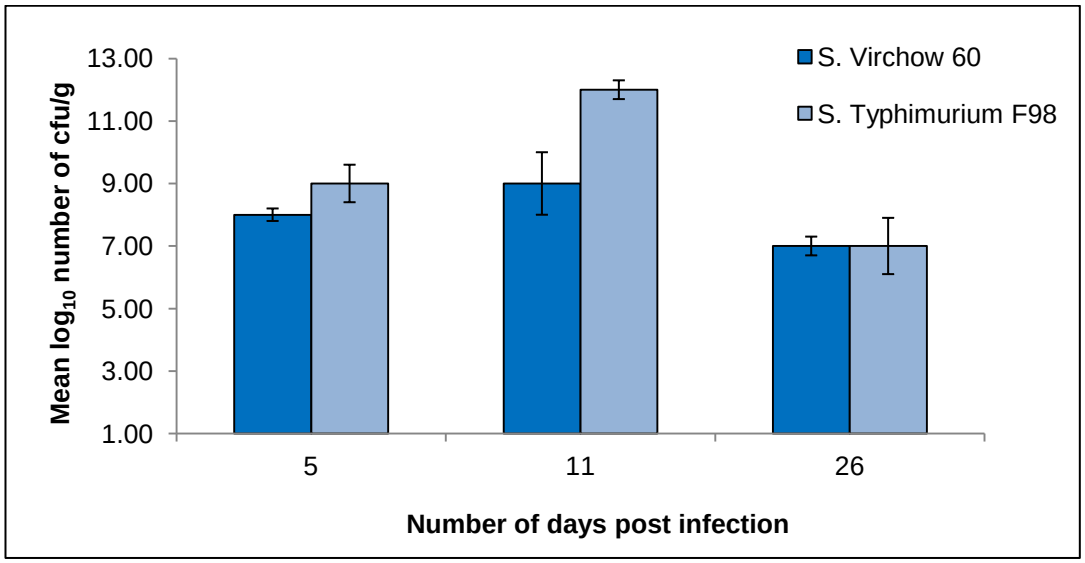

Figure 4.2: Experiment 2: Log_{10} caecal content counts at 5, 11 and 26 DPI. Error bars represent the standard error of the mean, which was calculated from 5 birds per time point.

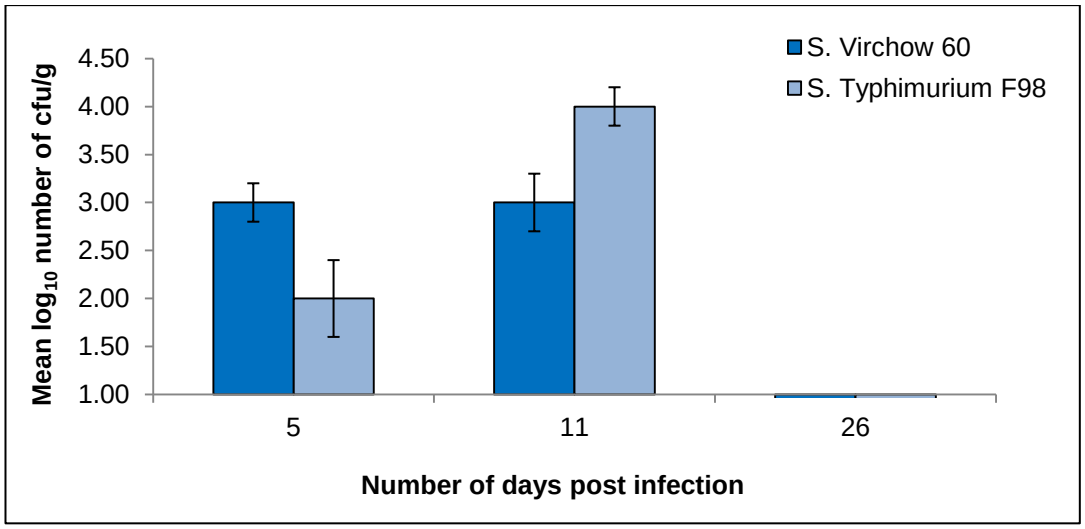

Figure 4.3: Experiment 2: Log_{10} spleen counts at 5, 11 and 26 DPI. Error bars represent the standard error of the mean, which was calculated from 5 birds per time point.

4.3.3 Histology

Tissue sections of the ileum and spleen were cut and stained with haematoxylin and eosin to determine structural and cellular changes to the tissue in response to *S.* Virchow infection. At 5 DPI, mild oedema of the ileum lamina propria could be observed in chickens infected with *S.* Virchow. The results were similar for the *S.* Typhimurium F98 infected group where, in addition, lymphocytic infiltration, mild lymphocytic exocytosis and mild villi fusion could also be observed. At 11 DPI, mild lymphocytic infiltration of the ileum lamina propria of *S.* Virchow infected chickens could be observed, which could still be observed in *S.* Typhimurium F98 infected chickens. In addition to mild lymphocytic infiltration, mild hyperaemia could be observed in some of the *S.* Typhimurium F98 infected chickens at 11 DPI. At 26 DPI, the ileum exhibited mild lymphocytic infiltration in the lamina propria, villus fusion and mild hyperaemia in the *S.* Virchow infected chickens (Figure 4.4). Mild lymphocytic infiltration was observed in the *S.* Typhimurium infected group. At 5 and 11 DPI, no histological abnormalities were observed in the spleen of *S.* Virchow and *S.* Typhimurium F98 infected chickens. At 26 DPI, an increased number of lymphocytes and macrophages could be seen in the spleen of *S.*

Virchow and *S.* Typhimurium F98 infected chickens, indicating an immune response had occurred.

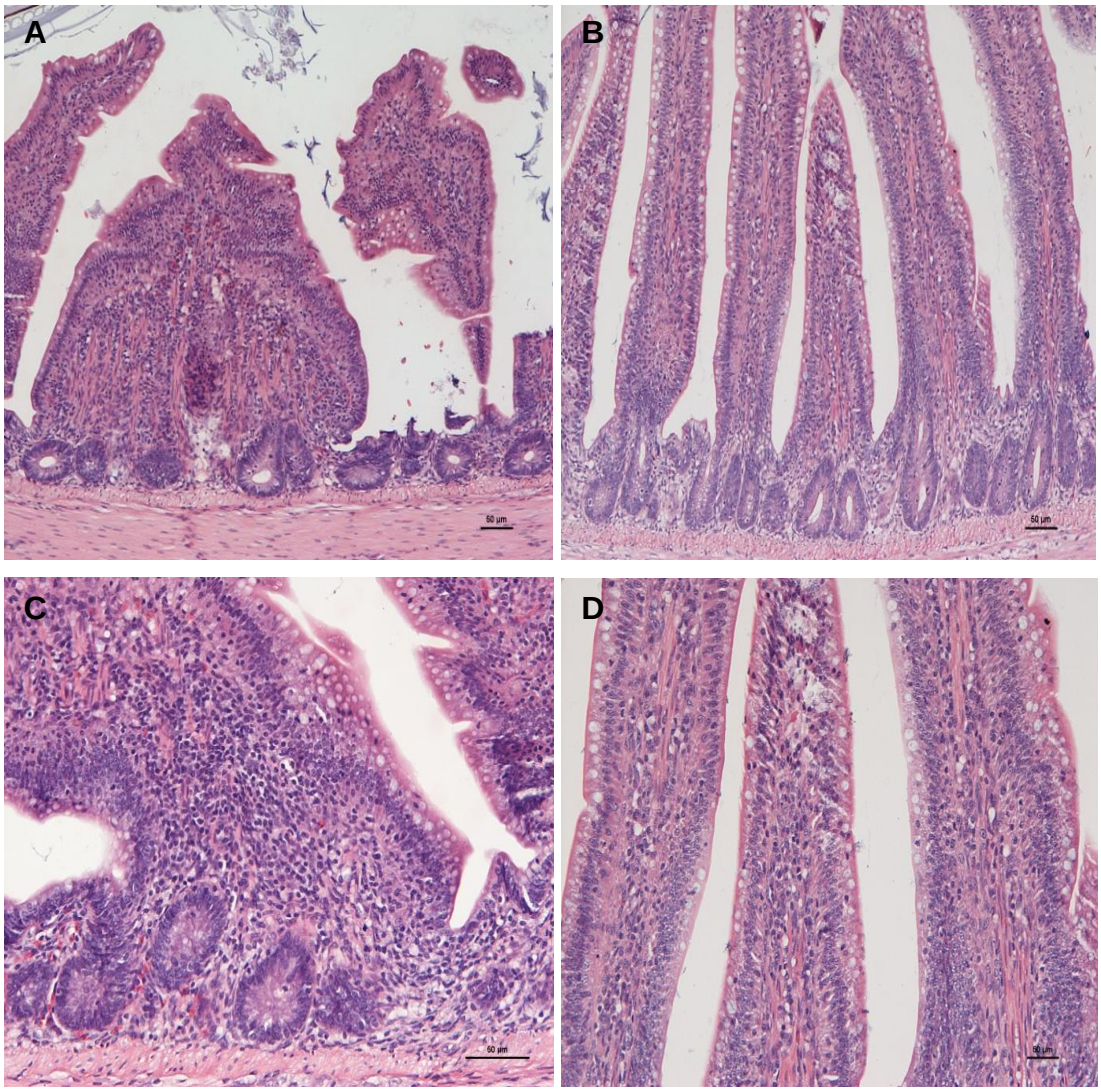

Figure 4.4: Photomicrographs of H&E stained sections of the ileum showing some of the effects of *Salmonella* infection. A = *S.* Virchow infected chicken, ileum shows villi fusion (10X magnification). B = Uninfected chicken, ileum of healthy chicken (10X magnification). C = *S.* Virchow infected chicken, showing early lymphocyte infiltration (20X magnification). D = Uninfected chicken, showing no lymphocyte infiltration (20X magnification).

4.4 Discussion

This study has shown that *S.* Virchow has similar infection biology to that of *S.* Typhimurium in chickens. *Salmonella* serovars can be split into 2 broad groups based on their host range and infection biology. *S.* Typhimurium and *S.* Enteritidis infect a broad range of hosts and typically cause limited gastroenteritis by colonising the caeca (Morgan et al., 2004, Barrow et al., 1987). However, serovars *S.* Gallinarum and *S.* Pullorum, which are host-restricted to poultry, cause rapid severe systemic infection that may result in high mortality rates (Shivaprasad, 2000). The data presented here indicates that *S.* Virchow falls within the former, milder phenotype of these groups.

During experiments 1 and 2, *S.* Virchow was found in the caecal contents at a range of $\log_{10}$ 5.5-7.4 cfu/g at 3 DPI and at $\log_{10}$ 8.0 cfu/g at 5 DPI respectively. These counts were similar to those of *S.* Typhimurium F98 in this study and to those seen with F98 in other studies at similar time points post infection (Withanage et al., 2004, Beal et al., 2004). The highest counts for *S.* Virchow and *S.* Typhimurium F98 in the caecal contents were at 11 DPI, as by 26 DPI the counts had begun to decline. The high bacterial caecal content counts from this study show that *S.* Virchow can colonise the chicken intestine to high levels and is similar in this regard to *S.* Typhimurium F98, unlike host-specific serovars such as *S.* Gallinarum and *S.* Pullorum, which are less concentrated in the intestine and instead target GALT (Jones et al., 2001, Wigley et al., 2001).

S. Virchow showed the ability to colonise the spleen as early as 3 DPI during experiment 1 and 5 DPI during experiment 2, although the bacterial counts from the spleen were considerably lower than those from the caecal contents. The isolates were cleared from the spleen by 26 DPI, whereas they were still found at high levels in the caecal contents at this time point. Slightly higher numbers of *S.* Virchow have been found in the spleen during a previous study; however, this can be attributed to the use of an intravenous inoculation route, rather than an oral route (Gantois et al., 2008). During this experiment, Gantois et al (2008) found that *S.* Virchow had similar colony

counts in the spleen compared with *S.* Typhimurium (Gantois et al., 2008). These findings are consistent with the findings during this study and both indicate *S.* Virchow is characteristic of a broad-range serovar. In contrast to the low level of systemic colonisation seen with broad-range serovars *S.* Typhimurium, *S.* Enteritidis and *S.* Virchow, host-restricted serovars *S.* Gallinarum and *S.* Pullorum can cause high and rapid systemic colonisation that may lead to septicaemia (Shivaprasad, 2000).

During the period the chickens were infected they showed no clinical signs of disease and there was no mortality or morbidity due to *S.* Virchow and *S.* Typhimurium F98 infection. Clinical signs exhibited by chickens during infection with *Salmonella* during other studies have included anorexia, distress with ruffled feathers, lethargy, disinclination to drink and eat, diarrhoea, vent staining and emaciation (Barrow et al., 1987, Withanage et al., 2004, Desmidt et al., 1997, Okamura et al., 2001). Clinical symptoms of *Salmonella* infection and high mortality rates, other than with *S*. Gallinarum or S*.* Pullorum infection, are usually observed when the chicks are less than a week old (Desmidt et al., 1997). In older chickens, clinical symptoms are usually absent and *Salmonella* is shed in the caecal contents asymptomatically. This can result in horizontal transmission to other chickens, contamination of poultry meat at slaughter and subsequently entry into the food chain, resulting in human salmonellosis (Corry et al., 2002, Davies and Breslin, 2001, Kim et al., 2007, Marin and Lainez, 2009).

During experiment 2, pathological findings included blood in the caecal contents and inflammation of the ileum in the *S.* Virchow infected group and mild hepatosplenomegaly and inflammation of the ileum in the *S.* Typhimurium F98 infected group, by 11 DPI. These findings and others have been described in previous studies during *S.* Typhimurium and *S.* Enteritidis infection of chickens (Desmidt et al., 1997, Henderson et al., 1999).

Histological analysis of the ileum showed mild oedema of the lamina propria, mild lymphocytic infiltration, moderate villus fusion and mild multifocal hyperaemia throughout the experiment, in chickens infected with *S.* Virchow.

Similar results were seen with the *S.* Typhimurium infected group, indicating the chickens are similarly susceptible to *S.* Virchow infection as to *S.* Typhimurium infection. These findings have been described in other studies during *Salmonella* infection of chickens (Desmidt et al., 1997, Desmidt et al., 1998).

Although the results indicate that *S.* Virchow displays the pathogenesis of broad-range serovars rather than host-restricted ones, which would cause rapid severe systemic disease and high morbidity and mortality rates, *S.* Virchow is rarely isolated from species other than man and chickens (Fashae et al., 2010, Bonalli et al., 2011). In comparison, broad-range serovars such as *S.* Typhimurium may be isolated from many species including humans, chickens, pigs, sheep, cattle and domestic animals (DEFRA, 2008c, Fashae et al., 2010, DEFRA, 2010). Additionally, previous studies have shown *S.* Virchow to be an invasive serovar in humans, particularly in children, the immuno-compromised and the elderly (Weinberger et al., 2004, Schifferdecker et al., 2009, Matheson et al., 2010, Ispahani and Slack, 2000). Therefore, although *S.* Virchow demonstrated a similar biology of infection in poultry to *S.* Typhimurium in this study and has many of the features associated with broad-range serovars, in reality *S.* Virchow may have a narrower host range.

This study has shown that *S.* Virchow exhibits similar infection biology to *S.* Typhimurium in the chicken. *S.* Virchow can be isolated at high levels from the caecal contents and can be isolated from systemic sites, although it is cleared fairly quickly from the spleen. During this study *S.* Virchow did not make the chicks clinically ill, although evidence of structural changes and inflammation in the ileum were found during post mortem and histological analysis. The results from this study show that *S.* Virchow behaves differently in chickens than in humans. Previous research has shown *S.* Virchow can often cause invasive disease in humans, whereas the results from this study show it causes a more mild infection in chickens (Eckerle et al., 2010, Schifferdecker et al., 2009).

The main route for *Salmonella* infection in chickens is the faecal-oral route; therefore, oral inoculation during these experiments was the most relevant method, as it indicates what is most likely to happen during uncontrolled infection on farms. The results indicate *S.* Virchow can be shed for a long period in the faeces, with the chicken showing no clinical symptoms; therefore, increasing the likelihood of it entering the food chain and causing human salmonellosis and in some cases invasive disease, leading to septicaemia. This is of particular concern as previous studies have shown an increasing resistance of *S.* Virchow to antimicrobial drugs that are used regularly to treat invasive disease, making entry of *S.* Virchow into the food chain a greater risk to human health than other less invasive serovars (Meakins et al., 2008, Weinberger and Keller, 2005, Weinberger et al., 2006).

Chapter 5
Immunological Studies

5.1 Introduction

Poultry meat and eggs are the main source of human salmonellosis worldwide (DEFRA, 2008c, Mead, 2004, Currie et al., 2005, DEFRA, 2010, Braden, 2006). Therefore, methods to control *Salmonella* infection in food production animals are of great importance to prevent entry into the food chain. Developing vaccines to increase resistance of poultry to *Salmonella* infection through stimulation of the immune response is a useful tool for controlling infection. Understanding the immune response of poultry against *Salmonella* infection is essential for the development of new vaccines and to improve the efficacy of current vaccines against infection (Berndt and Methner, 2004).

A large amount of research has been done to characterise the immune responses produced by poultry against *Salmonella* infection. The immune response produced in the chicken intestinal tract is of great interest, as this is where *Salmonella* initially invades and colonises its host. Subsequently, *Salmonella* is shed in the faeces, usually asymptomatically and spreads by horizontal transfer to other chickens. The chicken intestinal tract is a tubular structure enclosed by a single layer of polarized epithelial cells, which are attached to a basement membrane (Smith, 2008). The epithelial cells form protruding villus structures, which are interspersed by indentations called crypts (Smith, 2008). The intestinal gut is composed of the small intestine (duodenum, jejunum and ileum) and the large intestine (caecum and a short colon). Research has examined the immune responses to *Salmonella* throughout the intestinal gut.

The immune responses produced by chickens against *Salmonella* infection depend on the infecting serovar, the age of the host and host genetics (Setta et al., 2012b, Beal et al., 2005, Schokker et al., 2012, Berthelot-Herault et al., 2003). Infection and colonisation of the avian gut by broad-range *Salmonella* serovars causes an acute inflammatory response including secretion of several cytokines/chemokines, followed by an influx of heterophils (Chappell et al., 2009, Henderson et al., 1999, Kaiser et al., 2000, Meade et al., 2009).

Several *in vitro* and *in vivo* studies have documented changes in pro-inflammatory cytokines in the gut, in response to *Salmonella* infection. *S.* Typhimurium infection of 4 day old broiler chickens stimulates an up-regulation of IL-1β, IL-6 and IFN-γ mRNA and a down-regulation of IL-10 mRNA in the small intestine (Fasina et al., 2008). Similar findings in the small intestine have been found by infecting newly hatched and 1 week old SPF Rhode-Island Red chicks with *S.* Typhimurium (Withanage et al., 2005b, Withanage et al., 2004). However, differences in cytokine and chemokine production have been found depending on the invading serovar. During infection of 1 day old chicks *S.* Enteritidis and *S.* Typhimurium are more invasive and virulent than *S.* Hadar and *S.* Infantis (Berndt et al., 2007). During this study, *S.* Enteritidis and *S.* Typhimurium were shown to be stronger inducers of the chicken immune response during infection. Expression of IFN-γ, IL-2, IL-12, IL-18, LITAF, IL-8 and MIP-1β mRNA in the caecum were up-regulated in all of the infected groups, however this was generally most pronounced in the *S.* Enteritidis infected group (Berndt et al., 2007). This is in agreement with other studies that have found *S.* Enteritidis to be a strong inducer of the inflammatory response in the caecal tonsil (Setta et al., 2012b). Differences in cytokine and chemokine mRNA expression in chickens depending on the infecting serovar have also been shown using primary CKC cells (Kaiser et al., 2000). Infection with broad-range serovars *S.* Typhimurium and *S.* Enteritidis stimulate a significant increase in IL-6 by the CKC cells, in contrast to host-restricted *S.* Gallinarum, which has little effect (Kaiser et al., 2000). The failure of *S.* Gallinarum to induce an inflammatory response is thought to be due to its host specific nature and prevents it from being limited to the gut (Kaiser et al., 2000).

Heterophils have been shown to express an array of cytokines and chemokines in the gut following *Salmonella* infection, showing they have a role in immuno-regulation (Kogut et al., 2003). Following phagocytosis of *S.* Enteritidis, mRNA expression of IL-6 and IL-8 increases in heterophils (Kogut et al., 2003). The production of IL-8 by heterophils indicates that they can promote their own recruitment to sites of inflammation (Kogut et al., 2003). LPS on its own can also stimulate heterophils to produce cytokines and

chemokines in a TLR-4-dependant manner (Kogut et al., 2005). Up-regulation of IL-1β, IL-6, IL-18, CXCLi1, CXCLi2, CCLi4 and the CXC receptor occurs following LPS stimulation of heterophils (Kogut et al., 2005).

Following an acute inflammatory response there is a strong influx of macrophages and T lymphocytes into the gut (Van Immerseel et al., 2002, Schokker et al., 2012). *S.* Enteritidis infection of one day old chicks stimulates a significant increase in T cells expressing CD3, CD4 and CD8 in the gut by 7 DPI (Asheg et al., 2002). CD3 is part of the TCR complex and triggers signal transduction following antigen recognition (Viertlbock, 2008). CD4 and CD8 are co-expressed with CD3 on subsets of the T cell population. CD4 is primarily expressed on Th cells, whereas CD8 is primarily expressed on cytotoxic T cells (Viertlbock, 2008). CD8 consists of 2 chains and can be expressed in two forms including CD8αα homodimer and CD8αβ heterodimer, although the majority of chicks express the CD8αβ heterodimer (Viertlbock, 2008). Infection of 1 day old chicks with *S.* Enteritidis and other serovars including *S.* Typhimurium, *S.* Hadar and *S.* Infantis have also been shown to increase CD4, CD8α and CD8β expression, as well as that of γδ T cells in the gut (Berndt et al., 2007). In chickens, a small subset of T cells express γδ TCRs, rather than αβ TCRs. The γδ T cells can be further subdivided based on their expression of CD8 (Berndt et al., 2006). During *Salmonella* infection, up-regulation of CD8α and CD8β chains occurs, particularly the CD8α chain (Pieper et al., 2011). Characterisation of γδ T cells has shown they exhibit a wide range of functions including antigen presentation and they also provide a link between innate and adaptive immune responses (Brandes et al., 2005, Scotet et al., 2008). During infection, γδ TCR^+ cells show an increase in IFN-γ expression, suggesting they contribute to Th1 responses (Pieper et al., 2011).

