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Identification and characterisation of
Salmonella enterica serovar Typhimurium
factors playing a role in the colonisation of the
porcine gut

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A thesis submitted for the degree of Doctor of Philosophy
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Declaration

I declare that this thesis and the experiments described herein are my own work, except where otherwise stated. No part of this thesis has been submitted for a degree at this or any other university.

J. Elvidge

Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ADP	Adenosine Di-Phosphate
AHVLA	Animal Health and Veterinary Laboratories Agency
AMP	Adenosine mono-phosphate
APC	Antigen presenting cell
ATP	Adenosine Tri-Phosphate
ATR	Acid Tolerance Receptor
BBSRC	Biotechnology and Biological Sciences Research Council
BCR	B cell receptor
BGA	Brilliant Green Agar
BPEX	British Pig Executive
CAP	Catabolite activator protein
CFU	Colony forming units
CSPO	Control of <i>Salmonella</i> in poultry order
CTL	Cytotoxic lymphocytes
CV	Crystal violet
DC	Dendritic cells
DIVA	Differentiating Infected from Vaccinated Animals
DMEM	Dulbecco's Modified Eagle Medium
ECL	Enhanced chemi-luminescence
EFSA	European Food Safety Authority
EHEC	Enterohemorrhagic <i>Escherichia coli</i>
ELISA	Enzyme Linked ImmunoSorbent Assay
EPEC	Enteropathogenic <i>Escherichia coli</i>
ERK	Extracellular-signal-regulated kinases
ETEC	Enterotoxogenic <i>Escherichia coli</i>
FACS	Florescence activated cell sorting
FAE	Follicle Associated Epithelium
FAK	Focal Adhesion Kinase
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FSA	Food Standards Agency
GALT	Gut associated lymphoid tissue
GAP	GTPase-Activating Protein
GEF	Guanine Exchange Factors
GLM	General linear model
GTPase	Enzyme that hydrolyses guanosine triphosphate
HBSS	Hanks Balanced Salt Solution
H-NS	Histone-like nucleoid-structuring protein

HPA	Health Protection agency
HRP	Horse radish peroxidase
IcLN	Ileocecal lymph nodes
IFN	Interferon
Ig	Immunoglobulin
IGHD	Ig heavy diversity
IGHJ	Ig heavy junction
IGHV	Ig heavy variable
IL-	Interleukin
IM	Intramuscular
iNOS	Inducible nitric oxides
Ipaf	Ice protease activating factor
KIR	Killer cell Ig-like receptor
LB	Luria Bertani
LPS	Lipopolysaccharide
LS	Low shedding
M cell	Microfold cell
MAPK	Mitogen Activated Protein Kinase
MCP	Monocyte chemotactic protein
MEM HEPES	Minimum essential media with 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
MHC	Major histocompatibility complex
MIP	Macrophage Inflammatory Protein
MLN	Mesenteric lymph nodes
MOI	Multiplicity of infection
MyD88	Myeloid Differentiation factor 88
Nal ^r	Naladixic acid resistant
NCP	National control plan
NF	Nuclear Factor
NK	Natural killer
NLR	Nod like receptor
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
PAMP	Pathogen Associated Molecular Pattern
PBS	Phosphate buffered saline
PBST	PBS Tween
PC	Principal components
PFA	Paraformaldehyde
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear
PP	Peyer's patches