Differences in the host genetics influence the cellular responses against *Salmonella* infection in the gut (Schokker et al., 2012). Macrophages in fast growing chickens produce a faster and stronger response compared to slow growing chickens, showing they have an enhanced response against *Salmonella* infection compared to slow growing chickens (van Hemert et al.,

2007). A decrease in $CD4^+$ cells and an increase in $CD8^+$ cells in the intestine were also found in this study, which is unusual as most studies report an increase in both CD4 and CD8 in the gut following *Salmonella* infection. However, differences in the number of each cell type has been found for T cell markers depending on location in the digestive tract, infection dose, infecting serovar, time post infection, age of the host and genetic background (van Hemert et al., 2007, Berndt et al., 2007).

Antibodies to major markers, including Bu1a and Bu1b, have been developed to stain for chicken B cells. During *S.* Enteritidis infection of 1 day old chicks, B cells increase in the caecum by 10 DPI (Asheg et al., 2002). Staining for specific immunoglobulins in the caecum, following *S.* Typhimurium infection of 1 day old chicks, showed an increase in IgA and IgM from 3 DPI (Berndt and Methner, 2004). Only a few studies have measured antibody secretions in the gut, as measurements of antibody production during *Salmonella* infection are usually analysed in serum (Beal and Smith, 2007). IgA can be detected in intestinal secretions by 3 weeks post infection (WPI) in 1 week old chickens infected with *S.* Enteritidis (Berthelot-Herault et al., 2003). A decline in *S.* Enteritidis colonisation in the gut follows a peak in IgA concentration in intestinal secretions, suggesting intestinal IgA antibodies contribute to the elimination of it from the gut in the later stages of infection (Berthelot-Herault et al., 2003). During this infection IgM and IgG were detected in only very low concentrations in intestinal secretions (Berthelot-Herault et al., 2003). Reports have also found an increase in IgA, IgG and IgM in the small intestine during *S.* Typhimurium infection (Brito et al., 1993). An increase in these immunoglobulins, particularly IgA, has been found in bile, which is drained from the liver directly into the duodenum (Brito et al., 1993, Rose et al., 1981, Lebacq-Verheyden et al., 1974). Although an increase in B cells occurs in the gut following *Salmonella* infection, some studies have shown B cells and antibodies are not required for clearance of infection (Desmidt et al., 1998, Beal et al., 2006a), therefore suggesting a cell-mediated immune response is more important for clearance of *Salmonella* infection.

The chicken intestinal tract contains GALT, which is also found in the mammalian intestinal tract. Mammalian GALT is highly organised and well characterised, unlike chicken GALT, which has been shown to form less well defined aggregates throughout the intestinal tissues. The best studied components of the avian GALT includes the bursa of Fabricius, Peyer's patches and caecal tonsils (Casteleyn et al., 2010). The bursa of Fabricius is a primary lymphoid organ located at the dorsal side of the proctodeum (Casteleyn et al., 2010). The bursa mainly consists of lymphoid follicles and is encapsulated by connective tissue (Casteleyn et al., 2010). It is densely populated with B lymphocytes and macrophages (Casteleyn et al., 2010). Peyer's patches are a secondary lymphoid organ and up to six can be found scattered throughout the avian gut (Olah, 2008, Casteleyn et al., 2010). Some Peyer's patches can be consistently found, for example in the distal ileum and the proximal ileum (Vaughn et al., 2006). Microscopic evaluation of Peyer's patches has shown that they consist of a large amount of organised lymphoid follicles, which mainly contain B cells (Casteleyn et al., 2010, Vaughn et al., 2006). T lymphocytes can be found in the interfollicular regions (Casteleyn et al., 2010).

The avian gut contains 2 caecal tonsils located at the end of the caeca, near the caeco-ileal junction. The caecal tonsils are composed of several lymphoid nodular units, separated by thin connective tissue (Kitagawa et al., 1998). The function of the caecal tonsils is still quite uncertain, but it has been shown that follicle-associated tissue of the tonsils contains M cells. The function of avian M cells may be to take up particles present in the gut, therefore bringing them closer to lymphocytes present in the caecal tonsil (Kitagawa et al., 2000, Casteleyn et al., 2010). Caecal tonsils appear in the late embryonic stage and most of their development occurs after hatching (Gomez Del Moral et al., 1998). Avian caecal tonsils will reach adult histological condition 4 days post hatching, but will continue to grow until 6 weeks post hatching (Gomez Del Moral et al., 1998). During development of the caecal tonsils, changes in the number of B and T lymphocytes are observed (Gomez Del Moral et al., 1998). In the first two weeks of life the caecal tonsils contain mainly T lymphocytes however, by the time the birds

are 6 weeks old, the chicken caecal tonsils are dominated by B lymphocytes (Gomez Del Moral et al., 1998).

Cytokine and chemokine changes occur in the caecal tonsil following *Salmonella* infection in chickens. IFN-γ mRNA is significantly up-regulated by 3 DPI following *S.* Typhimurium infection of 1 week old chickens (Withanage et al., 2005b). IFN-γ T cell mediated responses are thought to play a central role in *Salmonella* clearance. IL-6 also increases in the caecal tonsils later on, by 14 DPI (Withanage et al., 2005b). The late increase in IL-6 has been suggested to be associated with lymphocyte or macrophage development, regulation and activation, rather than initiation of the inflammatory response (Withanage et al., 2005b). A significant up-regulation in the gene expression of IL-10 and the chemokines CXCLi1 and CXCLi2 in the caecal tonsil also occurs following *Salmonella* infection, although this varies slightly depending on the age of the chicken and the infecting serovar (Setta et al., 2012b). The up-regulation of IL-10 following *Salmonella* infection is in contrast to other findings that have reported a down-regulation of it (Fasina et al., 2008).

Following *Salmonella* infection of chickens, changes in immune cell markers occur in the caecal tonsil. Macrophages and B cells increase in the caecal tonsil in response to infection by a range of serovars including *S.* Enteritidis, *S.* Infantis, *S.* Gallinarum and *S.* Pullorum (Setta et al., 2012b). Sasai et al (2000) infected 16 day old chickens with either a low or a high dose of *S.* Enteritidis to determine changes in caecal tonsil lymphocyte subpopulations between 0 and 6 DPI (Sasai et al., 2000). IgM^+, and IgG^+ B lymphocytes increased in the caecal tonsil, whereas no changes were found in IgA^+ B lymphocytes (Sasai et al., 2000). Additionally, $CD3^+$ and $CD8^+$ T lymphocytes decreased in the caecal tonsils, whereas $CD4^+$ T lymphocytes increased. The increase in $CD4^+$ T lymphocytes was suggested to stimulate immunoglobulin class switching, as a significant increase in IgG^+ B lymphocytes was observed soon after CD4 up-regulation (Sasai et al., 2000). Additionally, the group infected with a low dose of *S.* Enteritidis sometimes exhibited a greater immune response compared to the group infected with a high dose, indicating that a high infection dose of *S.* Enteritidis can cause

immuno-suppression in the caecal tonsil (Sasai et al., 2000). Changes in caecal tonsil lymphocyte subpopulations in older chickens infected with *S.* Enteritidis have also been studied (Holt et al., 2010). Following infection at 34 and 41 weeks of age, $CD4^+$ and $CD8^+$ T lymphocytes increased in the caecal tonsil; however, $CD4^+$ cells were more prevalent (Holt et al., 2010). Changes in TCR subtypes were also found. The predominant TCR was αβ Vβ1, followed by αβ Vβ2, whereas γδ was sparse throughout the tissue (Holt et al., 2010). The less dramatic changes in T cell subsets found during this experiment compared to Sasai et al 2000, could be attributed to the different age of the chickens infected in the two different studies and therefore the differences in the maturity of the immune system between young and older chickens (Holt et al., 2010).

Following intestinal invasion, *Salmonella* is thought to be taken up by the host's macrophages and transported via the lymphatic system to systemic sites such as the spleen (Chappell et al., 2009). Macrophages express TLRs and become activated via TLRs upon bacterial invasion of the host (Iqbal et al., 2005a, Iqbal et al., 2005b). Uptake of *Salmonella* into macrophages occurs by phagocytosis and by bacterial invasion (Setta et al., 2012a). Following activation, macrophages produce ROS and NO against *Salmonella* infection and also up-regulate several cytokines and chemokines including IL-1β, IL-6, CXCLi1 and CXCLi2 (Setta et al., 2012a, Okamura et al., 2005, Wigley et al., 2006). However, differences in cytokine and chemokine expression have been found depending on whether the macrophages are isolated from chickens genetically resistant or susceptible to systemic salmonellosis (Wigley et al., 2006). Differences have also been found depending on the infecting serovar, suggesting that *Salmonella* serovars modulate key cytokines and chemokines differentially (Setta et al., 2012a). Intra-macrophage survival has been shown to be essential for systemic *Salmonella* infection (Jones et al., 2007, Jones et al., 2001, Wigley et al., 2002b). *S.* Pullorum is thought to modulate IL-10 production by macrophages, as IL-10 has an inhibitory effect on macrophages producing IFN-γ and ROS, creating a favourable environment for bacteria to survive

within these cells, enabling persistent infection (Bogdan et al., 1991, Uchiya et al., 2004, Wigley et al., 2005).

The spleen is considered to be an important secondary lymphoid organ and provides a site where lymphoid and non-lymphoid cells can interact (Olah, 2008). The spleen is surrounded by a thin layer of collagen and reticular fibres and consists of red and white pulp (Olah, 2008). Haematopoiesis is restricted to the red pulp and after this ceases, the main function of the red pulp is to filter out senescent erythrocytes (Olah, 2008). The white pulp constitutes the largest secondary lymphoid organ and receives antigens from the blood circulation (Olah, 2008). T lymphocyte subpopulations are present in the spleen and changes in the dynamics of the subpopulations, after *Salmonella* infection, has been investigated. Upon *Salmonella* infection $CD4^+$ T lymphocytes significantly decrease in the spleen at 7 DPI and $CD3^+$ and $CD8^+$ T lymphocytes significantly decrease at 14 DPI (Asheg et al., 2002). A decrease of cells in the spleen and circulation has been suggested to be due to cell trafficking to the intestinal gut. An increase in γδ T lymphocytes has been found to occur in the spleen following infection; however, as with other T cell markers, an increase occurs more rapidly in the caecum (Pieper et al., 2011).

The humoral response to *Salmonella* infection has been well studied in chickens. High titres of specific antibodies can be found in chicken serum after infection. A classical pattern of an initial rise of IgM followed by IgG and IgA with all three classes peaking at 13 DPI has been shown (Withanage et al., 2005b, Beal et al., 2004). Following the 13 DPI peak, IgM steadily declines to concentrations found in uninfected birds, whereas IgG and IgA remain elevated for at least 69 days (Beal et al., 2004). Elevated levels of serum IgA has been shown to correlate with secretory IgA in the gut lumen, suggesting a possible mechanism of clearance of *Salmonella* from the gut (Beal et al., 2004, Rose et al., 1981, Brito et al., 1993). Immunohistochemical markers to detect B cell changes in the chicken during *Salmonella* infection have also been used to study the humoral response. During *S.* Enteritidis challenge of 1 day old chicks, a significantly raised number of $Bu1b^+$ cells

were found in the caecum (Asheg et al., 2002). An increase of $Bu1b^+$ cells was also seen in the spleen during infection (Asheg et al., 2002). More specific staining for B cells in the spleen has shown IgA^+ and IgM^+ B lymphocytes increase following *S.* Enteritidis infection (Sasai et al., 1997). Less dramatic changes in $Bu1b^+$ cells in the caecum of chickens infected with *S.* Enteritidis have been shown (Van Immerseel et al., 2002). Markers targeting specific Ig expressing cells in chickens have also been used. Berndt et al (2004) showed a significant increase of IgM^+ and IgA^+ cells in the caecum in chickens infected with *S.* Typhimurium (Berndt and Methner, 2004). The studies outlined showed a significant humoral response as well as a cell-mediated response occurs during *Salmonella* infection in chickens. This suggests that the raised humoral response may be important for clearing *Salmonella* infection from the host. However, other studies performed on bursectomised (B cell-free) chickens have shown B cell lymphocytes are not required to clear *Salmonella* infection (Desmidt et al., 1998, Beal et al., 2006a).

The aims of this study were to determine the dynamics of the humoral, cell-mediated and cytokine responses during primary infection of 7 day old chickens with *S.* Virchow. The humoral immune response was determined by ELISA using serum collected during infection and also by immunohistochemical staining for B cells. The cell-mediated immune response was determined by immunohistochemical staining. Changes in key cytokines and chemokines were detected using RT-PCR.

5.2 Materials and Methods

5.2.1 Soluble antigen preparation

See Chapter 6.

5.2.2 ELISA

See Chapter 6.

5.2.3 Western blot

See Chapter 6.

5.2.4 Immunohistochemistry – Sample preparation

During the poultry infection experiment outlined in Chapter 4, spleen, ileum and caecal tonsil were embedded on cork in OCT and snap frozen in liquid nitrogen for immunohistological analysis. Serial 10 µM sections of each sample were cut, using a cryostat; at least 8 sections were cut for each sample. Sections were adhered to poly-l-lysine coated slides (VWR International) and air dried. Sections were incubated in 100% acetone, for 10 minutes at room temperature, to remove OCT from the slide. Slides were stored at -80ºC until staining.

5.2.5 Immunohistochemistry – Staining

The sections were incubated in 1 x TBS for 15 minutes at room temperature, to remove any excess OCT. To reduce non-specific binding of the reagents to endogenous peroxidases, the sections were incubated for 15 minutes in 360 ml methanol containing 6 ml of hydrogen peroxide. The sections were washed in sterile distilled water for 5 minutes and washed briefly a second time, before putting the slides on Shandon cover plates using 1 x TBS. The chamber of the plates was filled with 1 x TBS and incubated for 5 minutes. Following this, 100 µl of horse serum was added to each chamber and the sections were incubated for 10 minutes, to reduce any non-specific binding of the secondary antibody. Specific mouse monoclonal anti-chicken antibodies were added separately to individual sections. Antibodies for chicken CD3, CD4, CD8α, CD8β, MHC II, KuL01, γδ TCR and Bu1a (Southern Biotechnology, Cambridge, UK) were included. Antibodies were diluted 1:100 in 1 x TBS, except for Bu1a, which was diluted 1:50. Sections were incubated overnight at 4ºC with 100 µl of primary antibody. The sections were washed by filling the chambers with 1 x TBS and incubating at room temperature for 5 minutes. A Vectastain Elite ABC kit (Vector Laboratories, Peterborough, UK) was used for the detection of stained cells. The biotinylated horse anti-mouse secondary antibody was diluted 1:100. The sections were incubated with 100 µl of the secondary antibody for 30 minutes at room temperature. Following

the incubation, the sections were washed with 1 x TBS and incubated with ABC (avidin biotin complex) solution for 30 minutes. The plate chambers were filled with 1 x TBS and incubated for 5 minutes, before removing the slides from the plates and washing them briefly 3 times in sterile distilled water. The specific colour reaction in the sections was developed by incubating the slides with 0.2 g 3'3-diaminobenzidine in 400 ml imidazole buffer (280 ml 0.1M imidazole : 120 ml 0.42M hydrogen chloride, pH 7.18-7.21) for 30 minutes on a magnetic stirrer (Berndt and Methner, 2004, Asheg et al., 2002, van Hemert et al., 2007). The sections were washed 3 times in sterile distilled water, for 5 minutes per wash, and then counter-stained in haematoxylin for 1 minute. The sections were washed under running water for 5 minutes. To dehydrate the sections, they were incubated in 96% ethanol, followed by 2 incubations in 100% ethanol for 1, 2 and 3 minutes respectively. To remove excess ethanol, the slides were incubated in 3 containers of xylene for 2, 3 and 3 minutes respectively. The slides were mounted in DPX using 40mm cover slips.

5.2.6 Immunohistochemistry – analysis

Slides were analysed using a Nikon eclipse microscope, version 5.03 and NIS-elements BR 3 software.

Five images of the spleen and caecal tonsil were taken at X40 magnification for each sample. The first image was taken at the centre of the tissue. The magnification was then changed to X20 to move the slide up, down, left and right of the central image, for the other four images. Cell numbers were manually counted for all five images and then an average cell count was taken for each chicken. A different approach was taken for the $Bu1a^{+}$ cells as these cells formed follicles in the caecal tonsil. The area of every stained follicle present on the tissue was measured to determine if the follicles changed size during infection. An average follicle area was taken for each chicken.

No one part of the ileum was more heavily stained than other parts; therefore, cell counts were determined in the villi. Images were taken at X20

magnification of villi that covered the length of the computer screen. An average of 10 images was taken and an average cell count was determined for each chicken.

5.2.7 Cytokine expression by $2^{-\Delta\Delta CT}$ RT-PCR

Changes in IL-1β, IL-6, IL-4, cxcLi2 and IFN-γ mRNA were measured in the spleen and caecal tonsil, using a Rotor-Gene Q (Qiagen, UK). RNA samples were prepared from tissues stored in RNAlater at -20°C, using the RNeasy mini kit and following the manufacturer's instructions (Qiagen, UK). Primers and probes for 28S and cytokine specific amplification have been previously described and are listed in Table 5.1 (Kaiser et al., 2000, Setta et al., 2012a). One-Step RT-PCR was performed using the Rotor-Gene Probe RT-PCR Master Mix (Qiagen: includes RT stage) in a final concentration of 1 x 0.25 µl Rotor-Gene RT mix, 0.8 µM of both the forward and reverse primers, 0.2 µM of the probe and 1 µl RNA made up to a total volume of 25 µl with RNase-free water. The following cycling conditions were used for amplification: 50°C for 10 minutes, 95°C for 5 minutes, followed by 40 cycles of 95°C for 5 seconds and 60°C for 10 seconds.

5.2.8 Analysis of $2^{-\Delta\Delta CT}$ RT-PCR

For the RT-PCR experiments, each sample was run in triplicate and an average C_T value was taken for each group. The threshold for C_T values was set between 0.20 and 0.23. C_T values were normalised firstly to the endogenous control and then to the uninfected control group (Livak and Schmittgen, 2001). Expression levels in the infected groups were represented as the fold-change in expression compared to the uninfected control.

Table 5.1: Primer and probe sequences for $2^{-\Delta\Delta CT}$ RT-PCR. F = forward primer, R = reverse primer, P = probe.

Target		Probe/Primer Sequence (5'-3')
28S	F	GGCGAAGCCAGAGGAAACT
	R	GACGACCGATTTGCACGTC
	P	(FAM)-AGGACCGCTACGGACCTCCACCA-(TAMRA)
IL-1β	F	GCTCTACATGTCGTGTGTGATGAG
	R	TGTCGATGTCCCGCATGA
	P	(FAM)-CCACACTGCAGCTGGAGGAAGCC-(TAMRA)
IL-6	F	GCTCGCCGGCTTCGA
	R	GGTAGGTCTGAAAGGCGAACAG
	P	(FAM)-AGGAGAAATGCCTGACGAAGCTCTCCA-(TAMRA)
CXCLi2	F	GCCCTCCTCCTGGTTTCAG
	R	TGGCACCGCAGCTCATT
	P	(FAM)-TCTTTACCAGCGTCCTACCTTGCGACA-(TAMRA)
IL-4	F	AACATGCGTCAGCTCCTGAAT
	R	TCTGCTAGGAACTTCTCCATTGAA
	P	(FAM)-AGCAGCACCTCCCTCAAGGCACC-(TAMRA)
IFN-γ	F	GTGAAGAAGGTGAAAGATATCATGGA
	R	GCTTTGCGCTGGATTCTCA
	P	(FAM)-TGGCCAAGCTCCCGATGAACGA-(TAMRA)

5.3 Results

Following *Salmonella* infection of 7 day old chicks (Chapter 3), 5 birds from each group were killed by cervical dislocation at 5, 11 and 26 DPI. At post mortem, samples were collected for immunohistochemistry, RT-PCR and ELISA, to determine the cellular, humoral and cytokine response following infection.

5.3.1 Immunohistochemistry

To characterise the cellular immune response to *S.* Virchow infection, the spleen, ileum and caecal tonsil tissues were stained for several cell markers

including CD3, CD4, CD8α, CD8β, MHC II, KuL01, γδ TCR and Bu1α (Figures 5.1-5.3).

In the spleen, several of the markers increased or decreased in the infected groups compared to the uninfected group (Figure 5.4). The cell counts in the *S.* Virchow and *S.* Typhimurium infected groups were very similar in the spleen throughout the infection for all markers. At 5 DPI, $CD3^+$ cells decreased in the spleen in groups 1 and 2, compared to group 3, although had returned to similar levels by 11 and 26 DPI (P = >0.201). $CD4^+$ cells were significantly higher in the spleen in groups 1 and 2 compared to group 3 at 5 and 11 DPI (P = <0.05) and were still higher at 26 DPI. $CD8\alpha^+$ cells were higher in groups 1 and 2 compared to group 3 at 5 DPI. At 11 and 26 DPI, $CD8\alpha^+$ cells had increased even further in the infected groups, being significantly raised in group 1 at both time points (P = <0.034) and in group 2 at 26 DPI (P = <0.026). $CD8\beta^+$ cells were similar in all 3 groups at 5 and 11 DPI; however, at 26 DPI the cells in groups 1 and 2 were significantly higher than in group 3 (P = <0.025). $KuL01^+$ cells were up-regulated in groups 1 and 2 compared to group 3, at 5 and 11 DPI, being significantly higher at 11 DPI (P = <0.003). At 26 DPI, $KuL01^+$ cell counts were similar to group 3. At 5 DPI γδ TCR^+ cells were significantly lower in groups 1 and 2 compared to group 3 (P = <0.009). At 11 and 26 DPI, γδ TCR^+ cells were similar to group 3. Cell counts could not be determined for MHC II and Bu1a staining in the spleen. Hardly any specific staining could be found for either marker.