PR-39	Peptide regulator 39
PRR	Pattern recognition receptor
PRRSv	Porcine reproductive and respiratory virus
PS	Persistent shedding
QPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROI	Reactive oxygen intermediates
RpoA	RNA polymerase subunit
RT	Room temperature
RV	Rappaport-Vassiliadis
<i>S. Typhimurium</i>	<i>Salmonella enterica</i> subspecies <i>enterica</i> serovar Typhimurium
SCV	<i>Salmonella</i> Containing Vacuole
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide electrophoresis
SE	Standard error of the mean
SEM	Scanning Electron Microscopy
SIP	<i>Salmonella</i> Invasion Protein
SLA	Swine leukocyte antigen
SOP	<i>Salmonella</i> Outer Protein
SPI	<i>Salmonella</i> pathogenicity island
SV40	Simian virus 40
T3SS	Type three secretion system
TCR	T cell receptor
TEER	Trans-epithelial electrical resistance
Tfh	T follicular helper cells
TLR	Toll-Like Receptor
TNF-	Tumour necrosis factor
TPS	Two-Partner Secretion
V(D)J	Variable Diversity Junction
VLA	Veterinary Laboratories Agency
WAS-	Wiskott-Aldrich syndrome
WHO	World Health Organisation
WRS	Wilcox rank sum
WSR	Wilcox signed rank
WT	Wild-type
ZAP	Zoonosis Action Plan
ZNCPig	Zoonosis National Control Plan for <i>Salmonella</i> in pig meat

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Abstract

Salmonella is an important food borne pathogen. Over 100,000 cases of human *Salmonella* infection are reported in the European Union each year, resulting in an economic burden estimated to be around 3 billion Euros per year (EFSA, 2012). In a European Food Safety Authority (EFSA) survey between 2006 and 2007 *S. Typhimurium* was the most common serovar of *Salmonella* isolated from pig carcasses (EFSA, 2008a). Pigs can be asymptomatic carriers of *S. Typhimurium* (Berends et al., 1996) and contaminated pork contributes significantly to the number of human infections. It has been estimated that the porcine *Salmonella* reservoir contributes between 10-20% of human salmonellosis cases per year (VLA, 2010).

In addition to improvements in biosecurity and husbandry practices, immune-prophylaxis is an important method to reduce the prevalence of food borne pathogens such as *Salmonella* in reservoir species. An understanding of the molecular basis of bacterial colonisation and persistence in the reservoir host is crucial to rational vaccine design and targeting relevant species. *S. Typhimurium* expresses multiple surface factors involved in adherence and colonisation of gut epithelium in several host species. The aim of this project was to identify factors involved in *S. Typhimurium* colonisation of the porcine gut. The work presented here specifically focuses on the role of flagella in the colonisation of porcine gut epithelium.

Flagella are motility organelles possessed by many bacterial species. Flagella can also function as surface adhesins, shown in *Escherichia coli* O157:H7 (Mahajan et al., 2009), and *Pseudomonas* (De Bentzmann et al., 1996, Lillehoj et al., 2002). Flagellin is the major flagellar filament structural protein approximately 50kDa in size. *Salmonella enterica* has the ability to switch between two alternate, antigenic forms of its flagellin filament protein, expressing either FliC or FljB (Macnab, 1996). The biological relevance of these two types of flagella filament protein is still not understood. It has been postulated that the presence of a second phase type of flagella may offer an advantage to the bacteria by avoiding recognition by the immune system. However, studies have shown that both FliC and FljB flagella activate Toll-

like receptor-5 (TLR-5) mediated by nuclear factor (NF)- κ B signalling (Simon and Samuel, 2007b).

One specific objective of this research was to compare the role of flagellar phase types in *S. Typhimurium* adherence and colonisation of porcine gut. To this end a porcine colonic primary epithelial cell culture and *ex vivo* tissue explants were developed as *in vitro* infection models. Primary colonic cell cultures were phenotypically characterised using specific markers for epithelial and M cells. In addition to primary epithelial cell culture, porcine intestinal epithelial cell line, IPEC-J2, was also used for specific flagellar interaction studies.

The role of flagella in interaction of *S. Typhimurium* to porcine intestinal epithelium was tested using *S. Typhimurium* strain SL1344 and flagella mutant derivative strains. Flagella mutant strains exhibited reduced binding to porcine intestinal epithelial cells. Purified flagella proteins were also shown to bind porcine intestinal epithelial cells. Moreover, flagella specific anti-sera suppressed *S. Typhimurium* adherence to both porcine intestinal epithelial cells as well as porcine colonic explants.

The immuno-protective role of flagella as a potential *S. Typhimurium* vaccine candidate was tested during vaccine efficacy studies in pigs. Parenteral immunisation of pigs with purified FliC and FljB flagella proteins induced production of both IgG and IgA antibodies. The vaccination of pigs with *Salmonella* flagella provided some protection against challenge as fewer ileum tissue samples from the pigs in the vaccinated group tested positive for *Salmonella*. The intestinal contents from the vaccinated pigs tested for *Salmonella* post mortem appeared to also have lower levels of *Salmonella* compared to un-vaccinated controls, though these were not significantly different between groups.

This project has identified flagella as one potential subunit of a multivalent subunit vaccine to help control salmonellosis in the porcine reservoir.

1. Introduction

1.1. Food-borne pathogens

A study by Mead et al 1999 compiling evidence from several sources, estimated that foodborne pathogens account for approximately 76 million illnesses, 325,000 hospitalisations and 5,000 deaths in the United States each year (Mead et al., 1999). The same study showed that pathogens *Salmonella*, *Listeria* and *Toxoplasma* accounted for more than 75% of food-related deaths per year (Mead et al., 1999). In the European Union, over 100,000 cases of human *Salmonella* infection are reported each year. The resulting economic burden is estimated to be around 3 billion Euros per year (EFSA, 2012).

Salmonella enterica subspecies *enterica* serovar Typhimurium is one serovar widely associated with human infections. *S. Typhimurium* is most commonly transmitted to humans from contaminated animal products. Disease symptoms include fever, abdominal pain, diarrhoea, nausea, and sometimes vomiting. However, in young children, the elderly and people with compromised immune systems the disease can be life threatening (WHO, 2005). Epidemiological data from the Health Protection Agency (HPA) shows that in England and Wales the most common *Salmonella* serovars isolated from human infections between 2000 and 2010 were Enteritidis and Typhimurium (Figure 1.1).

1.2. *Salmonella enterica* subspecies *enterica* serovar Typhimurium

Salmonella is a gram negative bacterium with over 2500 identified serovars. *Salmonella* species are important food borne pathogens that cause gastroenteritis and enteric fever across a broad range of hosts. The genus *Salmonella* consists of 2 species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* represents a group of 6 subspecies with multiple serovars shown in Table 1.1. Typhimurium is one serovar within *Salmonella enterica*.

Table 1.1. *Salmonella* subspecies and serovars (Grimont and Weill, 2007).

Species	Subspecies number	Subspecies name	Number of Serovars
<i>S. enterica</i>			2557
	I	<i>enterica</i>	1531
	II	<i>salamae</i>	505
	IIIa	<i>arizonae</i>	99
	IIIb	<i>diarizonae</i>	336
	IV	<i>houtenae</i>	73
	VI	<i>indica</i>	13
<i>S. bongori</i>			22
Total number of serovars			2579

Salmonella enterica subspecies *enterica* serovar Typhimurium (*S. Typhimurium*) is one serovar most widely associated with cases of human infection. Disease symptoms usually develop after between 12 and 72 hours after infection in humans. Infected individuals may develop a fever, abdominal cramps, and diarrhoea. Infection typically lasts between 4 and 7 days and the majority of people recover without the need for medical intervention. However, some individuals may require hospitalisation due to severe dehydration resulting from diarrhoea (WHO, 2012, CDC, 2012).

1.3 Livestock reservoirs and transmission of *Salmonella* species

Some *Salmonella* serovars are host restricted and may only infect one or two closely related species. For example, *S. Choleraesuis*, *S. Dublin* serotypes are rarely associated with species other than pigs and cattle, respectively. Data collected between 1958 and 1967 indicated that 99% of *S. Choleraesuis* cases detected were associated with pigs and 95% of *S. Dublin* cases detected were associated with cattle (Wallis and Barrow 2005). Host restricted strains like *S. Choleraesuis* and *S. Dublin* typically result in systemic disease. However, serovars *S. Typhimurium* and *S. Enteritidis* are not as host restricted and result in gastroenteritis in a broad range of unrelated hosts (Smith and Halls, 1968, Wallis, 2005).