In the ileum, the cell markers increased in the *Salmonella* infected groups compared to the uninfected group (Figure 5.5). In some cases, a significantly greater response was seen in response to *S.* Virchow compared to *S.* Typhimurium. $CD4^+$ cell counts increased at each time point in groups 1 and 2 and were higher than $CD4^+$ counts in group 3. Although counts were higher in group 2 as well as group 1, considerably more $CD4^+$ cells were found in group 1 compared to groups 2 and 3. $CD4^+$ cell counts in group 1 were significantly higher at each time point compared to group 3 (P = <0.001). $CD8\alpha^+$ cells were higher at 5 and 11 DPI in groups 1 and 2 compared to group 3. At 11 DPI, a significant number of $CD8\alpha^+$ cells were found in group

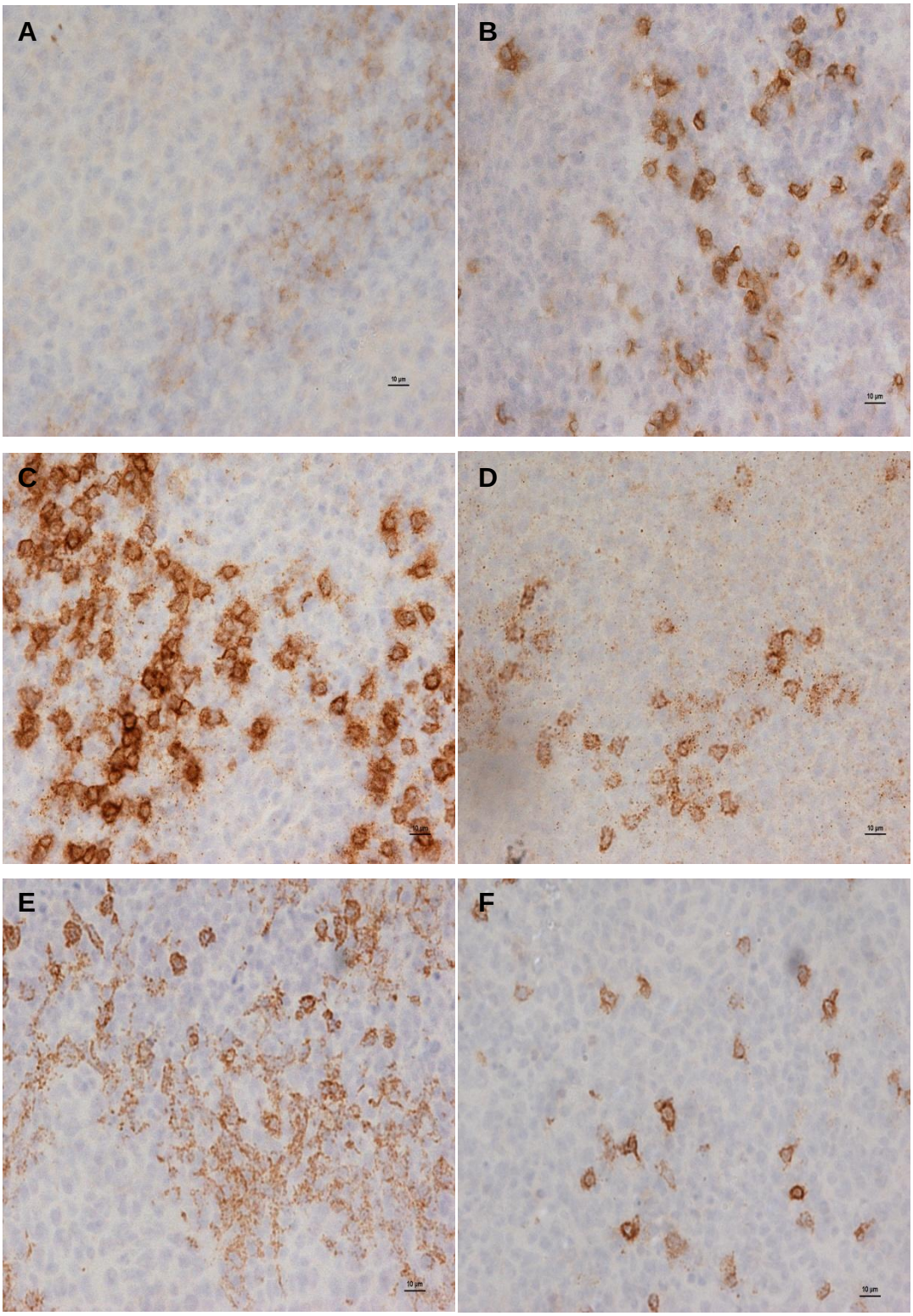

Figure 5.1: Immunohistochemistry staining showing $CD3^+$ (A), $CD4^+$ (B), $CD8\alpha^+$ (C), $CD8\beta^+$ (D), $KuL01^+$ (E) and γδ TCR^+ (F) positive cells in the spleen of chickens infected with *S.* Virchow at 5 DPI.

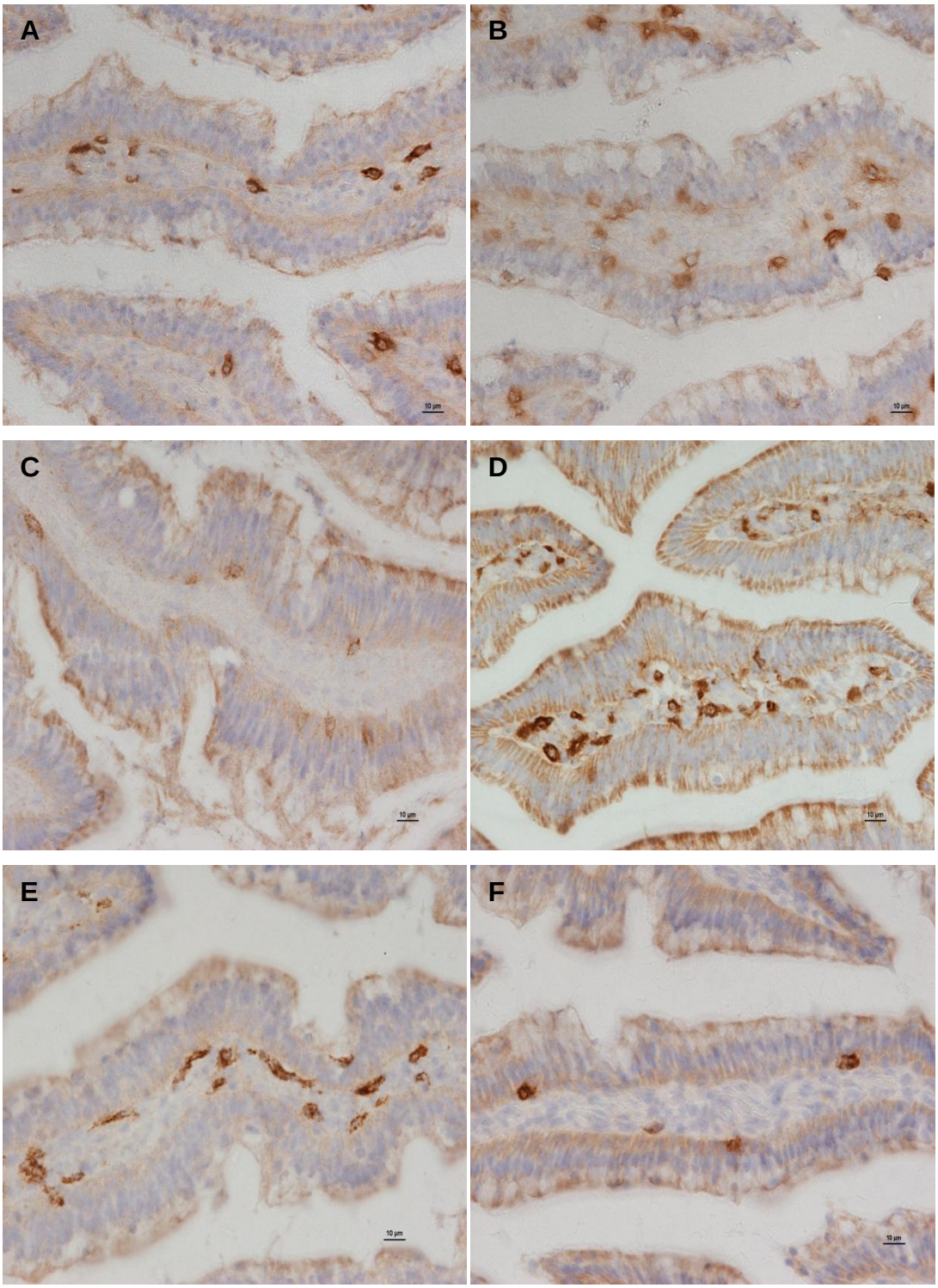

Figure 5.2: Immunohistochemistry staining showing $CD4^+$ (A), $CD8\alpha^+$ (B), $CD8\beta^+$ (C), MHC II^+ (D), $KuL01^+$ (E) and γδ TCR^+ (F) positive cells in the ileum of chickens infected with *S.* Virchow at 5 DPI.

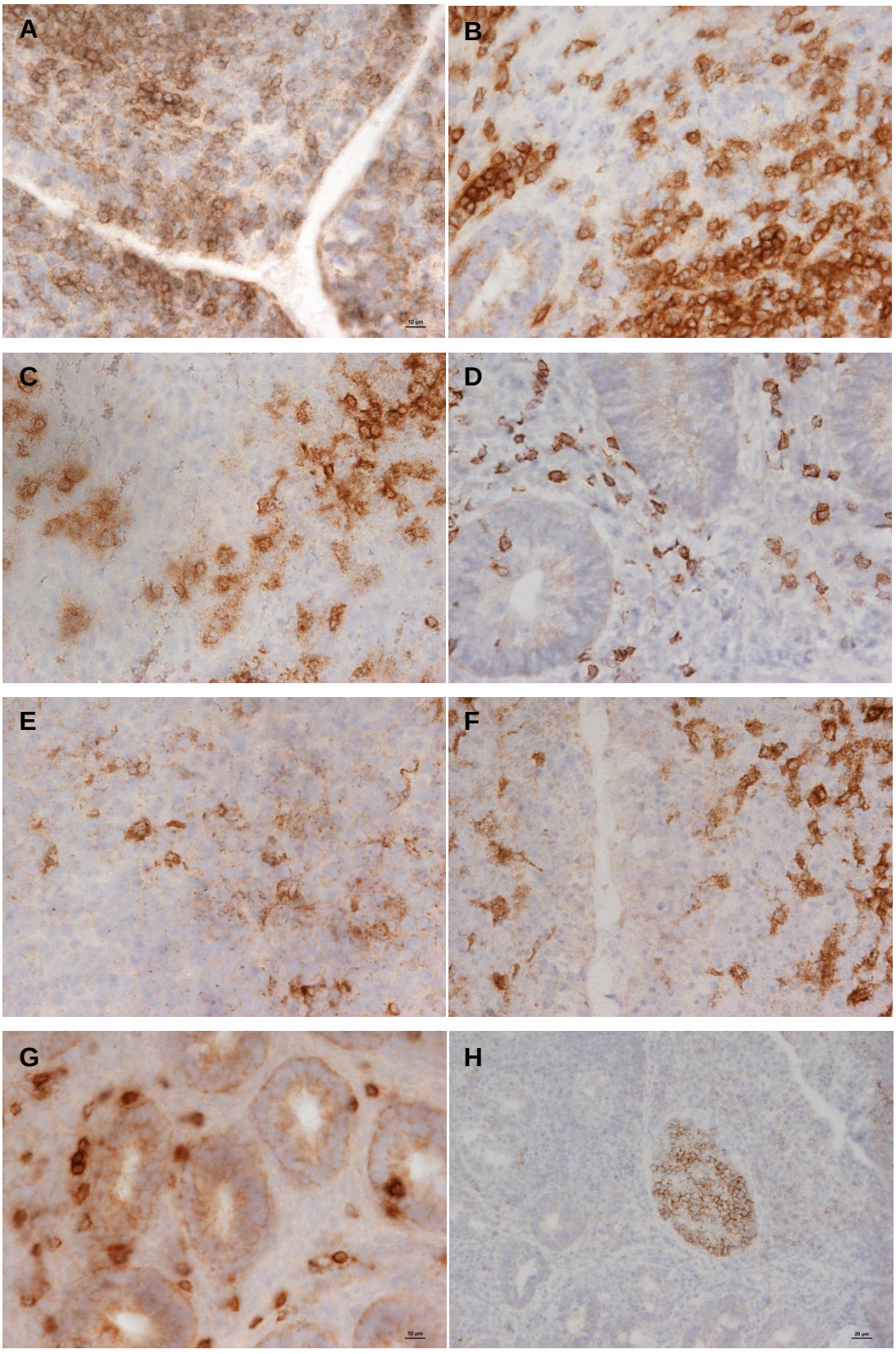

Figure 5.3: Immunohistochemistry staining showing $CD3^+$ (A), $CD4^+$ (B), $CD8\alpha^+$ (C), $CD8\beta^+$ (D), MHC II^+ (E), $KuL01^+$ (F), γδ TCR^+ (G) and $Bu1a^+$ (H) positive cells in the caecal tonsil of chickens infected with *S.* Virchow at 5 DPI.

1 compared to groups 2 and 3 (P = <0.001). At 26 DPI, counts in the infected groups were similar to those in group 3. CD8β$^+$ cells were higher in groups 1 and 2 compared to group 3, with group 1 having significantly higher counts than groups 2 and 3 at 11 DPI (P = <0.036). MHC II$^+$ cell counts were significantly higher in groups 1 and 2 compared to group 3 at 5 (P = <0.028) and 11 DPI (P = <0.011) and still remained higher at 26 DPI. At 5 DPI, KuL01$^+$ cell numbers were similar in the infected groups compared to the uninfected group. KuL01$^+$ cells significantly increased in both of the infected groups at 11 DPI (P = <0.016) and increased slightly more at 26 DPI (P = <0.016). γδ TCR$^+$ cells were similar in all 3 groups at 5 DPI. At 11 DPI, a significant increase was seen in group 1 compared to groups 2 and 3 (P = <0.001); however, this had decreased to the levels seen in the uninfected group by 26 DPI. No specific staining could be found in the ileum for CD3 and Bu1a.

In the caecal tonsil, the cell markers mainly increased in the infected groups during the infection period, however some cell markers decreased in number (Figure 5.6 & 5.7). At 5 DPI, CD3$^+$ cells were significantly higher in the infected groups compared to group 3 (P = <0.016). At 11 DPI, CD3$^+$ counts increased further in group 1, although by 26 DPI the counts in groups 1 and 2 were similar to those in group 3. CD4$^+$ cells were similar in all 3 groups at 5 DPI; however, by 11 DPI, CD4$^+$ cells were significantly higher in group 1 (P = <0.05) and also higher in group 2, compared to group 3. At 26 DPI, CD4$^+$ cell counts were similar in all 3 groups. CD8α$^+$ cell counts in the infected groups were similar to the uninfected groups at each time point, being slightly higher at 26 DPI. CD8β$^+$ cell counts were higher in groups 1 and 2 compared to group 3 at 5 DPI, but had returned to similar levels as group 3 at 11 and 26 DPI. MHC II$^+$ cells were significantly lower in groups 1 and 2 compared to group 3 at 5, 11 and 26 DPI (P = <0.045). KuL01$^+$ cells were significantly higher in group 1 compared to group 3 at all time points and significantly higher in group 2 at 26 DPI (P = <0.006). γδ TCR$^+$ cell counts were similar in group 1 compared to group 3 at all time points; however, group 2 cell counts increased above the uninfected group at 11 and 26 DPI. The area of all the

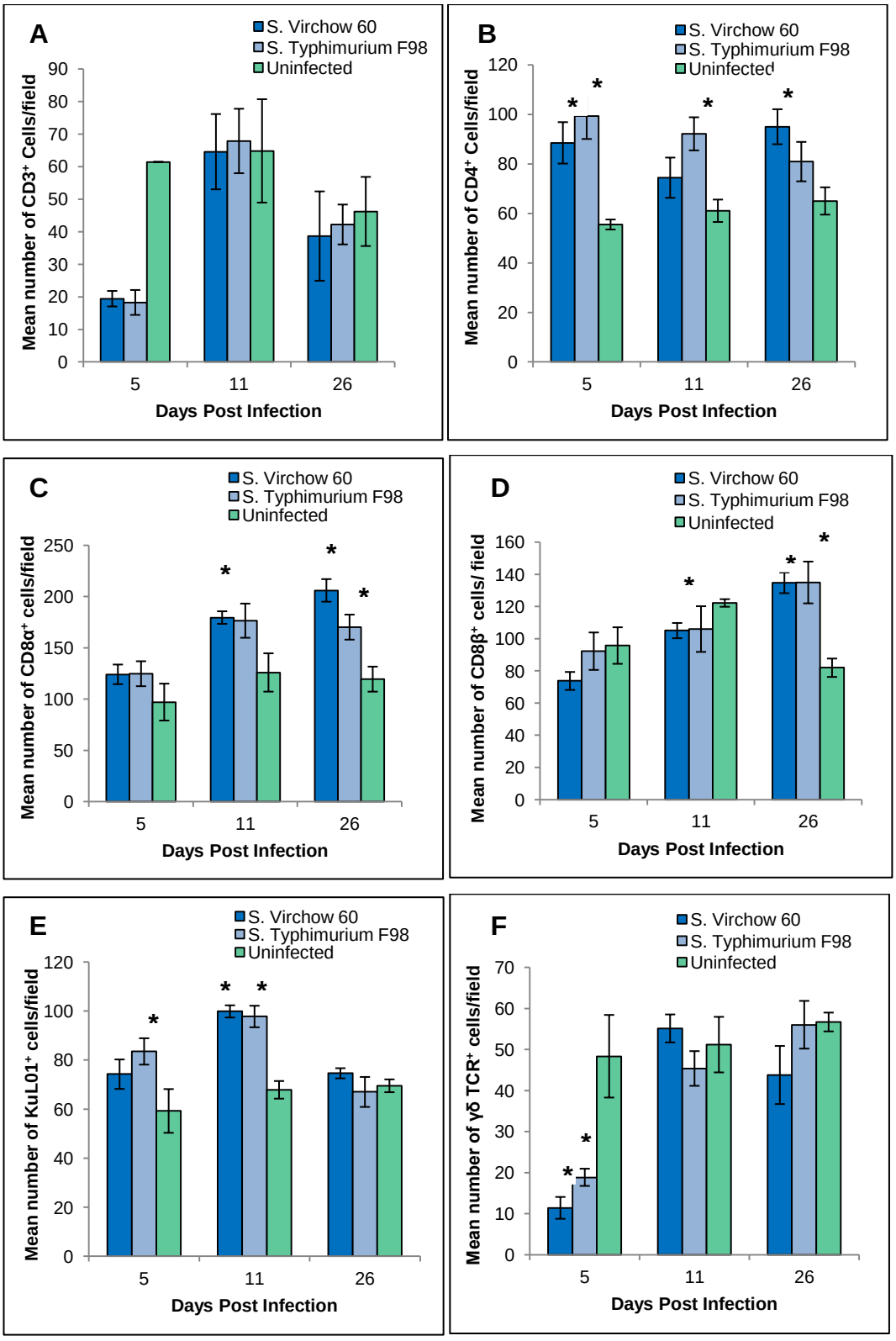

Figure 5.4: Numbers of CD3 (A), CD4 (B), CD8α (C), CD8β (D), KuL01 (E) and γδ TCR (F) positive cells in the spleen during *S.* Virchow and *S.* Typhimurium F98 infection. Error bars represent standard error of the mean. * = Significant difference compared to the uninfected group.

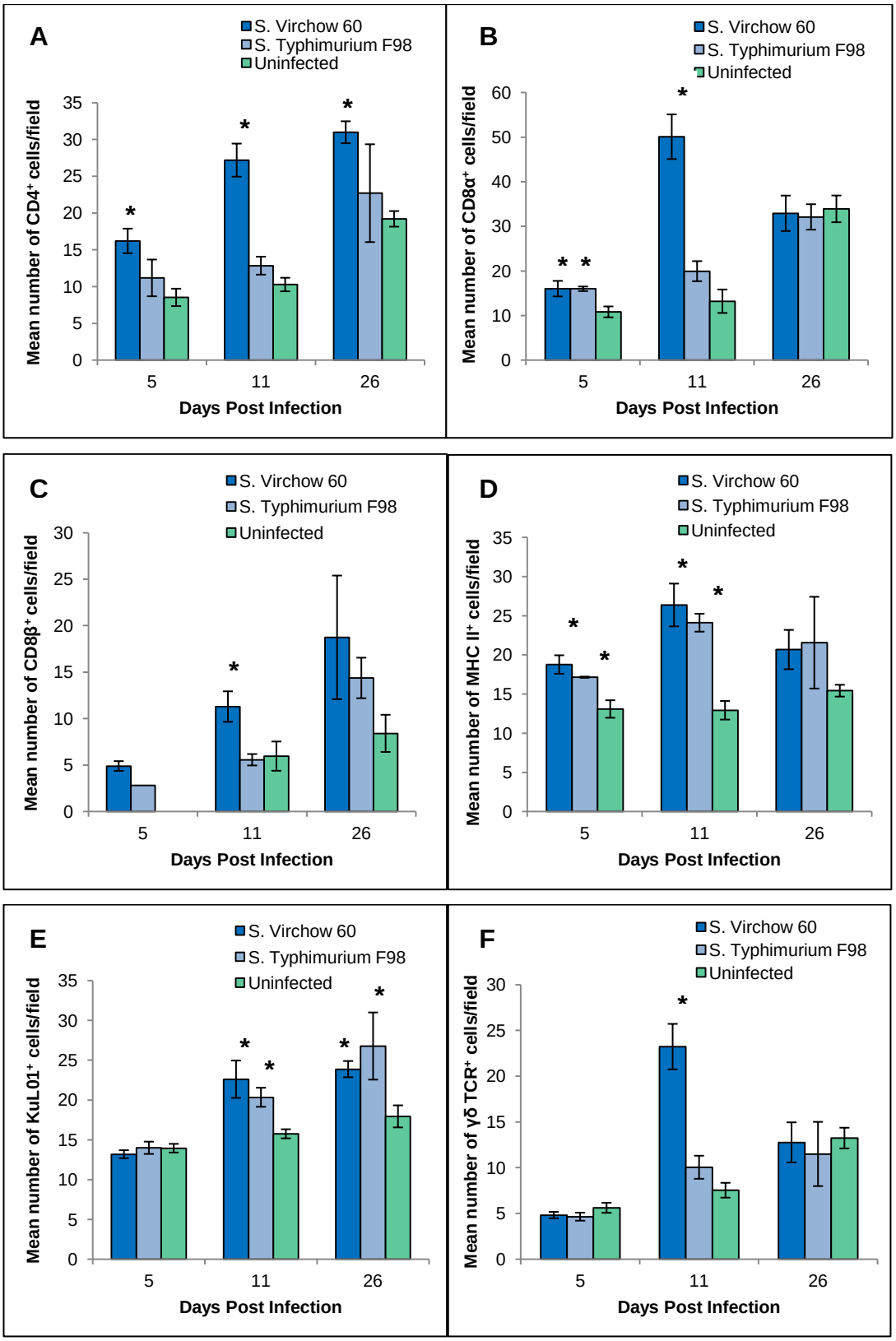

Figure 5.5: Numbers of CD4 (A), CD8α (B), CD8β (C), MHC II (D), KuL01 (E) and γδ TCR (F) positive cells in the ileum during *S.* Virchow and *S.* Typhimurium infection. Error bars represent standard error of the mean. * = Significant difference compared to the uninfected group.