1.3.1. Salmonellosis in Poultry

Salmonella enterica serovars Gallinarum and Pullorum cause fowl typhoid and pullorum disease respectively. These are systemic diseases of chickens and turkeys primarily but can also affect game birds. These serovars are host restricted and tend to only cause disease in birds. Following routine vaccination and strict control measures *S. Gallinarum* and *S. Pullorum* have largely been eradicated from the poultry industry of the Western world. However, these serovars still pose a threat to welfare and health of poultry in countries with less developed bio-security (Wallis, 2005). Epidemiological studies between 1968 and 1973 revealed that >40% of *Salmonella* isolations from poultry in the UK were *S. Typhimurium* (Wallis, 2005). In the 1980's however, serovar Enteritidis phage type 4 emerged as the predominant serovar. Zoonoses Regulation 2160/2003, came into force in December 2003. This order aims to reduce the prevalence of specific zoonotic infections at the production level, by requiring the implementation of species-specific *Salmonella* National Control Programmes (NCPs). Control of *Salmonella* in Poultry Orders (CSPO) implement this EC regulation in the UK and require producers to carry out routine testing for *Salmonella* at defined time points and in a specific manner as dictated by the CSPO.

The introduction of *Salmonella* NCPs and active immunisation of layer hens against *S. Enteritidis* in the UK which was introduced in 1998 and has resulted in a reduction of *Salmonella* isolates from poultry in the last few years (Wallis, 2005, HPA, 2011, AHVLA, 2011).

Data from the AHLVA 2011 *Salmonella* in livestock report describes 0.09% (1/1107) of poultry breeding flocks testing positive for a regulated *Salmonella* serovar. This is well within the limits set by the EC 2160/2003, which is 1%. Regulated serovars include the five most frequently isolated from human cases of salmonellosis. In 2003 these were: *S. Enteritidis*, *S. Typhimurium*, *S. Virchow*, *S. Hadar*, and *S. Infantis*.

Figure 1.1 shows the number of human cases of *S. Enteritidis* and *S. Typhimurium* between 2000 and 2010 in England and Wales (HPA, 2011). The number of *S. Enteritidis* cases has fallen. However, the number of *S. Typhimurium* cases has remained approximately the same despite the low number of *Salmonella* isolations from poultry.

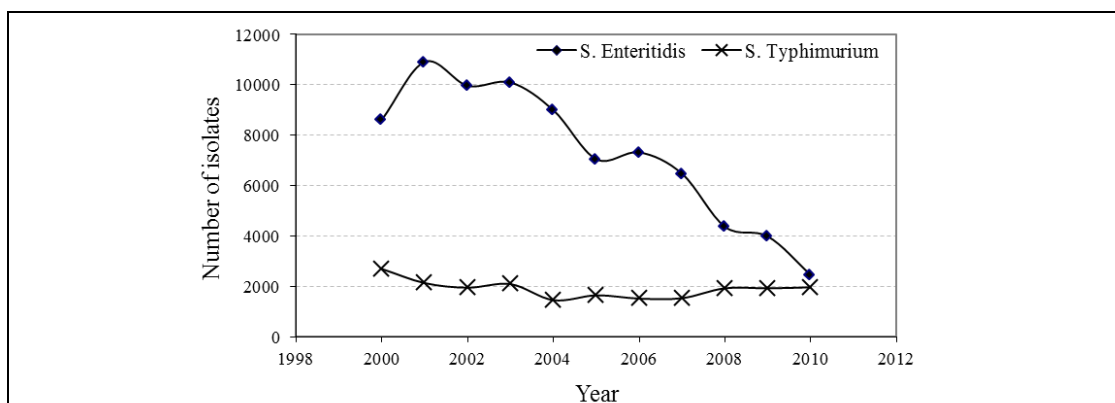


Figure 1.1. Human isolates of *S. Enteritidis* and *S. Typhimurium* (HPA, 2011)

The figure shows the number of human isolates of *S. Enteritidis* and *S. Typhimurium* between 2000 and 2010 in England and Wales (HPA, 2011). The number of *S. Enteritidis* cases has fallen possibly due to the introduction of poultry vaccines in 1998. The number of *S. Typhimurium* isolates however has remained approximately the same.