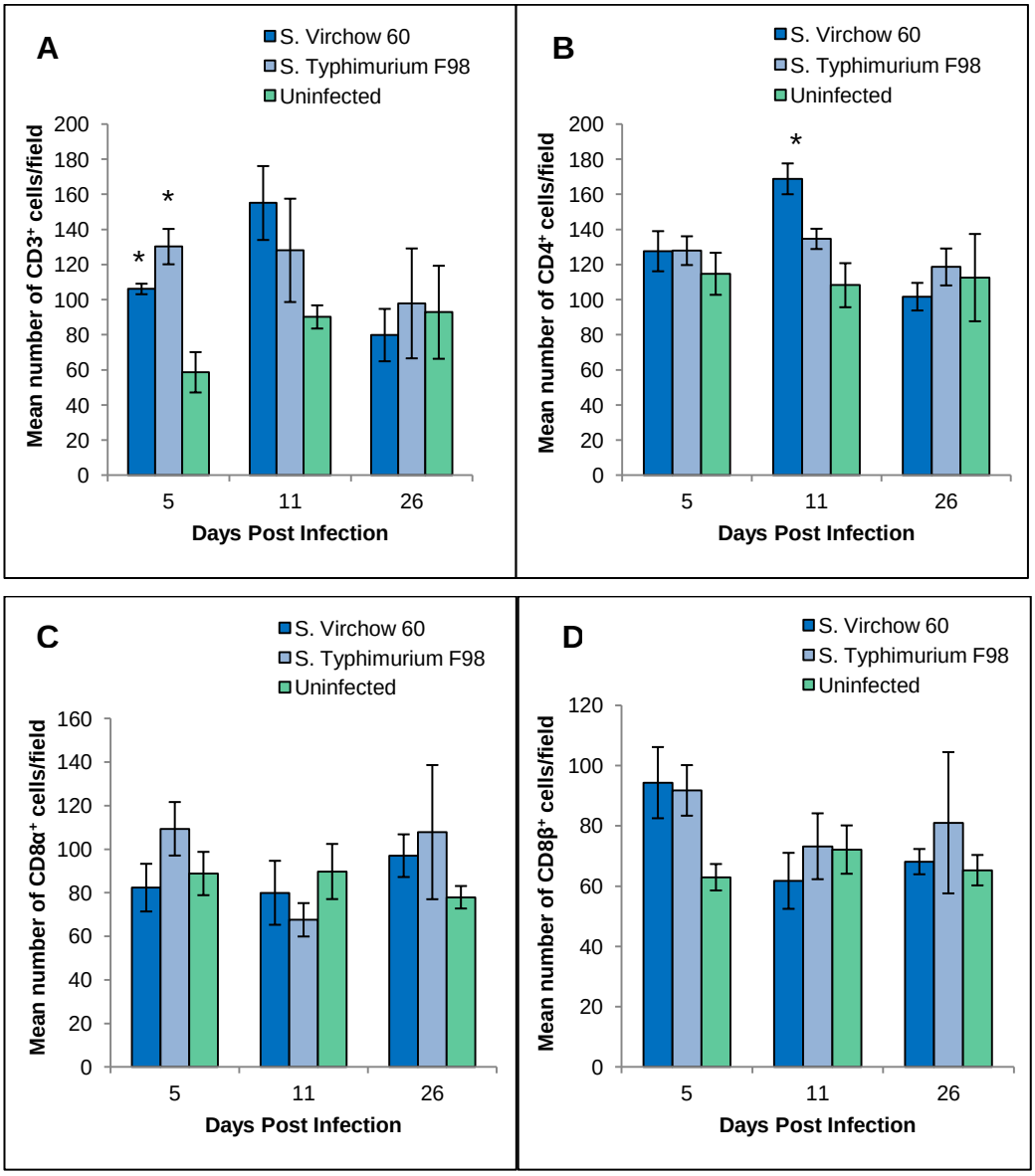

Figure 5.6: Numbers of CD3 (A), CD4 (B), CD8α (C) and CD8β (D) positive cells in the caecal tonsil during *S.* Virchow and *S.* Typhimurium infection. Error bars represent standard error of the mean. * = Significant difference compared to the uninfected group.

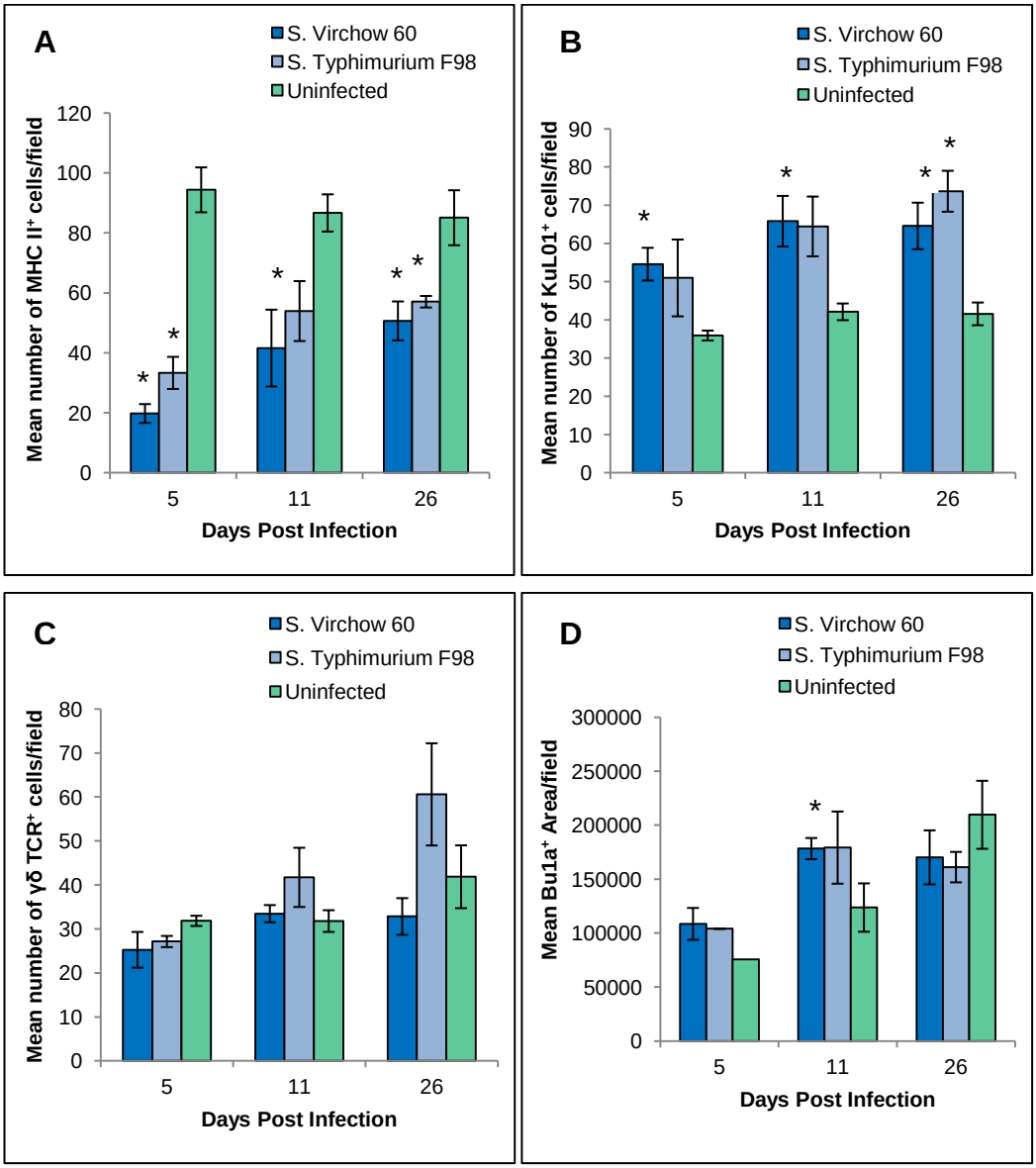

Figure 5.7: Numbers of MHC II (A), KuL01 (B), γδ TCR (C) and Bu1α (D) positive cells in the caecal tonsil during *S.* Virchow and *S.* Typhimurium infection. Error bars represent standard error of the mean. * = Significant difference compared to the uninfected group.

$Bu1a^+$ follicles in the tissue was measured and an average area was taken. At 5 and 11 DPI, the $Bu1a^+$ follicles were larger in the infected groups than in the uninfected group. At 26 DPI the follicles had returned to a similar size as the infected group.

5.3.2 Cytokine expression by comparative $2^{-\Delta\Delta Ct}$ RT-PCR

Expression of IL-1β, IL-6, IL-4, CXCLi2 and IFN-γ mRNA was measured in the spleen (Figure 5.8) and caecal tonsil (Figure 5.9) at 5, 11 and 26 DPI to determine changes in key cytokines and chemokines during *S.* Virchow infection.

In the spleen, IL-1β increased at 5 DPI in the *S.* Virchow and *S.* Typhimurium infected groups by 4.23 and 7.58-fold respectively, compared to the uninfected group. By 11 and 26 DPI, no difference in IL-1β expression was found between the groups. IL-6 also increased at 5 DPI by 5.9 and 7.62-fold in the *S.* Virchow and *S.* Typhimurium infected groups, respectively and had returned to levels found in the uninfected group by 11 DPI. CXCLi2 was up-regulated in the spleen by 2.99-fold, 1.71-fold and 1.22-fold at 5, 11 and 26 DPI, respectively in the *S.* Virchow infected group. In the *S.* Typhimurium infected group, CXCLi2 was slightly higher than in the *S.* Virchow infected group at 5 DPI, being 3.46-fold higher than levels in the uninfected group. Although at 11 and 26 DPI, CXCLi2 in the *S.* Typhimurium infected group was similar to the uninfected group and was 1.21-fold and 1.17-fold higher respectively. Spleen IFN-γ levels increased at different time points in the *S.* Virchow and *S.* Typhimurium infected groups. At 5 DPI, IFN-γ expression was similar to the uninfected group in the *S.* Virchow infected group. However, at 11DPI, IFN-γ had increased by 2.19-fold and was still 2.04-fold higher at 26 DPI. In the *S.* Typhimurium infected group, IFN-γ had increased by 1.89-fold at 5 DPI, although by 11 DPI IFN-γ had decreased to 1.21-fold and returned to the same level of expression as in the uninfected group, by 26 DPI. IL-4 was not expressed in the spleen at any time point during the infection period.

Some changes in mRNA expression were found in the caecal tonsil during infection, although there was considerable variation within groups for some cytokines and chemokines. IL-1β increased at 5 DPI in the *S.* Virchow infected group by 1.46-fold and then returned to expression levels seen in the uninfected group for the rest of the infection period. In the *S.* Typhimurium infected group no IL-1β expression was detected in 3 of the chickens. However, in the other 2 chickens expression increased by an average of 6.19-fold at 5 DPI. At 11 and 26 DPI, IL-1β expression was similar to that in the uninfected group. IL-6 increased slightly, by 1.25-fold, in 3 of the chickens in the *S.* Virchow infected group at 5 DPI and then remained similar to the uninfected group at 11 and 26 DPI. Caecal tonsil IL-6 did not change in the *S.* Typhimurium infected group for the duration of the infection experiment. CXCLi2 was higher in the *S.* Virchow infected group than the uninfected group at each time point. CXCLi2 expression was increased by 7.73-fold, 1.65-fold and 1.80-fold at 5, 11 and 26 DPI, respectively. The increase in expression was found in 3 birds at 11 DPI and 4 at 26 DPI, as a CXCLi2 increase was not detected in the other birds within the group. CXCLi2 increased by an average of 5.39-fold in 2 birds from the *S.* Typhimurium infected group at 5 DPI. At 11 and 26 DPI, the expression had declined back to similar levels to those found in the uninfected group, being 1.18-fold and 1.21-fold higher, respectively. IFN-γ expression did not change much in the *S.* Virchow infected group, although it had increased slightly by each time point. At 5, 11 and 26 DPI IFN-γ had increased by 1.29-fold, 1.34-fold and 1.56-fold respectively, compared to the uninfected group. In the *S.* Typhimurium infected group IFN-γ expression was similar to the uninfected group at 5 DPI, however had increased by 3-fold at 11 DPI. At 26 DPI IFN-γ expression had decreased and was 1.33-fold higher than the uninfected group. IL-4 expression was not detected in the caecal tonsil for the duration of the experiment.

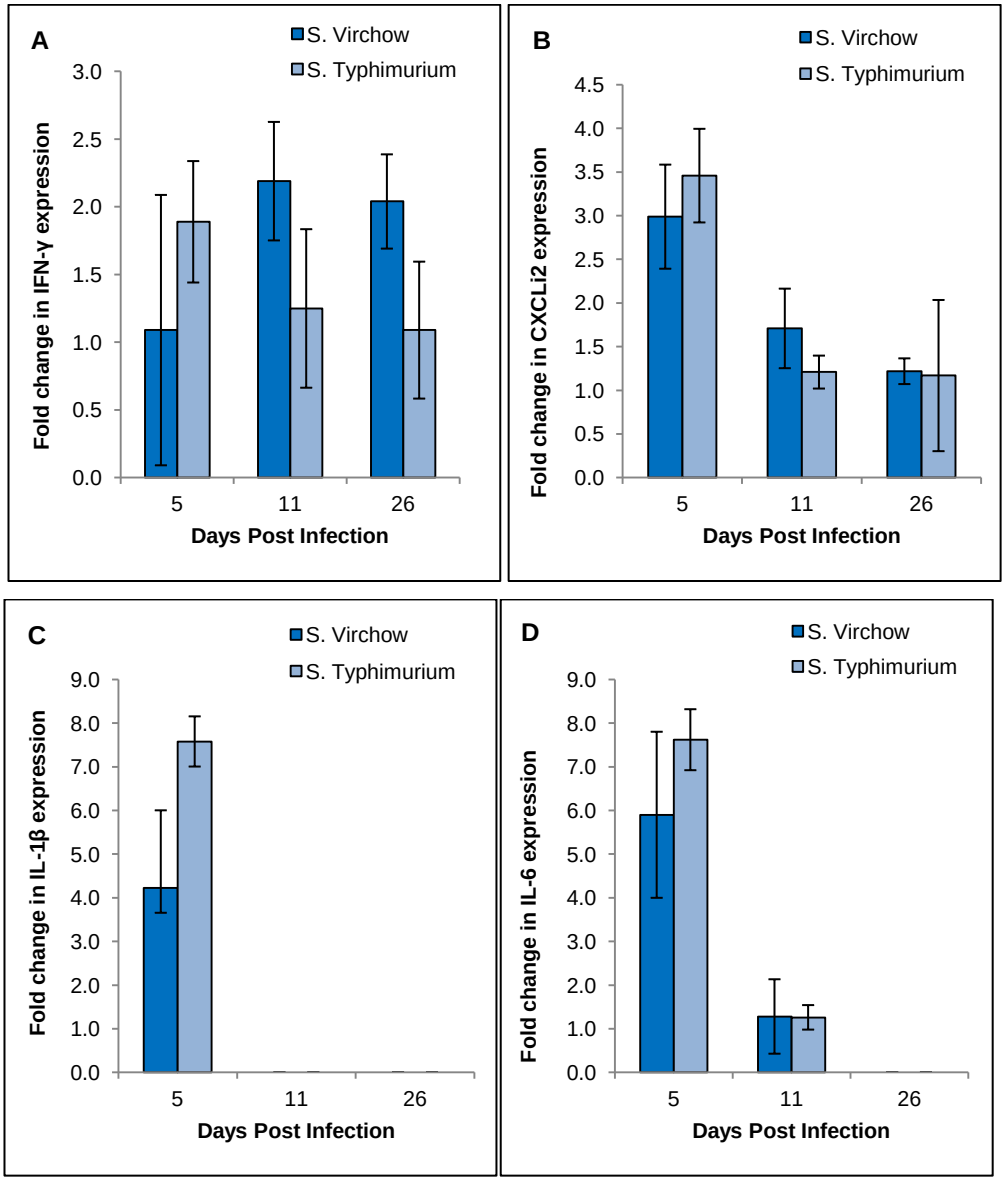

Figure 5.8: Relative expression of IFN-γ (A), CXCLi2 (B), IL-1β (C) and IL-6 (D) in the spleen of chickens infected with *S.* Virchow and *S.* Typhimurium F98 compared to uninfected chickens, at 5, 11 and 26 DPI. Error bars represent standard error of the mean.

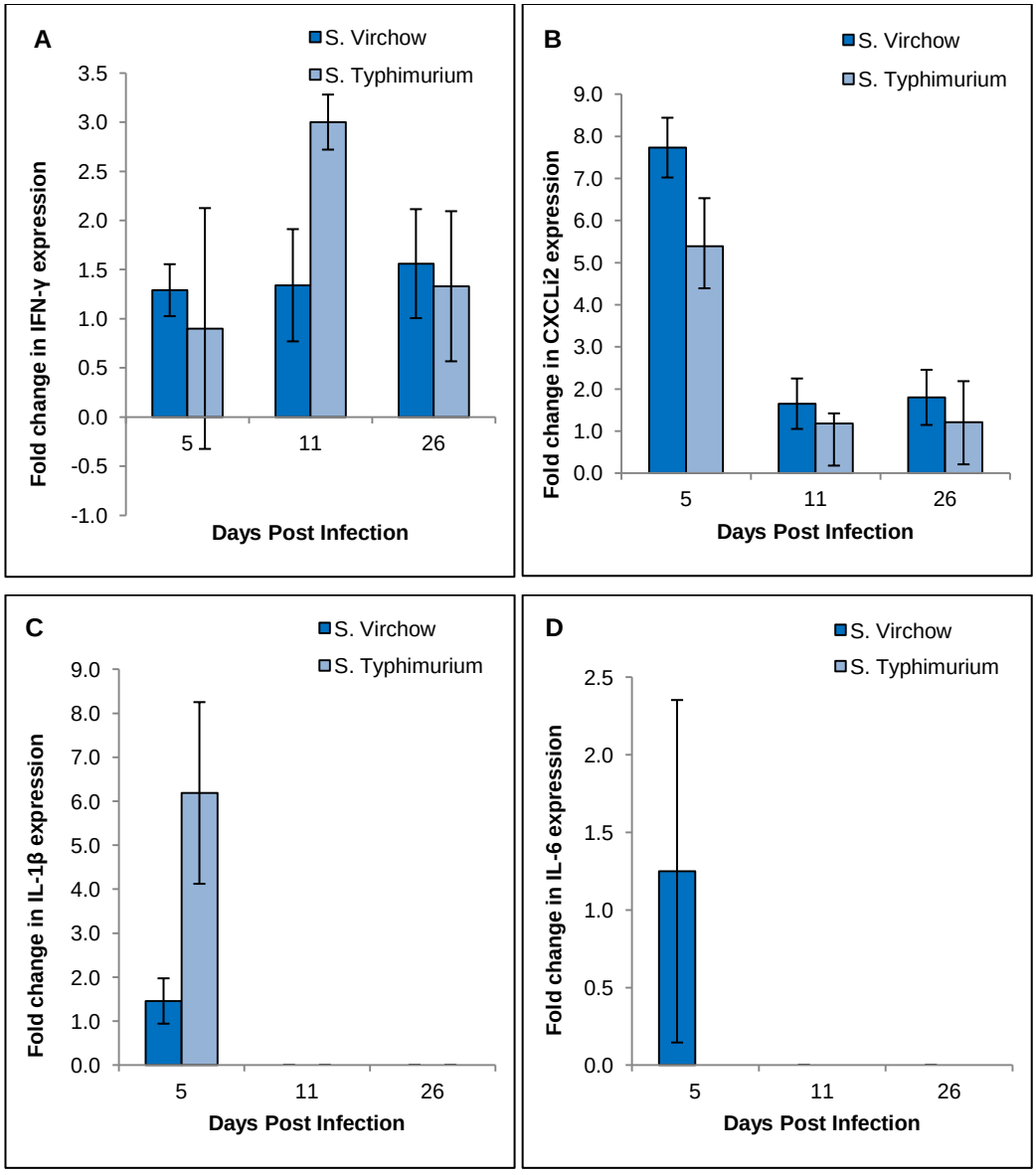

Figure 5.9: Relative expression of IFN-γ (A), CXCLi2 (B), IL-1β (C) and IL-6 (D) in the caecal tonsil of chickens infected with *S.* Virchow and *S.* Typhimurium F98 compared to uninfected chickens, at 5, 11 and 26 DPI. Error bars represent standard error of the mean.

5.3.3 ELISA

Serum was prepared from blood taken from the chickens at post mortem at 5, 11 and 26 DPI to measure specific IgA, IgM and IgG against *S.* Virchow and *S.* Typhimurium F98. At 5 DPI, the level of IgM was similar to that in uninfected chickens (*P* = >0.128) (Figure 5.10). Between 5 and 11 DPI, IgM rapidly increased in both infected groups, reaching a peak level at 11 DPI (*P* = <0.005). At 26 DPI, IgM had declined in both infected groups, towards levels found in the uninfected group (*P* = >0.508). IgA and IgG increased more slowly but to greater levels then IgM during infection with *S.* Virchow and *S.* Typhimurium. At 5 DPI, IgA in infected chickens was at similar levels to that in uninfected chickens (*P* = >0.093) (Figure 5.11). At 11 DPI, IgA levels in the infected chickens had slightly increased above those in uninfected chickens. Between 11 and 26 DPI, a sharper increase in IgA in both of the infected groups occurred. At 26 DPI, both of the infected groups had significantly greater IgA compared to the uninfected group (*P* = <0.030). At 5 DPI, IgG was at similar levels in the infected groups compared to the uninfected group (*P* = >0.113) (Figure 5.12). After 5 DPI, IgG increased steadily throughout the infection period. IgG levels were significantly higher in both of the infected groups compared to the uninfected group at 11 DPI (*P* = <0.026). At 26 DPI, IgG in the *S.* Virchow and *S.* Typhimurium infected group was higher than that in the uninfected group, with the levels in the *S.* Typhimurium group being significantly higher than levels in the uninfected group (*P* = <0.003).

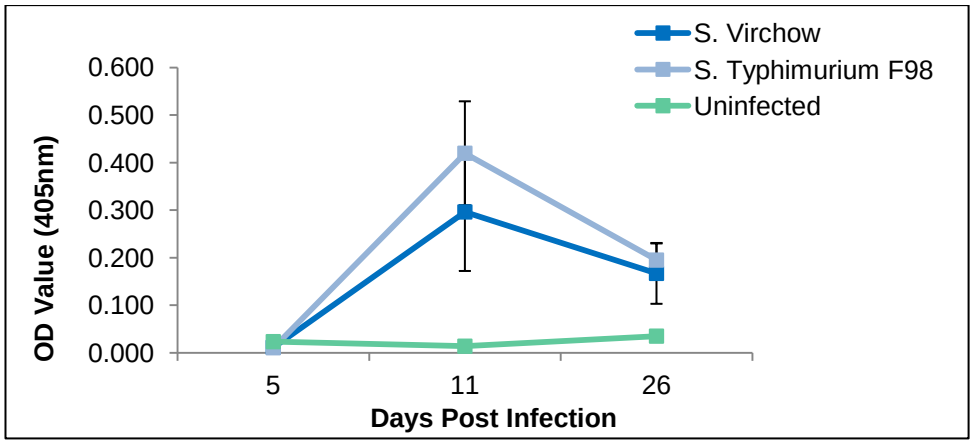

Figure 5.10: Serum IgM response to *S.* Virchow and *S.* Typhimurium F98 infection. Error bars represent the standard error of the mean.

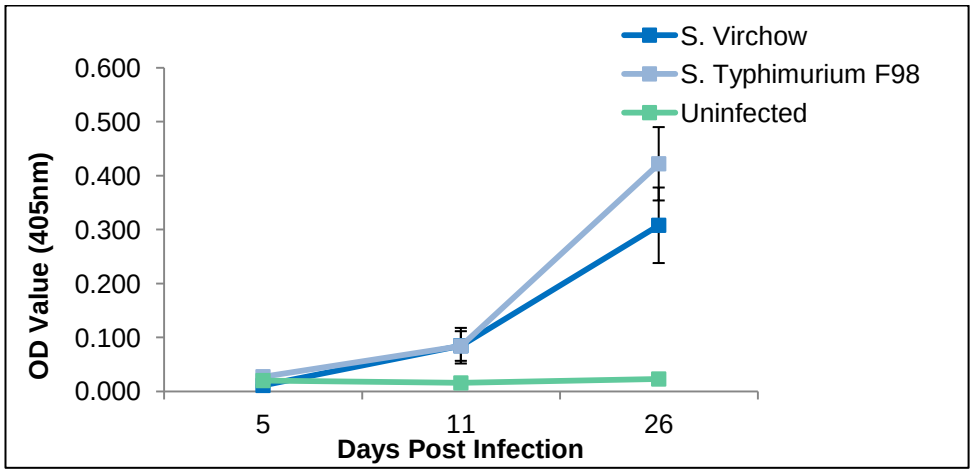

Figure 5.11: Serum IgA response to *S.* Virchow and *S.* Typhimurium F98 infection. Error bars represent the standard error of the mean.

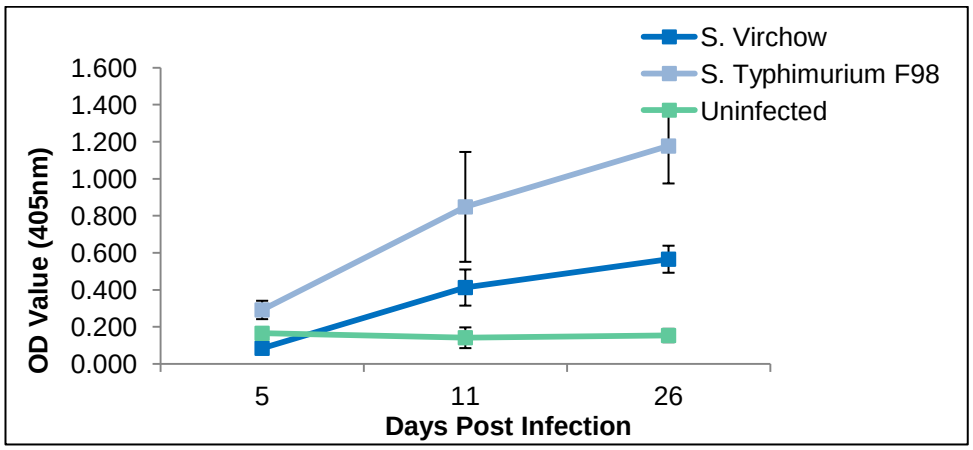

Figure 5.12: Serum IgG response to *S.* Virchow and *S.* Typhimurium F98 infection. Error bars represent the standard error of the mean.

5.3.4 Western blot

Coomassie brilliant blue staining solution was used to visualise the protein bands on the SDS-PAGE gel, to confirm the presence of the *Salmonella* proteins (See Chapter 6, Figure 6.6). Once the presence of proteins had been confirmed, the antigen preparation was separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane and incubated with chicken serum from all 3 groups to determine the reactivity and cross-reactivity of the serum with the proteins on the gel. A band, ~38 kDa in size, reacted and cross-reacted with the serum samples from the infected groups, as well as the uninfected group (Figure 5.13). Another band, ~80 kDa in size, reacted and cross-reacted with the serum samples from the infected groups, but not sera from the uninfected group (Figure 5.13).

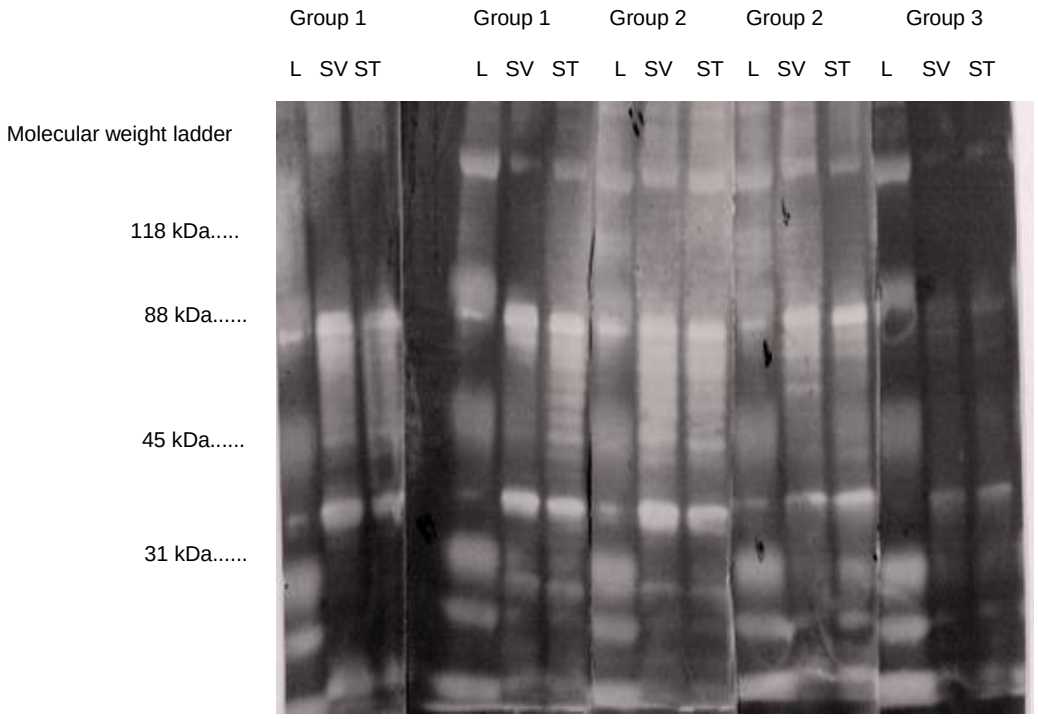

Figure 5.13: Western blot using chicken serum from 26 DPI. Two proteins of ~34 kDa and ~80 kDa reacted with chicken serum from each group. Each serum sample was incubated with *S.* Virchow and *S.* Typhimurium antigens, respectively. L = Broad-range ladder, SV = *S.* Virchow antigens, ST = *S.* Typhimurium antigens.

5.4 Discussion

The data presented here indicate that *S.* Virchow stimulates immune responses in chickens, similar to those seen against broad-range serovars. Upon infection with broad-range serovars, such as *S.* Typhimurium and *S.* Enteritidis, an acute inflammatory response is produced in the gut (Chappell et al., 2009). In contrast, host-restricted serovars do not cause an acute inflammatory response seen with broad-range serovars. A relationship has been shown between the invasiveness of broad-range serovars in the gut and the intensity of the immune response elicited by the serovar. Infection of 1 day old chicks with several broad-range serovars, including *S.* Enteritidis and *S.* Infantis, showed *S.* Enteritidis to be highly invasive and *S.* Infantis to be significantly diminished in systemic sites (Berndt et al., 2007). The high level of invasiveness seen with *S.* Enteritidis correlated with a strong immune cell influx into the gut, whereas a weak immune cell influx into the gut was found against *S.* Infantis (Berndt et al., 2007). During the infection experiment outlined in Chapter 4, slightly higher caecal content and spleen bacterial counts were found in the *S.* Typhimurium infected group compared to the *S.* Virchow group, although counts followed the same pattern. Although some differences were found in the immune responses overall the response against *S.* Virchow was similar to that against *S.* Typhimurium, showing that the serovars stimulate the immune system to similar intensities. Differences in the immune response depending on the infecting serovar have been shown in other studies (Setta et al., 2012b, Brito et al., 1993, Okamura et al., 2005).

Immunohistochemical analysis of the ileum, caecal tonsil and spleen detailed the cell-mediated immune response that occurs during *S.* Virchow infection. At 5 DPI, $CD4^+$, $CD8\alpha^+$ and $CD8\beta^+$ cells had increased in the ileum and remained elevated for the duration of the infection period. An increase in these cell markers in the gut following *Salmonella* infection is consistent with previous studies and shows a T helper as well as a cytotoxic T cell response (Berndt et al., 2007). An increase in $CD4^+$ and $CD8^+$ cells have also been shown in the ovaries and oviducts of laying hens following *S.* Enteritidis

infection (Withanage et al., 2003). In contrast, a decrease of $CD4^+$ cells has also been found in the gut following *S.* Enteritidis infection of 1-day old chicks and was suggested to be due to them not being immunologically mature (van Hemert et al., 2007). T cell influx into the gut following *Salmonella* infection has been shown to be variable depending on location in the GIT, infection dose, age at the time of infection and the genetic background of the chickens (van Hemert et al., 2007, Asheg et al., 2002, Beal et al., 2005, Berndt et al., 2007). Asheg (2002) found a significant increase in $CD3^+$, $CD4^+$ and $CD8^+$ cells in the gut of chickens infected with a high dose of *S.* Enteritidis, which was not as quick or prolonged in the group infected with a low dose of *S.* Enteritidis (Asheg et al., 2002).

MHC II^+ cells increased in the ileum throughout the infection experiment in both of the infected groups. The major histocompatibility complex (MHC) can be found on most cells and antigens are presented to T cells in combination with MHC (Kaufman, 2008). MHC can be divided into class I and class II molecules based on their structure (Kaufman, 2008). MHC class I molecules are found on nearly all cells and are recognised by $CD8^+$ cytotoxic T lymphocytes, whereas MHC class II molecules are found on APCs and are recognised by $CD4^+$ T helper cells (Kaufman, 2008). The continued increase in MHC class II^+ cells in the ileum in this study correlates well with the increase seen in $KuL01^+$ cells and suggests APCs have an important role in control of *S.* Virchow infection. Increased MHC II could also indicate local activation of macrophages, as a response to infection.

At 11 DPI, γδ TCR^+ cells increased in the ileum in both the *S.* Virchow and *S.* Typhimurium infected groups, although the number of these cells in the former group was significantly greater than in the *S.* Typhimurium infected group and the uninfected group. An increase in γδ TCR^+ cells in the caecum has been shown previously against *S.* Typhimurium infection; however, this increase was seen earlier, peaking at 4 DPI (Pieper et al., 2011). An earlier increase of γδ TCR^+ cells in the caecum has also been found in chicks infected with *S.* Enteritidis and *S.* Hadar (Berndt et al., 2007). The difference between time points may be due to different areas of the gut being sampled.

The changes in cell subpopulations were less prolonged in the caecal tonsil compared to the ileum and there were clear differences between the *S.* Virchow infected group and the *S.* Typhimurium infected group. $CD3^+$ and $CD8\beta^+$ cells increased early in the infected groups by 5 DPI, followed by an increase in $CD4^+$ cells at 11 DPI and $CD8\alpha^+$ cells by 26 DPI. During infection of 1 day old chicks with *S.* Enteritidis, $CD4^+$ cells were shown to increase in the caecal tonsil at 4 DPI (Sasai et al., 2000). Following this, an increase in IgG^+ B lymphocytes was seen at 6 DPI, suggesting $CD4^+$ lymphocytes are involved in Ig class switching in the caecal tonsil (Sasai et al., 2000). This mechanism could explain why, in this study, $CD4^+$ cells were only up-regulated in the caecal tonsil at 1 time point, which was at 11 DPI. The size of $Bu1a^+$ follicles in the caecal tonsil increased in area in the infected groups compared to the uninfected group at 5 and 11DPI, indicating the humoral immune response does have a role in clearance of *Salmonella* infection.

Throughout the infection experiment, MHC class II^+ cells decreased in the caecal tonsil, whereas $KuL01^+$ cells continually increased. An increase in $KuL01^+$ cells in the caecal tonsil following *S.* Enteritidis infection has been shown previously (Setta et al., 2012b) and could indicate antigen presentation to immune cells located in the caecal tonsil. No changes in γδ TCR^+ cells were found in the caecal tonsil of chickens infected with *S.* Virchow; however, an increase was found at 11 and 26 DPI in chickens infected with *S.* Typhimurium. Small numbers of γδ TCR^+ cells has also been found in the caecal tonsil during *S.* Enteritidis infection (Holt et al., 2010). The findings from this study show that although *S.* Virchow and *S.* Typhimurium induce a similar immune response in chickens, there are subtle differences.

At 5 DPI, $CD3^+$ and γδ TCR^+ cells decreased in the spleen in infected groups, before returning to levels seen in the uninfected group at 11 DPI. Following a decrease of γδ TCR^+ cells in the spleen, they had increased in the ileum at 11 DPI, which could indicate cell trafficking of γδ TCR^+ cells from the spleen to the gut. Although in this study $CD3^+$ staining of the ileum was not done, previous studies have shown that a decrease in $CD3^+$ cells in the spleen coincides with an increase in the ileum (Asheg et al., 2002). $CD4^+$ and

$CD8\alpha^+$ cells increased in the spleen in infected groups and remained elevated for the duration of the experiment. $CD8\beta^+$ cells did not increase in the spleen until 26 DPI in the infected groups. Varying results have been found for these subpopulation changes in the spleen and could be a result of age of the chickens when infected, infecting serovar or dose (Asheg et al., 2002, Sasai et al., 2000, Berndt et al., 2006).

Changes in IFN-γ, CXCLi2, IL-1β and IL-6 were found in the spleen and caecal tonsil during *S.* Virchow and *S.* Typhimurium infection. The magnitude of the response varied within groups, which can be seen by the standard error bars (Figure 5.8 & 5.9). This was particularly seen in the caecal tonsil as some chickens would exhibit a response, whereas others would not. Variability in cytokine and chemokine response in the caecal tonsil within the same group has been shown in previous studies and may be due to differences in immunological maturation from chicken to chicken (Sasai et al., 2000, Beal et al., 2004). Also, although RT-PCR is currently the most sensitive and reliable method to measure changes in mRNA expression, it may not directly correlate with bioactive protein in the cells (Kogut et al., 2005).

At 5 DPI, IL-1β and IL-6 increased in expression in the infected groups in the spleen and caecal tonsils. At 11 DPI, expression of both had returned to similar levels as in the uninfected group, except for IL-6 in the spleen, which was raised until 26 DPI. IL-1β and IL-6 are both pro-inflammatory cytokines (Weining et al., 1998, Ferro et al., 2005, Jones, 2005). Transient increases in IL-1β and IL-6 in the spleen and caecal tonsils have been shown in previous studies and a rapid decline in these cytokines could be due to an increase in the anti-inflammatory cytokine IL-10 or TGF-β_4, which prevent over-expression of pro-inflammatory cytokines (Kogut et al., 2003, Beal et al., 2005, Withanage et al., 2004, Withanage et al., 2005b, Beal et al., 2004). Late expression of IL-6 has been associated with lymphocyte and macrophage development rather than initiation of the inflammatory response and could explain why IL-6 expression was still up-regulated at 11 DPI in this study (Withanage et al., 2005b). A large increase in CXCLi2 was found in the

spleen and caecal tonsil of infected groups at 5 DPI and although expression had declined by 11 DPI, CXCLi2 remained higher than in the uninfected group until the end of the experiment. CXCLi2 is a pro-inflammatory chemokine that is highly homologous to human IL-8 and is important for early immune responses in the gut, including an influx of heterophils (Kogut et al., 2005, Kogut et al., 2003, Martins-Green, 2001). The increase in IL-1β, IL-6 and CXCLi2 in the spleen and caecal tonsils shows that like *S.* Typhimurium and other broad-range serovars, *S.* Virchow elicits a strong immune response in the chicken, causing a rapid inflammatory response upon infection, unlike host-restricted serovars such as *S.* Pullorum and *S.* Gallinarum.

IFN-γ expression was higher in both of the infected groups compared to the uninfected group at every time point, in both the spleen and caecal tonsil, throughout the experiment. IFN-γ enhances oxidative burst by macrophages against *Salmonella* infection (He et al., 2011). Elevated levels of IFN-γ support the idea that *S.* Virchow clearance is dependent on IFN-γ T-cell mediated responses. The increased level of IFN-γ, in combination with the lack of IL-4 expression (Th2 cytokine) (Avery et al., 2004), suggests *S.* Virchow clearance is primarily Th1 dominated.

Serum humoral responses followed a classical pattern of a rapid rise in IgM followed by a rise in IgG and IgA, against *S.* Virchow infection. The humoral immune response was slightly stronger against *S.* Typhimurium than *S.* Virchow throughout the infection experiment, although it did follow the same pattern in both infected groups. Serum IgA has been shown to correlate with secretory IgA in the gut (Rose et al., 1981). IgA and IgG continued to increase throughout the infection experiment. $CD4^+$ cells have been suggested to have a role in Ig class switching, which is indicated in this study by the increasing rise in $CD4^+$ cells in the gut and spleen as well as a late rise in the caecal tonsil, combined with the continued increase in IgA. Increased serum Ig and increased amounts of $Bu1a^+$ cells in the caecal tonsil suggest the humoral response has a role in *S.* Virchow clearance.

Western blot analysis showed the serum from the infected groups to react against 2 proteins, ~34 kDa and ~80 kDa in size (Figure 5.13). Serum from the uninfected group was also found to cross-react with the 34 kDa protein, but not the 80 kDa protein. Previous studies have found porins, heat shock proteins (HSPs) and fimbriae, which are all similar sizes to the proteins in this study, to react with serum from chickens infected with *Salmonella* (See Chapter 6).

This study has shown that *S.* Virchow induces an inflammatory response in chickens, similar to that found against *S.* Typhimurium. A wide range of changes representing the innate, humoral and cellular immune responses were found against *S.* Virchow and *S.* Typhimurium. Rapid up-regulation of CXCLi2, IL-6 and IL-1β cytokines are representative of the inflammatory response initiated by infection. Elevated levels of IFN-γ and increased expression of T lymphocyte cell markers show the importance of Th1-mediated immune responses against *S.* Virchow infection, whereas no IL-4 expression indicates the lack of a Th2-mediated response. Increased expression of serum immunoglobulins and $Bu1a^{+}$ cells show the humoral as well as the cell mediated immune response plays a role in clearance of *S.* Virchow infection. These findings provide valuable information about indicative and protective immune responses to S Virchow in chickens and should, after further studies, enable immunologically based preventative or therapeutic approaches.

Chapter 6
Immunological Protection Studies

6.1 Introduction

Control of the spread of zoonotic diseases in animals is important to prevent entry of pathogens into the food chain and subsequently disease and illness in humans. The main source of human salmonellosis is through the consumption of contaminated poultry meat and eggs (EFSA, 2012). However, control of *Salmonella* infection in poultry is difficult because once the chickens are more than a few days old they rarely exhibit any clinical symptoms of disease. Therefore, infected chickens can shed *Salmonella* in their faeces without detection, leading to horizontal transmission of the disease. In addition, after infection with serovars such as *S.* Enteritidis, chickens can become carriers. At the onset of lay, *S.* Enteritidis can spread to the reproductive organs and contaminate eggs by vertical transmission (Withanage et al., 2003).

The optimum method for controlling and preventing spread of *Salmonella* infection in poultry is through vaccination (Barrow, 2007). Effective vaccines for controlling the spread of infection are now even more important due to the emergence of multiple antibiotic-resistant bacteria (Barrow, 2007, Ngwai et al., 2006, Chu et al., 2012, Kingsley et al., 2009). Research has focused on identifying bacterial ligands that stimulate the host immune response, as potential vaccine candidates.