1.3.2. Salmonellosis in Cattle

Salmonellosis causes pyrexia, anorexia, reduced milk yield and diarrhoea. *Salmonella* serovars, *S. Dublin* and *S. Typhimurium* are the predominate causes of salmonellosis in cattle. *S. Dublin* results in systemic disease, which in pregnant cows generally results in abortion. However, most *S. Dublin* cases are encountered in young calves. In the 1960s salmonellosis in cattle reached a peak with >4000 incidents in 1969 (Sojka et al., 1977). Information from the AHVLA has identified *S. Dublin* as the most common serovar in cattle for 13 years in succession in the UK. In 2011, 712 *Salmonella* incidents were recorded by the AHVLA, 65% of which were *S. Dublin*, 12.9% were *S. Mbandaka* and 7.9% were *S. Typhimurium* (AHVLA, 2011).

S. Typhimurium infection can cause severe dehydration and anaemia in cattle as a result of severe gastroenteritis. Within the serovar *S. Typhimurium* a small number of strains predominate which are multi-drug resistant: PT29 in the 1960s, 204c in the 1990s and DT104 at present which are resistant to chloramphenicol, streptomycin, sulphonamides and tetracyclines. The strain DT104 is also associated with human infection (CDC, 2002).

While cattle infected with *Salmonella* display symptoms of disease such as diarrhoea, animals may be carrying the organism without displaying clinical symptoms. These are referred to as active carriers. The active carriage of *Salmonella* occurs before clinical enteritis or systemic infection. These infected animals may secrete *Salmonella* for years or even life. *Salmonella* may be excreted by these active carriers' continuously in concentrations greater than 10^5 CFU/g of faeces and thus can be detected by routine bacteriological examination. Cattle exhibiting clinical symptoms of salmonellosis can excrete up to 10^8 CFU/g of faeces. These animals contaminate the surrounding environment potentially causing infection of other livestock in the area. The most likely route of infection is orally but infection may also occur via the respiratory tract or conjunctiva (Wallis, 2005).

There is no statutory testing for *Salmonella* in cattle and so the only submissions to the AHVLA for *Salmonella* testing came from clinical cases in 2011 (AHVLA, 2011). This makes it difficult to judge the prevalence of *Salmonella* serovars present in this reservoir species. Serovar Dublin results in clinical symptoms, resulting in testing and as a result is reported as the predominant serovar. However, *Salmonella* serovars which do not result in clinical symptoms will not be detected, so it is possible other *Salmonella* serovars are present, though remain undetected.

1.3.3. Salmonellosis in Pigs

Pigs are affected by a host restricted serovar of *Salmonella*, *S. Choleraesuis* which results in systemic disease. In the 1950's and 1960's *S. Choleraesuis* was the predominant serovar isolated from pigs (Sojka et al., 1977). Now *S. Choleraesuis* is only isolated infrequently (Wallis, 2005). None of the *Salmonella* positive samples from pigs sent to the VLA for serotyping in 2011 were *S. Choleraesuis* (AHVLA, 2011). However, *S. Choleraesuis* remains a problem in the US and its decline in the UK was not due to any specific prevention measure (Wilcock, 1992). The disease symptoms of *S. Choleraesuis* include lethargy, pyrexia and respiratory symptoms including coughing. The mortality is high with infection of this serovar. *S. Choleraesuis* is unusual among host-restricted serovars as it had the ability to cause disease in pigs, calves, rabbits, guinea pigs as well as humans, which is a relatively wide range of animals.

In an EFSA survey between 2006 and 2007, 87 different serovars of *Salmonella* were found to affect pigs across Europe. *S. Typhimurium* was the most common serovar isolated with ~40% of positive pigs infected with *S. Typhimurium* (EFSA, 2008a).

S. Typhimurium typically causes disease in pigs aged between 6 and 12 weeks and rarely in adult pigs. Older animals frequently have subclinical infections that result in high transmission rates to surrounding animals if not detected. The initial clinical signs of infection include watery diarrhoea. Pigs become anorexic, lethargic, and febrile following infection though mortality is usually low. The mesenteric lymph nodes are usually swollen and intestinal lesions may be present as red, rough mucosal surfaces having grey/yellow debris. The contents of the colon and caeca are

bile stained often having black gritty material on the mucosal surface. In addition, intestinal necrosis might be observed, often associated with resolving lesions appear as sharply delineated button ulcers (Wallis, 2005, Wray and Wray, 2000).

Infection of pigs with *Salmonella* is mainly via the faecal oral route. Although, intranasal as an alternative route has been identified. *S. Typhimurium* has been isolated from both lungs and tonsils (Wood et al., 1989).

Studies have shown that isolation of *Salmonella* from pigs is 3-10 times higher post transport to abattoir compared to on farm (Hurd et al., 2002). It has been postulated that increased stress in pigs during transport induces *Salmonella* shedding (Hurd et al., 2002). In support of this hypothesis, studies using 6-hydroxydopamine to induce exogenous release of norepinephrine increased *Salmonella* shedding in pigs (Pullinger et al., 2010).

As part of the World Health Organisation (WHO) millennium goals the UK food standards agency (FSA) has aimed to reduce the number of *Salmonella* food-borne infection cases (Regulation (EC) No 2160/2003). To monitor prevalence of *Salmonella* in pigs in the UK the Zoonoses Action Plan (ZAP), *Salmonella* Programme was launched in June 2002. The overall aim of ZAP is to promote and enhance the safety of British pig meat through integrated action along the entire food-chain. The specific objective of the *Salmonella* Programme is to monitor trends in the levels of *Salmonella* on British pig farms so that appropriate actions can be taken to reduce the prevalence of *Salmonella* in pigs presented at abattoirs and therefore reduce risk to human population. The *Salmonella* action plan has recently been updated to the Zoonoses National Control plan for *Salmonella* in Pigs (ZNCP). This is now putting more focus on the individual farm and putting measures in place to deal with *Salmonella* control on an individual farm basis (BPEX, 2012).

Salmonella Typhimurium, VacT is a live attenuated vaccine strain primarily used to control *Salmonella* infection of poultry (Lohmann, Animal Health). Vaccination of pigs with this strain has also been shown to have some efficacy (Roesler et al.,

2004a). VacT immunisation protected piglets against colonisation and clinical salmonellosis, along with a significant decrease in colonisation of tissues and inner organs, as well as the shedding of *S. Typhimurium* (Roesler et al., 2004b). Vaccination of source animals is viewed as a key strategy to reduce *Salmonella* in pigs. An effective vaccine would limit initial colonisation and subsequently restrict *Salmonella* levels in food animals. Understanding *Salmonella* interaction with host cells may help identify factors important for *Salmonella* adherence and invasion. Once identified, characterised immunogenic factors could form part of a multivalent vaccine.

1.4. *Salmonella* infection of the host

Typical infection with *Salmonella* occurs via the oral route. *Salmonella* bacteria infect cells of the gastrointestinal tract of the host. However, *Salmonellae* must endure very challenging conditions to get to the intestine of the host, such as the low pH of the stomach, which in humans is as low as 1.5, high concentrations of bile salts, osmolytes, commensal bacteria metabolites, and low oxygen tensions (Álvarez-Ordóñez et al., 2011).