Ligands of TLRs are important stimulators of the host's immune response and potential vaccine candidates. TLRs are PRRs that recognise pathogens by their expression of conserved molecular structures known at pathogen associated molecular patterns (PAMPs) (Akira, 2004). TLRs induce signals through TIR (Toll/IL1-R) domains that interact with different adapter proteins such as MyD88, resulting in the activation of NF-kB and the mitogen-activated protein kinase signalling cascade (Barton and Medzhitov, 2003). Different PAMPs are ligands for specific TLRs; for example, LPS is a ligand for TLR4. Upon activation, TLRs induce responses such as an up-regulation of cell proliferation/maturation, production of cytokines and chemokines and production of other effector molecules (Akira, 2004). Expressed sequence

tags (EST) have been identified for several chicken TLRs and show that some are expressed in the majority of tissues and some are more restricted to certain tissues, such as immunological ones (Iqbal et al., 2005a). In addition, cells of the innate immune system express a broad range of TLRs, showing they are important for the early activation of the immune response (Iqbal et al., 2005a). A range of tissues derived from the chicken gut, including the duodenum, jejunum, ileum, caecum and colon, also express a wide range of TLRs, most likely due to the combination of somatic and immunological tissues and for early pathogen detection (Iqbal et al., 2005a).

Bacterial LPS has been identified as a potent stimulator of the host immune response and is recognised through TLR4. LPS, derived from *S.* Minnesota and *E.* coli, has been shown to mediate human cellular activation via TLR4 and be a main signalling molecule in human whole blood (Tapping et al., 2000). *Salmonella* LPS stimulation of the chicken immune response via TLR4 has also been demonstrated (Kogut et al., 2005). LPS activation of TLR4 expressed on chicken heterophils causes a 2-fold increase in heterophil degranulation and an increase in the expression of pro-inflammatory cytokines and chemokines (Kogut et al., 2005). LPS stimulation of heterophils also stimulates the processing of pre-cursor proteins to mature cathelicidin-2, which is released by heterophils and has potent antimicrobial and immuno-modulatory activities (van Dijk et al., 2009).

Bacterial flagellin is a potent stimulator of the host immune response and is recognised by TLR5 (Hayashi et al., 2001, Vo et al., 2007). TLR5 is expressed on a wide range of tissues and is expressed by cells of the immune system (Iqbal et al., 2005b). Exposure of chicken $TLR5^{+}$ cells to *S.* Typhimurium derived flagellin stimulates the up-regulation of the pro-inflammatory cytokine IL-1β (Iqbal et al., 2005b). In addition, a poultry infection experiment showed an aflagellated *S.* Typhimurium mutant colonised systemic sites to significantly higher levels than a WT flagellated strain, in the first 24 hours (Iqbal et al., 2005b). These results show how important recognition of a pathogen through identification of flagella is for the early activation of the host immune response. Poultry specific serovars, *S.*

Gallinarum and *S.* Pullorum lack flagella, which could be a contributing factor to their ability to cause rapid systemic infection and hardly any gut inflammation.

DNA motifs containing unmethylated CpG motifs are recognised by the chicken immune response via TLR21, the orthologue to mammalian TLR9 (Brownlie et al., 2009). The chicken immune system has also been shown to respond to CpG motifs through TLR15, which is absent in mammals (Ciraci and Lamont, 2011). Research has been carried out to investigate the effectiveness of CpG-ODN in stimulating the chicken immune response and increasing resistance to *Salmonella. In ovo* injection of eggs with CpG-ODN has been shown to result in a significant reduction of bacteria isolated from the caecal contents during *S.* Enteritidis infection and also an increase in heterophil function (Mackinnon et al., 2009). In addition, administration of CpG-ODN, IFN-γ, double-stranded RNA (Poly I:C) and squalene as an adjuvant, in combination with whole killed *Salmonella* as an experimental vaccine, gave significant protection against caecal colonization compared to the killed *Salmonella* vaccine without the adjuvant (Hartley et al., 2012).

Recently, research has focused on the development of adjuvants, which are potent immuno-stimulatory molecules (Lowenthal et al., 2000). The use of an adjuvant with killed *Salmonella* or with virulence sub-units is more beneficial than using live, attenuated vaccines, the main reason being that it is safer for consumers (Barrow, 2007). However the level of protection offered by a vaccine against infection depends of several factors including the strain of *Salmonella*, the route of administration, the infection dose, the age of the birds and the species of birds (Barrow, 2007, Hartley et al., 2012).

The use of virulence factors to stimulate the immune response and be included as potential vaccine candidates has also been researched. The SPI-1 T3SS injects virulence proteins into the host cell and is an important virulence factor for invasion (Schraidt et al., 2010, Jones et al., 2007). Vaccination trials in poultry with PrgI (a major SPI-1 needle component) and SipD (needle-tip) showed these proteins significantly decreased the bacterial

load in internal organs. However, they did not affect the bacterial load in the caecal contents and the number of chickens that were *Salmonella* positive in the vaccinated group compared to the unvaccinated group was the same (Desin et al., 2011). However, significantly higher antibody titres were found in the vaccinated group and western blot analysis revealed SipD protein to be immunogenic (Desin et al., 2011). SPI-1 T3SS proteins may therefore be important components of subunit vaccines. Further research using SPI-1 or SPI-2 mutants as vaccines, where the whole pathogenicity island had been removed revealed significantly less systemic spread in the vaccinated groups (Matulova et al., 2012). At 4 days post challenge, the number of birds positive for *Salmonella* in the caecum was similar in all of the groups, but by 14 days post challenge the SPI-1 vaccinated group had significantly fewer *Salmonella* positive chickens compared to the unvaccinated group (Matulova et al., 2012). Significantly higher concentrations of antibodies and significantly higher expression of cytokines were found in vaccinated birds at 4 days post challenge, showing vaccination resulted in a quicker immune response (Matulova et al., 2012).

IroN is an OMP involved in iron acquisition in iron-limiting environments and is an important *Salmonella* virulence factor. Immunization of chickens with iroN + Freund's incomplete adjuvant followed by intravenous challenge with *S.* Enteritidis revealed a significant difference in mortality rates between the vaccinated and unvaccinated groups (Kaneshige et al., 2009). This study suggests iroN is a good candidate for vaccine research. However, further research would need to be done to assess the protection offered against caecal and systemic colonisation.

Novel vaccine ideas using bacteriophages to protect chickens against *Salmonella* infection have also been published. Bacterial ghosts are produced by the activation of bacteriophages and contain the bacterial membrane, with the cell surface structures intact, but none of the cytoplasmic contents present (Jawale et al., 2012). Immunization of chickens via intramuscular, subcutaneous or oral routes with a *S.* Enteritidis ghost vaccine, resulted in significant stimulation of the humoral and cellular immune

response compared to controls vaccinated with PBS (Jawale et al., 2012). Groups vaccinated with the ghost vaccine were significantly protected against *S.* Enteritidis systemic colonisation (Jawale et al., 2012). Additionally, groups vaccinated via the intramuscular or subcutaneous routes were significantly protected against caecal colonisation (Jawale et al., 2012). As using whole bacteriophages has drawbacks, such as reduced efficacy under anaerobic conditions, the emergence of phage-resistant bacteria and the risk of horizontal transfer, the use of certain components such as tail spike proteins (Tsps) has been investigated (Waseh et al., 2010). Oral administration of P22 phage Tsp, 1 hour after infecting chickens with *S.* Typhimurium, significantly reduced colonisation of bacteria in the caecum, liver and spleen (Waseh et al., 2010). An *in vitro* assay showed presence of the Tsp in agar significantly inhibited the motility of *S.* Typhimurium, indicating a possible mechanism for inhibition *in vivo* (Waseh et al., 2010).

The development of vaccines against *S.* Enteritidis infection in laying hens has succeeded in significantly reducing human salmonellosis cases from infected eggs in several countries, including the UK (Cogan and Humphrey, 2003). Initially, the vaccines used in the UK were based on killed bacteria. Of these, an inactivated *S.* Enteritidis vaccine grown under iron-restricted conditions (Intervet Nobilis Salenvac) was shown to significantly reduce egg contamination, reduce the extent of diarrhoea and offer systemic protection after intravenous challenge (Woodward et al., 2002). Subsequently, a bivalent killed vaccine Nobilis Salenvac T (Intervet, Milton Keynes, UK), containing both *S.* Enteritidis and *S.* Typhimurium, grown under iron-restricted conditions was produced (Clifton-Hadley et al., 2002). This vaccine significantly reduced shedding of *S.* Typhimurium into the environment (Clifton-Hadley et al., 2002). Although the study showed the humoral immune response had been activated, no differences between the vaccinated and unvaccinated groups were found for systemic spread and clearance of *Salmonella*, suggesting not all aspects of the immune response had been activated (Clifton-Hadley et al., 2002).

Salmonella is an intracellular pathogen, therefore activation of the cellular immune response is considered essential. Live vaccines are considered to be greater stimulants of the cellular immune response, due to expression of all the appropriate antigens *in vivo* (Barrow, 2007). A number of live vaccines are available for commercial use. The AviPro® Megan® Egg live vaccine is available for commercial use in the USA, New Zealand and Dominican Republic (http://www.lah.de/Salmonellosis.131.0.html ?&no_cache=1&tx_kbshop_pi1[selected]=28). This vaccine significantly protects layers against *S.* Enteritidis colonization of the gut and systemic sites, including the spleen and reproductive tract (Hassan and Curtiss, 1997). Additionally, the vaccine protects against egg colonisation and during molt (Hassan and Curtiss, 1997). Protection against *S.* Enteritidis colonisation lasts throughout the laying period (Hassan and Curtiss, 1997). TAD *Salmonella* vac® E and TAD *Salmonella* vac® T are live vaccines, also available commercially (http://www.bnotharel.com/apage/523. php) and are the main vaccines currently used in the UK. A combination of these two vaccines has been shown to greatly reduce reproductive tract colonisation and internal egg contamination (Gantois et al., 2006). Disadvantages of live vaccines include public acceptability and consumer safety, due to the possibility of the live bacteria becoming virulent (Barrow, 2007). Indeed some countries including France do not permit the use of live *Salmonella* vaccines in poultry. Therefore, inactivated or sub-unit vaccines are often safer and preferred methods of protection.

Although bivalent vaccines are available commercially to protect against the two most common *Salmonella* serovars associated with human salmonellosis, the third most common serovar associated with human salmonellosis (*S.* Virchow) in the UK is associated with invasive disease in humans (Matheson et al., 2010, Ispahani and Slack, 2000, Langridge et al., 2009). Thus a greater understanding of the immune response produced by poultry against infection is essential for developing new vaccines, improving the efficacy of current vaccines and particularly developing multivalent vaccines, to protect poultry against several *Salmonella* serogroups.

The aims of this study were to determine if primary infection of *S.* Virchow in poultry offered any protection against secondary infection with this bacterium or any cross-protection against secondary infection with *S.* Typhimurium. During secondary infection, IgA, IgG and IgM antibody titres were determined in the serum, to characterise the humoral immune response to secondary infection. In addition the *Salmonella*-specific antigens with which the serum reacted and cross-reacted with were determined by western blotting.

6.2 Materials and Methods

6.2.1 Bacterial isolates and chickens

Forty-eight 1-day old SPF Rhode-Island Red chicks were obtained from the Institute for Animal Health, Compton, UK. Chicks were housed separately in 2 groups of 24, at a temperature of 30ºC, which was reduced to 20ºC at 3 weeks of age. Chickens were given *ad-libitum* access to water and a vegetable protein based diet (SDS, Witham, Essex, UK). All experiments were conducted within local ethical guidelines and according to national legislation.

S. Virchow 60 (See Chapter 2, Table 2.1) was selected to infect the chickens, based on the first two poultry infection experiments (Chapter 4). *S.* Typhimurium F98 was included in the infection experiment, during re-challenge, to determine cross-protection offered against secondary challenge with a heterologous serogroup. Bacteria were grown from -70ºC stocks, in 10ml LB broth, in an orbital shaking incubator overnight, at 37ºC at 150rpm.

6.2.2 Poultry infection experiment

At 7 days of age, chickens in Group 1 were infected orally with 10^8cfu/ml *S.* Virchow in 0.3ml LB broth. Group 2 remained uninfected, to act as an age-matched control. The chickens were checked twice daily, for any signs of morbidity and for any mortality. At 2, 4, 5, 7, 8, 10 and 11 WPI cloacal swabs were taken from 5 chickens in each group. Swabs were directly plated onto

BGA and then enriched in selenite broth for *Salmonella* detection. BGA plates and enriched swabs were incubated overnight at 37ºC and the enriched swabs were re-plated and incubated overnight at 37ºC. At 13 weeks post-primary infection (WPPI), group 1 and group 2 were both divided in half. Half of each group was (re-)challenged with 10^8cfu/ml *S.* Virchow and half with 10^8 cfu/ml *S.* Typhimurium F98. The infection regime is outlined in Table 6.1. At 3 and 5 DPI, half of each group were killed by cervical dislocation for bacteriological analysis.

Table 6.1: Infection protocol

Group	Primary Infection	Secondary Infection
1	*S.* Virchow	*S.* Virchow
2	*S.* Virchow	*S.* Typhimurium
3	Uninfected	*S.* Virchow
4	Uninfected	*S.* Typhimurium

6.2.3 Post mortem and bacteriology

At post mortem, caecal contents and spleen were taken aseptically and added to 1 x PBS in a 1:10 dilution. Spleen samples were homogenised using a MicroStomacher 80 (Seward, UK) and the caecal contents were vortexed to form a suspension. Spleen and caecal content samples were serial diluted in 1 x PBS to 10^{-5} and 10^{-11} respectively and plated onto BGA. The plates were incubated at 37^{o}C for 18 hours and the colonies counted. During post mortem blood was collected from the heart using 21mm needles, for ELISA and western blot and was centrifuged at 13000 x *g* for 5 minutes. The serum was removed and stored at -20°C.

6.2.4 Production of soluble *Salmonella* lysate antigen

Overnight cultures of 10 ml LB broth inoculated with *S.* Virchow 60 or *S.* Typhimurium F98 were prepared from frozen stocks stored at -70ºC and incubated in an orbital shaking incubator at 37ºC and 150 rpm. The overnight cultures were used to inoculate 100 ml LB broth, which was then incubated overnight at 37ºC and 150 rpm. Cultures were aseptically poured into sterile

tubes and centrifuged at 4080 x *g* for 25 minutes at 4ºC (Beal et al., 2004). Supernatant was poured off and the bacterial pellet was suspended in 20 ml 1 x PBS. Bacterial suspensions were then incubated in a waterbath at 65ºC for 5 hours. Suspensions were plated onto nutrient agar and incubated at 37ºC overnight to confirm that no viable *Salmonella* remained. Following this check, the bacterial suspensions were sonicated in 10 ml volumes in 20 second bursts on ice, a total of 10 times, allowing the suspension to cool for 1 minute between each burst. Bacterial suspensions were centrifuged at 4080 x *g* for 20 minutes at 4ºC. The suspension was then ultra-centrifuged at 30000 x *g* for 20 minutes at 4ºC (Beal et al., 2004). The supernatant was aseptically poured into sterile tubes and protein concentrations were measured using a Lowry kit. The soluble antigen preparations were aliquoted into 1 ml volumes and stored at -20ºC until used.

6.2.5 ELISA

Flat-bottomed 96-well plates were coated with 100 µl/well of *S.* Virchow 60 or *S.* Typhimurium F98 soluble antigen, diluted in carbonate-bicarbonate buffer (pH 9.6) to a concentration of 16.2 µg/ml and incubated overnight at 4°C (Withanage et al., 2005b). Following overnight incubation, the plates were washed three times with PBS Tween-20 (0.05%) and then incubated with 100 µl of 3% blocking buffer for 1 hour at 37°C. After the 1 hour incubation, the plates were washed once with PBS Tween-20 (0.05%). Chicken serum samples were diluted in blocking buffer for detection of IgA (1:25), IgM (1:400) and IgG (1:400). Plates were incubated with 100 µl of the diluted chicken serum for 1 hour at 37°C and then washed three times with PBS Tween-20 (0.05%). Specific antibodies were detected by incubating samples with 100 µl alkaline phosphatase conjugated to either goat anti-chicken IgA (1:20000) (Serotec, Oxford, UK), IgM (1:1000) (Serotec, Oxford, UK) or IgG (1:2000) (Serotec, Oxford, UK) diluted in blocking buffer, for 1 hour at 37°C. Plates were washed with PBS Tween-20 (0.05%) and incubated with 100 µl *p*-nitrophenyl phosphate in the dark for 30 minutes at room temperature. The reaction was stopped by addition of 100 µl 3N sodium hydroxide. Absorbance was determined using a microplate reader at 405 nm.

6.2.6 Western Blot

Soluble antigen preparations of *S.* Virchow 60 or *S.* Typhimurium F98 were mixed in a 1:1 ratio with Laemmli loading buffer (containing 1/20th β-mercaptoethanol) and heated at 100°C for 10 minutes. The proteins were separated by SDS-PAGE by loading 15 µl/well of the antigen preparations onto a gel and running the gel in 1 x electrophoresis running buffer at 100v for 90 minutes. A pre-stained broad range ladder (Bio-Rad) was included on the gel as a size marker. To confirm the presence of the proteins on the gel, it was agitated on a rocker at room temperature in Coomassie brilliant blue staining solution (Bio-Rad) for 2 hours. Following this, the gel was agitated in de-staining solution for 2 hours at room temperature and then incubated in fresh de-staining solution overnight.

After running the antigen preparations out on the gel, to perform a western blot (instead of staining the gel), the proteins were transferred to a nitrocellulose membrane, using an iBlot dry blotting system (Invitrogen, UK), according to the manufacturer's instructions. The membrane was incubated with 3% blocking buffer on a rocker, for 1 hour at room temperature. Following this, the membrane was incubated with chicken serum diluted 1:100 with 3% blocking buffer, overnight at 4°C. After the overnight incubation the membrane was washed 3 times with PBS Tween 20 (1%) for 5 minutes each time on a rocker at room temperature. IgG conjugated to alkaline phosphatase was diluted 1:500 in 3% blocking buffer and added to the membrane, which was then incubated for 1 hour on a rocker at room temperature. The membrane was washed three times with PBS Tween 20 (1%), before incubation with 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) (Abcam, UK), to stain identified bands (Desin et al., 2011).

6.3 Results

Following secondary (re)-challenge, 5 birds from each group were killed by cervical dislocation, at 3 and 5 DPI. At post mortem, spleen and caecal contents were taken for bacteriology and serum was taken to determine the humoral immune response.

6.3.1 Bacteriology

Chickens in group 1 were infected with *S.* Virchow 60 at 7 days old. Cloacal swabs were taken from groups 1 and 2 every 1-2 weeks. At 11 WPI, group 1 had cleared *S.* Virchow infection. Group 2 remained negative for *Salmonella* for the duration of primary infection of group 1.

Following challenge at 13 WPPI, the bacterial load was determined in the caecal contents and spleen at 3 and 5 DPI (Figure 6.1 and 6.2). *Salmonella* was detected in the caecal contents of all 4 groups at similar levels. Group 1 had the lowest caecal content counts, which were $\log_{10}$ 4.47 cfu/g and $\log_{10}$ 4.05 cfu/g, at 3 and 5 DPSI respectively. Bacterial recovery was lower than that for group 3, which was $\log_{10}$ 5.11 cfu/g at 3 DPI and $\log_{10}$ 5.07 cfu/g at 5DPI. Although the bacterial load in group 1 was lower than that in group 3, no significant difference was found between the groups at 3 (P = >0.070) and 5 DPI (P = >0.104). Bacterial counts for group 2 were $\log_{10}$ 5.06 cfu/g and $\log_{10}$ 5.39 cfu/g at 3 and 5 DPSI, respectively. The counts for group 2 were slightly lower than counts for group 4, which were $\log_{10}$ 5.71 cfu/g on 3 and 5 DPI. No significant difference was found between group 2 and group 4 at 3 (P = >0.320) and 5 DPI (P = >0.607). Bacterial load was lower in group 1 compared to group 2 but the difference was also not significant (P = >0.062). No *Salmonella* could be detected in the spleen for all 4 groups at 3 and 5 DPSI by direct plating. Therefore, the spleens were enriched in Selenite broth and scored as positive or negative (Figure 6.2). The enriched spleens of group 1 and group 2 were all negative at 3 and 5 DPSI. Group 3 spleens were negative at 3 DPSI; however, at 5 DPSI 57% of the group were positive for *S.* Virchow. Positive spleens were detected at both time points in group 4, with 20% positive at 3 DPSI and 40% positive at 5 DPSI.

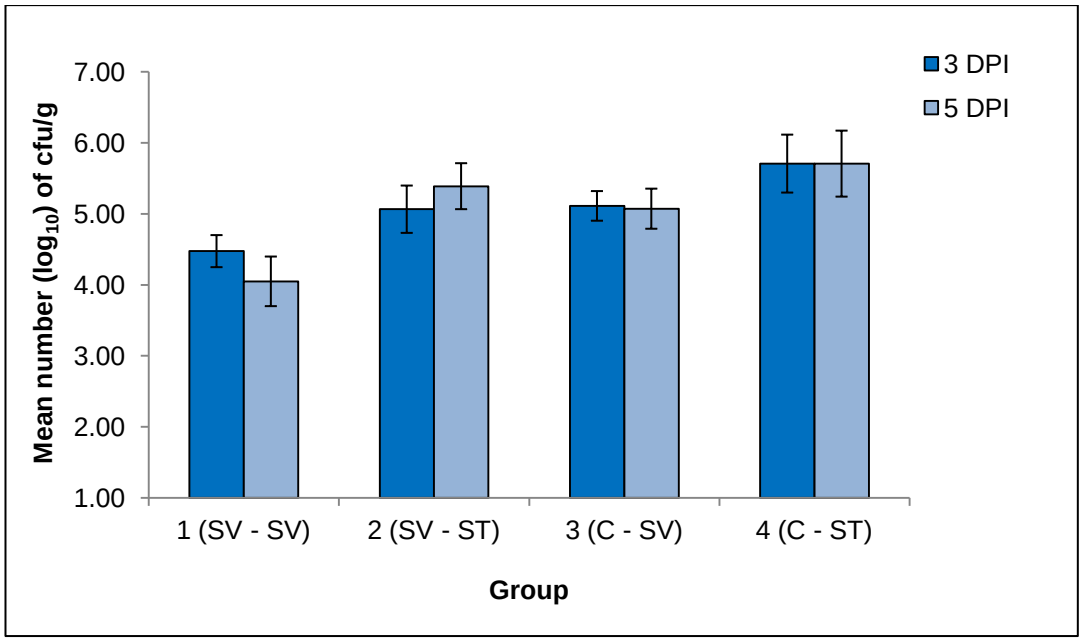

Figure 6.1: Mean $\log_{10}$ *Salmonella* numbers in the caecal contents at 3 and 5 DPI. Group 1 and 2 were primarily infected with *S.* Virchow and re-challenged with *S.* Virchow and *S.* Typhimurium, respectively. Group 3 and 4 were kept uninfected and then challenged with *S.* Virchow and *S.* Typhimurium, respectively. Error bars represent the standard error of the mean, which was calculated from 5 birds per time point.

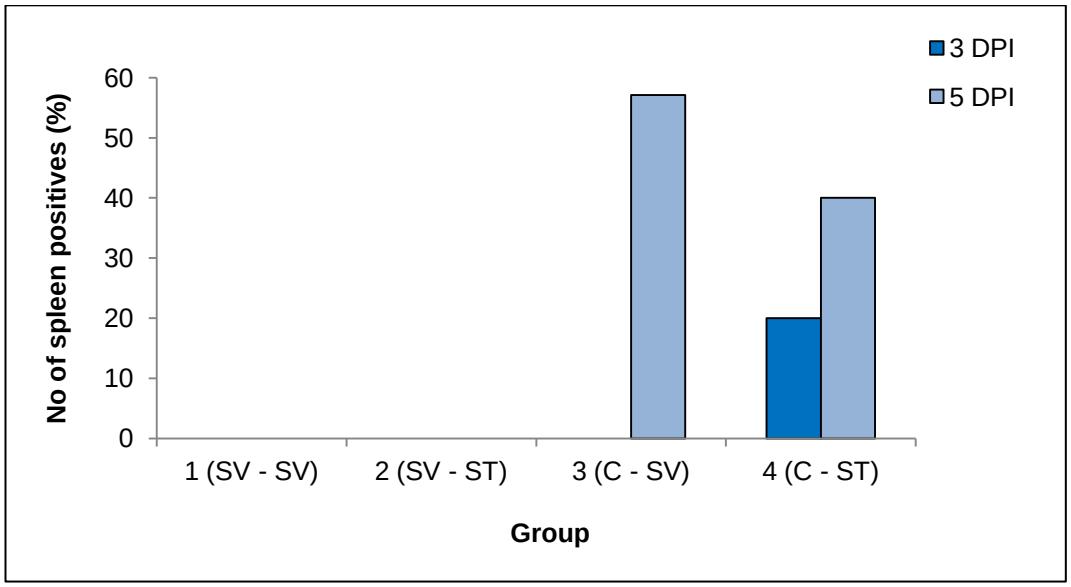

Figure 6.2: Percentage of positive spleens at 3 and 5 DPI. Group 1 and 2 were primarily infected with *S.* Virchow and re-challenged with *S.* Virchow and *S.* Typhimurium, respectively. Group 3 and 4 were kept uninfected and then challenged with *S.* Virchow and *S.* Typhimurium, respectively.

6.3.2. Humoral Response

During the post mortems, serum samples were collected to determine the humoral response produced by the chickens in response to primary and secondary *S.* Virchow and *S.* Typhimurium infection. Serum IgA antibodies increased in all four groups between 3 and 5 DPI (Figure 6.3). IgA antibody titres were considerably higher in group 1 compared to group 3, but this was not significant at either time point (P = >0.052). At 3 DPSI, IgA titres in group 2 were similar to those in both of the control groups (P = >0.166). However, a large increase in IgA was seen in group 2 between 3 and 5 DPSI. At 5 DPSI, the level of IgA in group 2 was significantly higher than in group 4 (P = >0.039). The IgA titre in group 1 was higher than in group 2 at both time points, particularly at 3 DPSI; however, the differences were not significant (P = >0.073).

Serum IgG antibodies increased in all 4 groups between 3 and 5 DPI (Figure 6.4). As with serum IgA, the serum IgG titres were highest in group 1 at both time points. IgG titres were greater in group 1 compared to group 3, with this difference significant at 5 DPI (P = <0.003). Group 2 IgG titres were higher at both time points compared to group 4 and significantly higher at 5 DPI (P = <0.039).

Serum IgM antibodies decreased between 3 and 5 DPI in all 4 groups (Figure 6.5). IgM titres were similar between all four groups, although the highest IgM titres were still seen in group 1, followed by group 2. No significant differences were found when comparing group 1 to group 3 (P = >0.186) and group 2 to group 4 (P = >0.061).

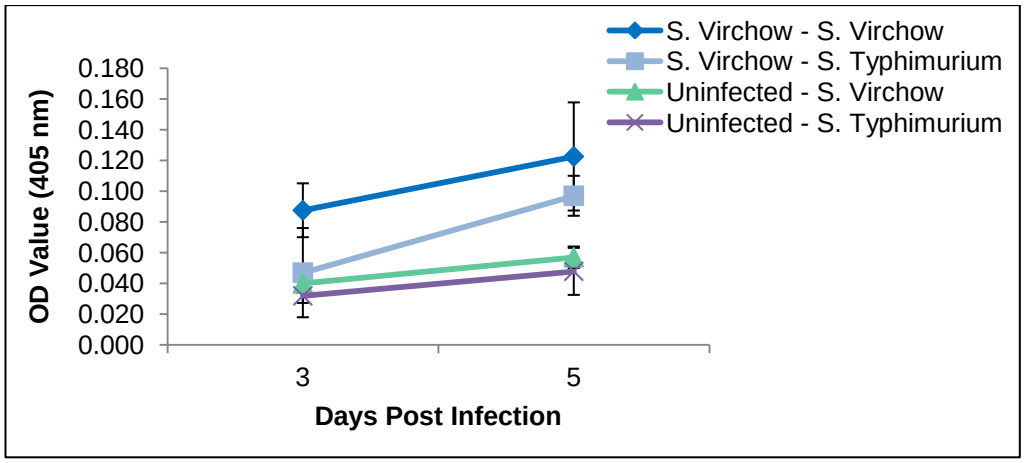

Figure 6.3: Serum IgA response at 3 and 5 DPSI. Error bars represent the standard error of the mean.

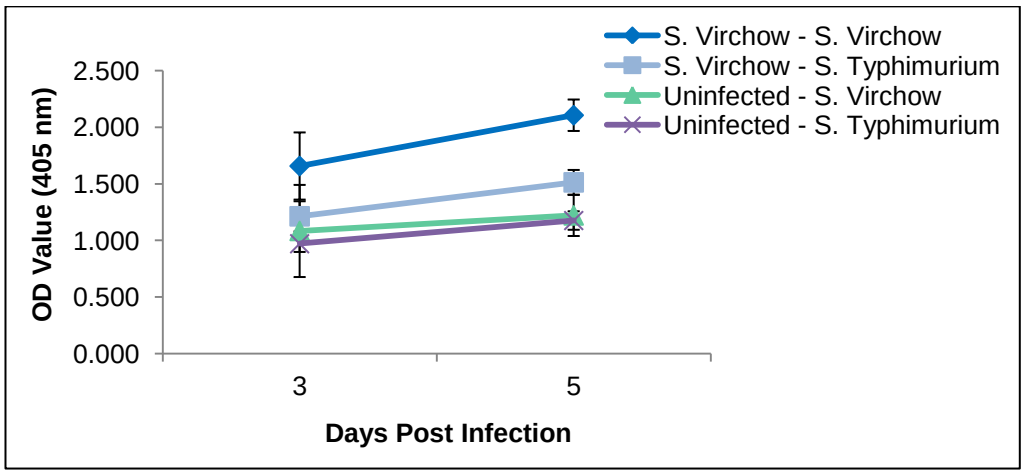

Figure 6.4: Serum IgG response at 3 and 5 DPSI. Error bars represent the standard error of the mean.

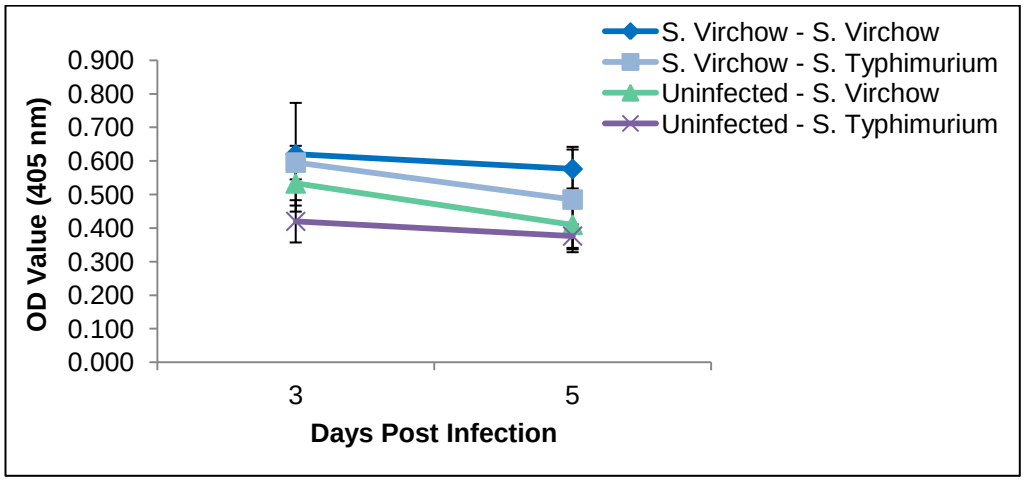

Figure 6.5: Serum IgM response at 3 and 5 DPSI. Error bars represent the standard error of the mean.

6.3.3. Western Blot

Coomassie brilliant blue staining solution was used to visualise the protein bands on the SDS-PAGE gel, to confirm the presence of the *Salmonella* proteins (Figure 6.6) (Steinberg, 2009, Sasse and Gallagher, 2009, Bradford, 1976). Once the presence of proteins had been confirmed, the antigen preparation was separated by SDS-PAGE, blotted onto a nitrocellulose membrane and incubated with chicken serum from all 4 groups to determine the reactivity and cross-reactivity of the serum with the proteins present on the gel. IgG secondary antibody, conjugated to alkaline phosphatase was used to detect proteins the serum reacted with. Two *S.* Virchow and *S.* Typhimurium proteins, ~38 kDa and ~80 kDa in size, reacted and cross-reacted strongly, with the chicken serum from all 4 groups at 3 and 5 DPI (Figure 6.7).

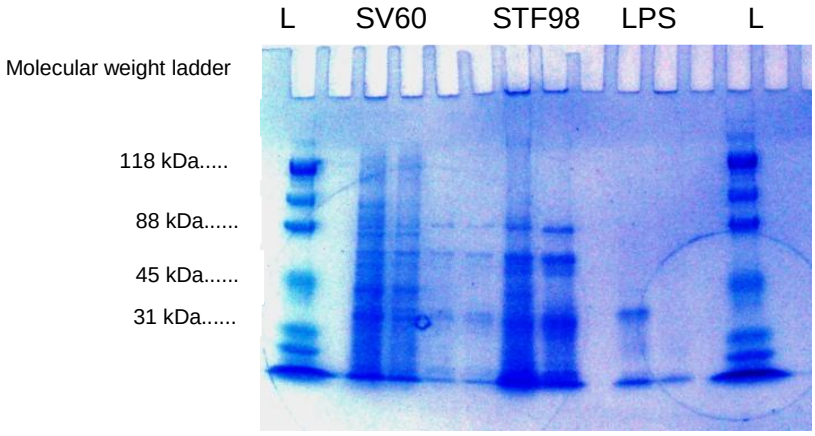

Figure 6.6: Coomassie brilliant blue staining of an SDS-PAGE gel. L = Broad-range molecular weight ladder, SV60 = *S.* Virchow antigen preparation, STF98 = *S.* Typhimurium F98 antigen preparation, LPS = *S.* Typhimurium lipopolysaccharide.

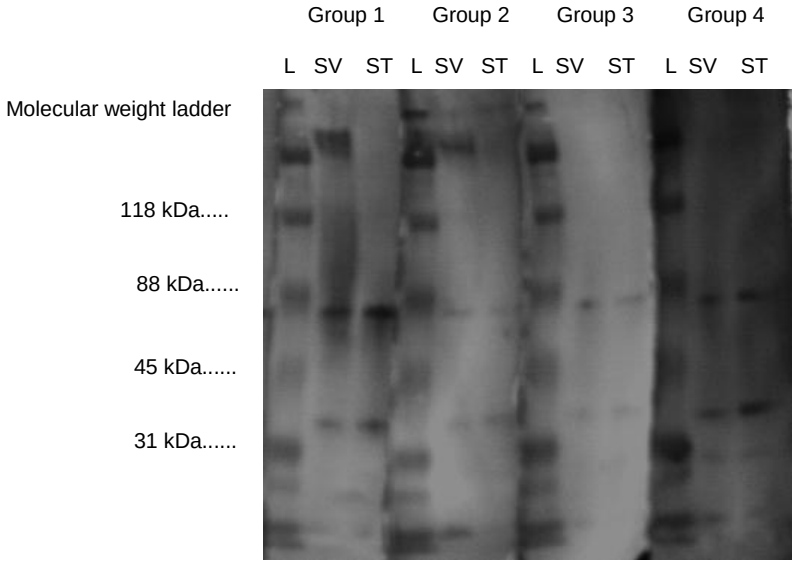

Figure 6.7: Western blot using chicken serum from 3 DPSI. Two proteins of ~34 kDa and ~80 kDa reacted with chicken serum from each group. Each serum sample was incubated with *S.* Virchow and *S.* Typhimurium antigens, respectively. L = Broad-range ladder, SV = *S.* Virchow antigens, ST = *S.* Typhimurium antigens.

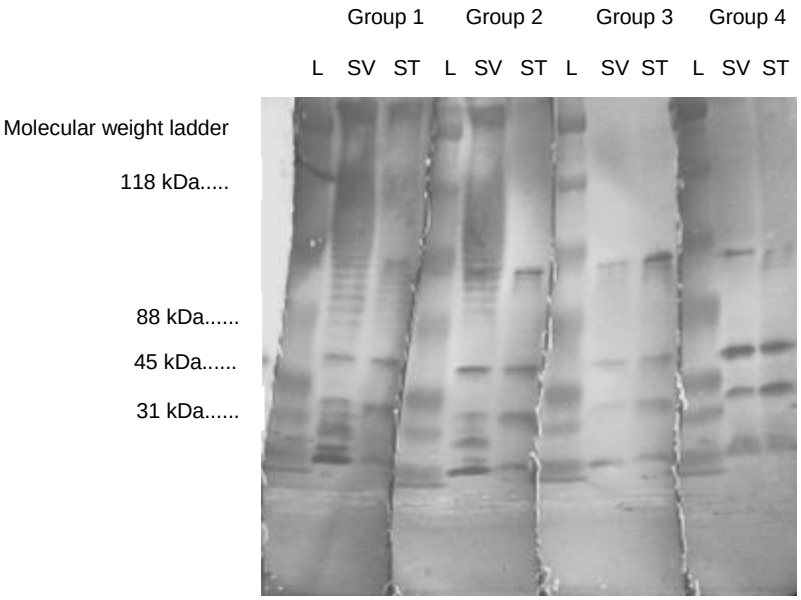

Figure 6.8: Western blot using chicken serum from 5 DPSI. Two proteins of ~34 kDa and ~80 kDa reacted with chicken serum from each group. Each serum sample was incubated with *S.* Virchow and *S.* Typhimurium antigens, respectively. L = Broad-range ladder, SV = *S.* Virchow antigens, ST = *S.* Typhimurium antigens.

6.4 Discussion

This study has shown that primary infection with *S.* Virchow offers some protection against secondary infection with this serovar. This was particularly noticeable at systemic sites, as no bacteria were detected in the spleen samples from group 1 (primary infection with *S.* Virchow, followed by secondary infection with *S.* Virchow) after enrichment. The bacterial load in the caecal contents of group 1 decreased from $\log_{10}$ 4.47 cfu/g at 3 DPSI to $\log_{10}$ 4.05 cfu/g at 5 DPSI, whereas in group 3 (uninfected, followed by infection with *S.* Virchow) the load was $\log_{10}$ 5.11 cfu/g at 3 DPSI and similarly $\log_{10}$ 5.07 cfu/g at 5 DPSI. Although the bacterial load in group 1 is lower than in group 3, no significant difference was found between the groups, indicating the protection offered against caecal colonisation is limited. Primary infection with *S.* Virchow offered less cross serogroup protection following secondary infection with *S.* Typhimurium. Although, no *Salmonella* was detected in the spleen showing protection offered against systemic invasion, bacterial load in the caecal contents was very similar in both groups, being only slightly lower in group 2 (primary infection with *S.* Virchow, followed by secondary infection with *S.* Typhimurium) compared to group 4 (uninfected, followed by infection with *S.* Typhimurium) (P = >0.320).

Serum antibody titres were measured to determine the humoral immune response against primary and secondary *Salmonella* infection. *Salmonella*-specific IgA, IgG and IgM were detected in all 4 groups but levels were always highest in group 1. These results show that the chicken immune system responds quicker and greater against secondary infection with *S.* Virchow compared to primary infection, indicating the development of an antigen-specific immune response. Additionally, the quicker and greater immune response observed in group 2 compared to group 4 indicate the immune response cross-reacts against *S.* Virchow and *S.* Typhimurium.

Research to investigate the possibility of multivalent vaccines that protect chickens against serovars across different serogroups has previously focused on *S.* Enteritidis and *S.* Typhimurium. A poultry infection study to

determine cross-protection and cross-reactivity of the immune response after homologous or heterologous re-challenge with *S.* Enteritidis and *S.* Typhimurium showed reduced counts in the caecal contents and the spleen compared to age-matched controls (Beal et al., 2006b). Cross-protection offered by primary infection with *S.* Typhimurium against secondary infection with *S.* Enteritidis was more effective than primary infection with *S.* Enteritidis followed by secondary infection with *S.* Typhimurium (Beal et al., 2006b). Therefore, although in the current study there is evidence of cross-protection against colonisation of systemic sites, primary infection with *S.* Typhimurium may have protected more effectively against secondary infection with *S.* Virchow. Similarly to the results here, this study found a high level of antibody cross-reactivity between the serovars (Beal et al., 2006b).

A vaccination study using the commercially available live *S.* Enteritidis vaccine Gallivac® Se or a combination of this with *S.* Enteritidis-*S.* Typhimurium inactivated vaccine Gallimune® Se+St, to immunize Lohmann Brown chickens, before infecting them with either *S.* Typhimurium or *S.* Enteritidis, found a significant reduction in liver and caecal content colonisation in the vaccinated groups (Springer et al., 2011). The results show that vaccination with live or live and attenuated vaccines can offer significant protection against *Salmonella* colonisation. Significantly higher levels of circulating antibodies were also found in the vaccinated groups compared to the unvaccinated groups (Springer et al., 2011). Previous studies assessed the protection offered by *S.* Typhimurium vaccines against serovars from several serogroups including groups B (*S.* Heidelberg), C (*S.* Kentucky) and E (*S.* Anatum), as well as serogroup D (*S.* Enteritidis) (Jiang et al., 2010). The vaccine vector used in this study, strain x9241-*tHP,* reduced colonisation and invasion of *S.* Heidelberg and *S.* Typhimurium and cross-protected against *S.* Enteritidis (Jiang et al., 2010).

Western blot analysis revealed 2 major proteins of ~38 kDa and ~80 kDa, which reacted strongly with serum *Salmonella*-specific antibodies from all four groups (Figure 6.7 & 6.8). Serum from the *S.* Virchow infected group cross-reacted with *S.* Typhimurium antigen and vice versa, also showing the

cross-reactivity of the immune system against the two serovars (Figure 6.7 & 6.8). Previous research has focused on the identification of immunogenic antigens as possible vaccine candidates.

OMPs have often been identified as highly immunogenic antigens, probably due to being more exposed to the host's immune cells than intra-cellular antigens. Gram-negative bacterial porins are OMPs found in abundance on the cell surface. They form hydrophilic channels that allow restricted entry of required nutrients into the cell (Achouak et al., 2001). Porins have been shown to be highly immunogenic in several hosts including chickens, humans and mice. Bacterial porins have been identified as ~34 kDa in molecular weight (Gomez-Verduzco et al., 2010). Therefore, one of the immunogenic proteins in this study could have represented porins and makes these worthy of further study, as identified by other researchers. To determine passive immunity against *S.* Gallinarum 200 broiler breeder hens were subcutaneously immunized with either 10 µg or 30 µg of *S.* Gallinarum porins. Eggs were then collected and hatched and the chicks were challenged with different doses of *S.* Gallinarum (Gomez-Verduzco et al., 2010). Determination of serum IgG responses showed a significantly higher concentration in immunized hens and their eggs compared to non-immunized ones, showing porins on their own are potent stimulators of the chicken immune response (Gomez-Verduzco et al., 2010). Immunized chicks also had a survival rate of 53 to 70% dependant on the dose of *S.* Gallinarum, whereas all of the chicks in the non-immunized group died after challenge with *S.* Gallinarum (Gomez-Verduzco et al., 2010).

The possibility of using *S.* Typhi porins to immunize humans against typhoid fever has been investigated (Salazar-Gonzalez et al., 2004). Fifteen healthy male volunteers were immunized with *S.* Typhi porins at a concentration of 20 µg. Venous blood was collected before vaccination and 7 and 14 days post vaccination, to determine specific antibody responses and cytokine profiles. An increase in porin-specific IgG and IgM was observed in immunized volunteers at 7 and 14 days post immunization (Salazar-Gonzalez et al., 2004). An increase in IFN-γ at 7 and 14 days post

immunization was also seen, indicating a Th1 response had been induced (Salazar-Gonzalez et al., 2004). These results show *S.* Typhi porins are immunogenic in humans and can stimulate the humoral and cellular immune responses (Salazar-Gonzalez et al., 2004).

Bacterial porins have been shown to react strongly with serum from uninfected chickens, as well as chickens naturally infected with *S.* Enteritidis (Ochoa-Reparaz et al., 2004). As bacterial porins are conserved in many bacterial species this reaction was suggested to be due to immune cross-reaction between *S.* Enteritidis and other enterobacteria the chickens have come into contact with (Ochoa-Reparaz et al., 2004, Simonet et al., 1996). During this study the 34 kDa band reacted with serum from an uninfected control (Data not shown). Due to the conserved nature of porins, this may have been due to exposure of the chicken immune system to gut flora and the antigens they express.