Salmonella have developed survival strategies to cope with these challenging conditions including an acid tolerance response (ATR). The ATR is directed by several mechanisms that help the bacteria survive the drastic change to low pH in the stomach of the host. The intracellular pH of gram negative bacteria is pH 7.6-7.8. This pH is maintained by pumps that remove protons from the cytoplasm in a low pH environment (Foster et al., 2000). In addition, *Salmonellae* use inducible lysine decarboxylase and arginine decarboxylase systems to increase intracellular pH. Following external cues indicating low external pH *Salmonellae* activate transcription of the *cadBA* operon. In the presence of lysine, the CadA enzyme converts lysine to cadaverine with the consumption of a proton. The CadB antiporter exports cadaverine in exchange for extracellular lysine. Thus the intracellular pH is increased (Álvarez-Ordóñez et al., 2011). Furthermore, *Salmonella* produce acid shock proteins to prevent/repair damage as a result of acid stress. The alternative sigma factor RpoS has been shown to control expression of at least 10 acid shock

proteins. Most acid shock proteins are involved in; cell regulation, molecular chaperoning, energy metabolism, transcription, translation, fimbriae synthesis, regulation of cell envelopes, colonisation and virulence (Álvarez-Ordóñez et al., 2011, Foster, 2000).

Other adaptations to acid conditions include modification of the bacterial membrane. *Salmonella* change the fatty acid composition of the membrane in response to low pH. Exposure to acid pH has been shown to result in a decrease of unsaturated fatty acids and increase in saturated fatty acids and cyclic fatty acids (Álvarez-Ordóñez et al., 2011).

The role of bile and bile salts in the digestive tract serves to digest lipids. *Salmonella* have developed mechanisms by which to resist digestion in bile. The shortening or loss of O antigen, results in a rough colony phenotype and increased bile sensitivity, indicating a role for LPS in resistance to bile (Álvarez-Ordóñez et al., 2011).

The lumen of gastrointestinal tract has a relatively high salt concentration, 0.3M NaCl. This increase in salt concentration might result in osmotic shock. *Salmonella* react to changes in osmolarity by stimulating uptake of potassium (and glutamate). This is followed by dramatic increase in the cytoplasmic concentration of so-called compatible solutes (Álvarez-Ordóñez et al., 2011).

Oxygen availability in the large intestine decreases and is largely anaerobic. *Salmonella* possesses cytoplasmic oxygen sensor, Fnr. Fnr binds promoter sequences and interacts with RpoA (RNA polymerase subunit), these interactions result in increased efficiency of transcription of genes important for anaerobic metabolism (Álvarez-Ordóñez et al., 2011).

The gastrointestinal tract is colonised by commensal microorganisms. *Salmonella* must compete with commensal bacteria to gain access to the epithelia. In addition, these commensal microorganisms produce metabolites such as bacteriocins and short chain fatty acids with anti-*Salmonella* activity. The specific mechanisms by which

Salmonella escapes destruction by bacteriocins has not yet been elucidated (Álvarez-Ordóñez et al., 2011).

Antimicrobial peptides such as defensins are encountered by *Salmonella* in the small intestine. Alternative sigma factor σ^E from *Salmonella* has been shown to increase resistance to killing by bactericidal/permeability-increasing protein derived peptide P2 and murine α -defensin cryptdin-4 and it is possible that σ^E provides resistance to killing by other antimicrobial peptides as well, such as the bacteriocins produced by commensal microorganisms, though this has yet to be tested (Álvarez-Ordóñez et al., 2011).