Shigella dysenteriae type 1 porins have been shown to be immuno-stimulatory in mice peritoneal cavity B-2 cells (Ray and Biswas, 2005). *S. dysenteriae* porins were found to up-regulate TLR2 and TLR6 expression on B-2 cells, as well as up-regulate B-2 cell expression of CD80, NF-KB, IgM, IgG2a and IgA (Ray and Biswas, 2005). Overall, research so far has shown bacterial porins to be highly immunogenic and therefore good candidates for vaccine research. Evidence from the literature suggests the 34 kDa protein reacting with chicken serum in this study could be porins. Further investigation of this protein as a potential candidate for a vaccine may therefore be useful.

Microbial HSPs are a highly conserved group of proteins that have been shown to be involved in the pathogenesis of disease and have been shown to be immuno-stimulatory (Lo et al., 2004). High titres of antibodies against Hsp 60 have been found in egg yolks from hens naturally infected with *S.* Enteritidis (Dera-Tomaszewska et al., 2003). The protective role of HSP 60 has been investigated. Following immunization of mice with HSP 60 + Freund's adjuvant, the mice were challenged with *S.* Typhi (Paliwal et al.,

2008). Immunized mice had significantly greater serum IgG following infection, compared to uninfected mice. Immunization with HSP 60 also conferred 70-90% protection against lethal doses of *S.* Typhi or *S.* Typhimurium (Paliwal et al., 2008). Additionally, lymphocyte cells isolated from immunized mice showed significantly greater proliferation and splenocytes showed significantly greater production of IL-4 and IFN-γ, compared to cells from uninfected mice (Paliwal et al., 2008). The identity of the larger protein that reacted with the chicken serum from infected chickens is still uncertain; however, a HSP is a possibility.

OMPs of 82.3 and 75.6 kDa have been shown to be expressed during attachment of *S.* Enteritidis to human intestinal cells (Fadl et al., 2002). Polyclonal antibodies against these 2 OMPs significantly reduce the binding of *S.* Enteritidis to these cells (Fadl et al., 2002). Additionally, infection of chickens with *S.* Enteritidis, following immunization with these OMPs, significantly reduced colonization in the intestine of the chickens (Khan et al., 2003). Therefore the ~80 kDa protein detected in this study could also be fimbriae or another OMP involved in attachment and invasion.

Overall, the data in this study show there is some protection and cross-protection offered by primary infection with *S.* Virchow against secondary infection with *S.* Virchow or *S.* Typhimurium, particularly against invasion of systemic sites. However, the aim of a vaccine is to reduce the spread of infection and the main route of infection for *Salmonella* is faecal-orally. No significant reduction of bacterial load in the caecal contents of group 1 and group 2 were found compared to group 3 and group 4 respectively. This suggests that primary infection with *S.* Virchow does not offer adequate protection or cross-protection against caecal shedding of secondary infection and therefore the spread of infection.

High antibody titres and sometimes significantly high antibody titres were detected in the sera of chickens from group 1 and group 2 compared to the age-matched controls. This shows that during secondary infection with *S.* Virchow, a stronger and quicker antigen-specific immune response is

produced and also the immune system cross-reacts against *S.* Virchow from serogroup C and *S.* Typhimurium from serogroup B. During western blotting, serum from chickens infected with *S.* Virchow strongly cross-reacted against *S.* Typhimurium antigens and vice versa. The cross-reactivity of the chicken immune system against *S.* Virchow and *S.* Typhimurium suggests that there is potential for a multivalent vaccine that will protect against both serogroups. The reactivity and cross-reactivity against ~34 kDa and ~80 kDa proteins suggest these two proteins may be good potential vaccine candidates. However, research of the efficiency of vaccines has so far shown that while they do offer some protection against colonisation, they only succeed in reducing colonisation in the intestine, rather than preventing it. This is possibly due to killed or subunit vaccines not activating all aspects of the immune response (Barrow, 2007). Therefore, to reduce *Salmonella* infection in chicken flocks other methods of prevention such as disinfection programs and rodent control are needed as well as vaccines.

Chapter 7
General Discussion

Although *S.* Virchow is a less common cause of human salmonellosis then *S.* Enteritidis and *S.* Typhimurium, it has increased in prevalence since the 1990s, it has shown increased resistance to antimicrobials and it is associated with invasive disease in humans (Langridge et al., 2009, Matheson et al., 2010, Bertrand et al., 2006, Hopkins et al., 2007). The aims of this study were to compare 12 *S.* Virchow strains isolated in England from different sources, using molecular techniques, to determine their genetic relatedness and to characterise the infection biology of *S.* Virchow *in vitro* using cell invasion assays and *in vivo* in poultry. Of particular importance was the aim to investigate the immune responses produced by poultry as a consequence of *S.* Virchow infection, for the potential of developing a vaccine to control the serovar in poultry and towards the development of multivalent vaccines that protect across all serogroups.

Molecular techniques have been used in several studies to characterise *S.* Virchow in countries where its prevalence is high and it has been shown to be particularly associated with invasive disease (Solnik-Isaac et al., 2007, Weinberger et al., 2006, Bonalli et al., 2011). PFGE results from these studies suggest that *S.* Virchow is a relatively clonal serovar over a widespread area. During this study molecular characterisation of 12 *S.* Virchow isolates from different sources in England was undertaken. PFGE revealed similar findings to those obtained in previous studies in different countries, indicating that *S.* Virchow is a relatively clonal serovar. Additionally, MLST analysis agreed with the PFGE, identifying 11/12 isolates as ST 16. Comparison of the *S.* Virchow isolates from this study to others submitted to the *Salmonella* MLST online database revealed ST 16 as a predominant ST, suggesting that it is widespread throughout Europe. Further analysis of the *S.* Virchow isolates through constructing eBURST diagrams showed ST 16 is likely to have evolved from ST 303, which was identified as the founder ST. As ST 16 is the most prevalent ST this could indicate the evolutionary development from ST 303 to ST 16 could confer an advantage for survival.

Understanding the evolution of infectious agents such as *Salmonella* is important for determining why a strain has become more virulent or resistant to treatment. Horizontal gene transfer of virulence genes or genes conferring antimicrobial resistance can occur amongst bacterial species and can result in epidemics (Bertrand et al., 2006). Another tool used to study the evolution of infectious agents, as well as the biology and mechanisms of host adaptation, is complete genome sequencing. Complete genome sequences are available for a number of *Salmonella* isolates including *S.* Typhi CT18, *S.* Typhimurium LT2, *S.* Pullorum RKS5078, *S.* Enteritidis PT4 isolate P125109 and *S.* Gallinarum 287/91 (Feng et al., 2012, Parkhill et al., 2001, McClelland et al., 2001, Thomson et al., 2008). These serovars represent those that target a broad host range and those that are host-adapted, which has allowed comparisons between the 2 groups to understand how these serovars have evolved to be as they are. Although the host-adapted serovars are adapted to different hosts, they have all evolved to barely colonise the intestinal tract and to cause severe systemic infection (Shivaprasad, 2000). Genomic sequencing and analysis has found that host-adapted serovars possess a significantly higher number of pseudogenes, in addition to insertions and deletions, compared to serovars that can target a broad host range (Thomson et al., 2008, Feng et al., 2012, Parkhill et al., 2001). A large number of pseudogenes known in host-restricted serovars were identified virulence genes and are therefore, thought to account for the main differences in host range between *Salmonella* serovars.

A reference whole genome sequence has been generated by the Wellcome Trust Sanger Institute (Cambridge) for *S.* Virchow, although it has currently not been published (http://www.sanger.ac.uk/resources/downloads/bacteria/salmonella.html). Comparison of the *S.* Virchow genome to other *Salmonella* serovars would provide great insight into the mechanisms of its pathogenesis. Although this study has shown *S.* Virchow to have similar infection biology to *S.* Typhimurium in chickens some differences between the 2 serovars are apparent. Firstly, *S.* Typhimurium is isolated from a wide range of hosts including humans, mice, chickens, pigs, cattle and sheep (DEFRA, 2008c, DEFRA, 2010). *S.* Virchow, although it could cause an

infection in these hosts is rarely reported to be isolated from hosts other than humans and chickens (Bonalli et al., 2011, Fashae et al., 2010). Therefore, although this study has characterised *S.* Virchow as a broad range serovar, it is likely to be more host-adapted than *S.* Typhimurium. Secondly, although *S.* Virchow causes similar infection biology in chickens compared to *S.* Typhimurium F98, it has been shown to be highly invasive in humans and has a significantly higher invasive index compared to *S.* Typhimurium (Langridge et al., 2009, Jones et al., 2008). It would be very interesting to see if *S.* Virchow showed some evidence of genome degradation or an increase in pseudogenes, seen in other serovars, to explain its relative host-adaptation compared to *S.* Typhimurium and its tendency to cause invasive disease in humans.

In vitro experiments using cell lines are used regularly to begin to understand the mechanisms that the pathogen uses to interact with the host. Gene knockout mutants are often used to infect cell lines, to determine if the particular gene has an effect on the virulence of the pathogen, before the mutant is used for *in vivo* experiments (Jones et al., 2001). Therefore *in vitro* experiments can be used to provide a baseline for *in vivo* experiment hypotheses. Throughout this study *S.* Virchow was found to encode 12 virulence genes (*prgH, sopB, sopE, invA, sitC, spiC, sifA, misL, orfL, pipD, iroN* and *pefA*), which have all been associated with adhesion, invasion and persistence in the host (Hughes et al., 2008, Dione et al., 2011, Skyberg et al., 2006). It would be interesting to see if mutants in some of these genes, particularly *pefA* and *sopE* that are absent from some serovars, impair *S.* Virchow in the invasion and persistence assays that were performed in Chapter 3. Additionally, if complete genome sequencing identified any virulence genes present in *S.* Virchow that were absent in *S.* Typhimurium, it would be exciting to see if they affected *S.* Virchow's pathogenesis in these cell models.

The *in vitro* cell models in this study have shown that *S.* Virchow can invade and persist in human and avian cells for at least 24 hours. *In vitro* human cell models are regularly used and are especially useful for attaining an insight

into host-pathogen interactions, as *in vivo* experiments are inevitably very limited (Vo et al., 2007, Shah et al., 2011, Nandakumar et al., 2009, Salazar-Gonzalez et al., 2004). The results from Chapter 3 have shown *S.* Virchow is invasive and persistent in human Caco2 cells. Such behaviours are required to invade the mononuclear-phagocyte system and cause systemic infection. Therefore, these results are consistent with *S.* Virchow's invasive nature in humans. *S.* Virchow infection of Caco2 cells induced a significantly higher inflammatory response compared to *S.* Typhimurium F98 and LPS, although the mechanisms for this remain unclear, as *S.* Typhimurium showed similar levels of invasiveness. Upon identifying potential genes for *S.* Virchow virulence, it would be interesting to see if the any of these mutants affected *S.* Virchow in the Caco2 cell model, where it was shown to be significantly more inflammatory than *S.* Typhimurium F98.

Invasion and persistence of *Salmonella* in avian macrophage cells has been studied widely and is of great interest, as macrophages are part of the innate immune response against the pathogen, but are also an environment *Salmonella* can survive in during systemic and persistent infection (Wigley et al., 2005, Wigley et al., 2002b, Chappell et al., 2009). The results from Chapter 3 show that *S.* Virchow can invade HD11 macrophage-like cells and persist in them; however, counts declined slightly by 24 hours, suggesting the time it can persist in avian macrophage cells may be limited. It may have been useful during this study to do a longer time point, for example cell counts at 48 hours, to further characterise *S.* Virchow's persistence in avian macrophage cells. The concentration of nitrites produced against *S.* Virchow was higher at 8 and 24 HPI than produced against *S.* Typhimurium F98, indicating that *S.* Virchow is inflammatory in avian cells.

Although *in vitro* studies using cell lines have undoubtedly been valuable for studying pathogen-host interactions, *in vitro* experiments have limitations and the necessity of *in vivo* infection models has been highlighted by some studies. An understanding of how *Salmonella* proliferates, spreads and distributes in organs can only truly be determined using *in vivo* infection models, which has recently been achieved using advanced molecular and

bioimaging techniques in combination with mathematical models (Mastroeni et al., 2009). Fluorescence microscopy studying the intracellular distribution of *Salmonella* has shown the infection loci within an organ only consists of a low number of bacteria and following replication the bacteria do not increase in numbers within the loci, they spread to new sites (Sheppard et al., 2003). This distribution pattern of *S.* Typhimurium has been suggested to be a mechanism of evading the immune response, as the distribution occurs in parallel with the escalation of the host immune response (Sheppard et al., 2003). The main mechanism of bacterial growth is thought to be through necrotic cell death of host cells and therefore release of the bacteria into the extracellular space (Sheppard et al., 2003, Mastroeni et al., 2009, Brown et al., 2006). A proposed model in which the burst rate of infected cells is independent of the net growth rate of the bacteria is consistent with this idea of intracellular bacterial distribution (Brown et al., 2006, Mastroeni et al., 2009).

The *in vivo* findings in Chapter 4 show that *S.* Virchow has similar infection biology to *S.* Typhimurium in chickens. *S.* Virchow colonised the caeca at high levels and caused transient systemic infection, although bacterial counts were always slightly lower than *S.* Typhimurium. These results indicate that *S.* Virchow is characteristic of a broad-range *Salmonella* serovar, rather than a host-restricted one, which would cause rapid, severe systemic infection (Shivaprasad, 2000). During infection, the chickens exhibited no clinical symptoms of being unwell, indicating *S.* Virchow and *S.* Typhimurium have low pathogenicity in chickens. However, both *S.* Virchow and *S.* Typhimurium have been shown to be invasive and cause systemic disease depending on the strain and the host they are infecting (Gulig and Doyle, 1993, Kingsley et al., 2009, Sato et al., 2000, Schifferdecker et al., 2009).

Previous studies have used whole genome microarrays to identify genes expressed by *Salmonella* in the intestine of the host and have found that expression is different to that *in vitro* in broth (Dhawi et al., 2011, Harvey et al., 2011). Additionally, expression of genes in the intestine and during systemic invasion by *Salmonella* has been shown to be conserved when the

bacteria invade different species and also to be host-specific (Carnell et al., 2007, Morgan et al., 2007, Morgan et al., 2004). It would be interesting to use whole genome microarrays to determine what virulence genes *S.* Virchow expresses in the chicken intestine during infection and to compare this with expression in other hosts and *in vitro*.

A large body of research has focused on understanding the immune response produced by poultry against *Salmonella* infection; however, the response can vary according to the infecting serovar, the host's age, the host's genetic background and the dose of the infecting serovar, making generalisation of findings impossible (Beal et al., 2005, Berthelot-Herault et al., 2003, Schokker et al., 2012, Setta et al., 2012b). The results in Chapter 5 show that *S.* Virchow stimulates an immune response in 7 day old chickens similar to that caused by *S.* Typhimurium F98, indicating further that *S.* Virchow is characteristic of a broad-range serovar. Evidence of an acute inflammatory response was found in the ileum of chicks infected with *S.* Virchow including, an increase in cytokines IL-1β and IL-6 and an increase in the chemokine CXCLi2 by 5 DPI. Following this, an increase in IFN-γ and an increase in T cells positive for CD4, CD8α, CD8β, MHC II, KuL01 and γδ TCR were found in the ileum, indicating an innate immune response, a T helper as well as a cytotoxic T cell response and an IFN-γ T cell mediated response had occurred. Immune responses were found in the caecal tonsil and systemically in the spleen, which were also representative of an inflammatory immune response and an IFN-γ T cell mediated response. An increase in IFN-γ and lack of IL-4 throughout the experiment suggests the immune response against *S.* Virchow is primarily Th1-mediated rather than Th2-mediated, as shown with other serovars during infection of chickens (He et al., 2011). However, despite the lack of IL-4 expression a strong humoral immune response occurred during *S.* Virchow infection, shown by a classical pattern of a rise in serum IgM, followed by a rise in IgA and IgG. Additionally, staining of the caecal tonsil with the B cell marker Bu1a showed an increase in the number of positive cells during *S.* Virchow infection.

The findings in Chapter 5 present valuable information showing the immune responses produced by chickens against *S.* Virchow and could be built on to enable immunological preventative or therapeutic approaches. Although RT-PCR has the drawback of measuring mRNA expression levels, which may not necessarily correlate with the amount of activate protein, it is a highly sensitive method that can be used widely to reliably quantify avian cytokines and chemokines and could be used further to characterise the immune responses against *S.* Virchow in poultry. IL-17 is a pro-inflammatory cytokine produced by a T cell subpopulation characterised as the Th17 lineage, which is distinct from the Th1 and Th2 lineages (Weaver et al., 2007). IL-17 has been identified in chickens and shares 37 – 46% amino acid sequence identity to the mammalian homologue (Min and Lillehoj, 2002). IL-17 has been shown to be produced in the caecum of chickens following *S.* Enteritidis infection and is thought to contribute to inflammation in the gut during infection and indicates the Th17 arm of the immune response is also involved in the chicken immune response against *Salmonella* (Crhanova et al., 2011). It would be interesting to see if this part of the immune response was also activated following *S.* Virchow infection. Additionally, the array of cytokines measured in this study did not include anti-inflammatory ones. Previous studies have found a rapid decline in inflammatory cytokines, such as IL-1β and IL-6, correlates with an increase in anti-inflammatory cytokines, such as IL-10 and TGF-β_4, which prevent over-expression of pro-inflammatory cytokines that could start to cause harm to the host (Kogut et al., 2003, Withanage et al., 2004, Withanage et al., 2005b).

An understanding of the host's immune response to infectious pathogens, such as *Salmonella*, is necessary to be able to develop effective preventative measures against the spread of infection. Vaccination is seen as the optimum method to control *Salmonella* infection in chickens (Barrow, 2007). Vaccines have been developed that have successfully reduced *S.* Enteritidis contamination of eggs and subsequently a bivalent vaccine has been developed for *S.* Enteritidis and *S.* Typhimurium that has reduced caecal shedding of the serovars (Cogan and Humphrey, 2003, Clifton-Hadley et al., 2002). Since the development of these vaccines, research has focused on

developing one with increased efficacy. A range of virulence factors have so far been selected as potential candidates for a vaccine including ligands of TLRs, SPI structural and effector proteins and OMPs; however, have demonstrated limited efficacy by reducing *Salmonella* colonisation and caecal shedding, but not eliminating the spread of the disease (Kogut et al., 2005, Matulova et al., 2012, Kaneshige et al., 2009).

The experiments in Chapter 6 were designed to determine if primary infection of *S.* Virchow offered any protection against secondary infection with *S.* Virchow or cross-serovar protection against *S.* Typhimurium F98 and to establish the differences in the immune response produced during secondary infection. The results revealed some protection against systemic invasion by both serovars; however, primary infection with *S.* Virchow offered limited or no protection against re-infection with this serovar or *S.* Typhimurium. Measurements of serum Ig revealed a stronger and greater immune response against secondary infection, as well as cross-reactivity between *S.* Virchow antigens and serum from chickens infected with *S.* Typhimurium and vice versa. Western blotting identified 2 proteins ~80 kDa and ~38 kDa that reacted and cross-reacted strongly against serum from infected chickens. It would be interesting to further characterise these proteins and to find out there immunogenic potential. Firstly, to identify what the 2 proteins are, which has been done previously using monoclonal and polyclonal antibodies (Ochoa-Reparaz et al., 2004). Following on from this, it would be worthwhile determining if the 2 proteins elicit any immune responses *in vitro* using avian epithelial cells, before trialling the proteins *in vivo* (Khan et al., 2003, Paliwal et al., 2008). Additionally, it would be of use to establish the T cell mediated immune response against these proteins. Previous studies have focused on the humoral immune response during trials of potential vaccine candidates and while this is useful cell-mediated immunity is considered more important for control of *Salmonella* infection (Barrow, 2007, Gomez-Verduzco et al., 2010).

Currently there is very little information available for *S.* Virchow. Previous studies have focused on the epidemiology of the serovar in countries where it

is more prevalent and have found it to be a particularly invasive serovar in humans (Weinberger et al., 2006, Weinberger and Keller, 2005, Weinberger et al., 2004, Matheson et al., 2010, Ispahani and Slack, 2000, Langridge et al., 2009). Additionally, some research has characterised the resistance of *S.* Virchow to antimicrobials, which is of concern because of its invasive behaviour in humans (Meakins et al., 2008, Bertrand et al., 2006, Ammari et al., 2009, Hopkins et al., 2007, Martin et al., 2001, Solnik-Isaac et al., 2007). The experiments outlined in this study aimed to characterise the genetic relationship of *S.* Virchow strains isolated in England, to determine the infection biology of it *in vitro* in avian and human cell lines and *in vivo* in chickens; to establish the consequent immune responses following infection and to investigate the potential of a vaccine through determining the immune protection offered by primary infection with *S.* Virchow.

S. Virchow isolates in England appear to be closely genetically related, which is consistent with findings in other countries. All of the isolates possess genes associated with increased adhesion, invasion and persistence during infection that are often absent in other serovars and could contribute to the virulence of *S.* Virchow. During *in vitro* experiments, *S.* Virchow stimulated significantly greater IL-8 production by Caco2 cells than *S.* Typhimurium, indicating it is highly inflammatory in human epithelial cells. *In vitro* and *in vivo* experiments consistently showed *S.* Virchow exhibits similar infection biology to *S.* Typhimurium F98 in poultry. *S.* Virchow colonises the intestinal tract to high levels following oral infection of 7 day old chickens. It also causes transient systemic infection shown by the bacterial counts in the spleen and consistent with the HD11 invasion assay, suggesting the ability of *S.* Virchow to persist in macrophage cells is limited. *S.* Virchow stimulated all aspects of the immune system including the innate, humoral and cell mediated responses and caused an increase in pro-inflammatory cytokines, characteristic on a broad-range serovar. Additionally, the immune response was typical of a Th1-mediated one, rather than a Th2-mediated response. Initial findings in Chapter 6 suggest the protection offered by *S.* Virchow against secondary infection is not adequate enough to prevent colonisation of the intestinal tract and caecal shedding of the serovar. However, evaluation

of the humoral immune response showed a strong and fast reaction to secondary infection compared to primary infection and therefore the potential of a vaccine candidate for *S.* Virchow warrants further investigation.

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APPENDIX 1

Salisbury, A. M., Bronowski, C., Wigley. P. (2011). *Salmonella* Virchow isolates from human and avian origins in England – molecular characterization and infection of epithelial cells and poultry. *J Appl Microbiol* **111**(6).