The specific mechanisms resulting in porcine *Salmonella* enteritis are not well understood. The majority of studies have focused on murine and bovine models of infection as reviewed by Tsohis et al 2011. However, it is generally accepted that the initial site of *S. Typhimurium* infection occurs in the distal small intestine (Ly and Casanova, 2007). *Salmonella* have been shown to enter and survive within enterocytes, M-cells and dendritic cells. In the mouse model of infection it has been shown that *S. Typhimurium* preferentially invades M-cells (Jones et al., 1994). Jones et al. showed that after 30min infection in ligated loops, *Salmonella* was found exclusively inside M-cells using transmission electron microscopy. However, evidence from cattle (Santos et al., 2002) and pigs (Reed et al., 1986, Bolton et al., 1999) showed no evidence of an M-cell to enterocyte preference. Santos et al. showed that in neonatal calves (ligated loops) infection of enterocytes and M-cells began within 15min, as both cell types contained *Salmonellae*. Bolton et al. recovered comparable numbers of *Salmonella* from both the Peyer's patches (PP) and the absorptive mucosae, 3h post infection of porcine ligated ileal loops.

In mice infection with *S. Typhimurium* results in systemic infection. In cattle and pigs however, *S. Typhimurium* does not disseminate systemically and remains in the gastrointestinal tract. It is possible the apparent preference for M-cells in the murine host is related to *S. Typhimurium* becoming systemic in this host. More studies will be required to elucidate the reasons for M-cell preference in the murine host.

Much is known about *Salmonella* enterocyte invasion, and the *Salmonella* effector proteins resulting in uptake, this is discussed in detail in section 1.4.1. However, less is known about their invasion of and survival within Microfold cells (M-cells) and dendritic cells, discussed in sections 1.4.3 and 1.4.5 respectively.

1.4.1. *Salmonella* infection of enterocytes

Enterocytes make up the majority of cells of the intestine. Their primary function is to absorb nutrients from the intestinal lumen. Enterocytes possess at the apical surface a covering of microvilli which are rigid and closely packed (Mooseker, 1985). These microvilli increase the surface area over which nutrients can be absorbed (Snoeck et al., 2005).

Salmonella induce their own uptake into enterocytes, and invade non-phagocytic cells by manipulating the host actin cytoskeleton, resulting in intense membrane ruffling and the formation large macropinosomes. *Salmonella* achieve this using a range of effector proteins injected via the type three secretion system (T3SS) encoded by *Salmonella* pathogenicity island 1 (SPI-1) (Ly and Casanova, 2007, Hernandez et al., 2004). Following internalisation the host cell membrane returns to normal and *Salmonella* reside within membrane bound vesicles termed *Salmonella* containing vacuoles (SCV). These vacuoles support bacterial replication. SCVs are modified by the bacteria to prevent maturation into or fusion with lysosomal compartments thus providing them with a protective environment in which to replicate (Hernandez et al., 2004, Ly and Casanova, 2007).

1.4.2. Molecular effectors involved in actin dynamics of enterocyte invasion

Salmonella entry into epithelial cells relies on the bacterium inducing macropinosome formation by actin re-organisation. *Salmonella* effector protein SipC is involved in actin re-organisation. The C-terminal cytoplasmic domain of SipC nucleates the assembly of actin filaments, leading to the rapid filament growth from the barbed ends. The N-terminus of SipC promotes actin assembly by bundling and

crosslinking existing actin filaments (Hayward and Koronakis, 1999). Effector protein SipA forms a complex with T-plastin increasing its actin-bundling activity. It has been shown that T-plastin is recruited to membrane ruffles and it is thought that the actin bundling activity by SipA and T-plastin is required for efficient bacterial entry (Zhou et al., 1999a). SipA also increases the nucleating activity of the C-terminal domain of SipC (McGhie et al., 2001) and binds to assembled filaments, preventing ADF/cofilin-mediated depolymerisation (McGhie et al., 2004). Furthermore, SipA protects actin filaments from fragmentation induced by the filament-severing protein gelsolin. SipA can even re-anneal severed filaments produced in the presence of gelsolin (McGhie et al., 2004). Structural studies have shown that SipA can act as a 'molecular staple' using two extended 'arm' domains to tether actin monomers on adjacent strands (Lilic et al., 2003). Although SipA is not required for bacterial internalisation, it has been shown to enhance its efficiency (Zhou et al., 1999b). Cells that were infected with SipA-deficient strains exhibited less focal membrane ruffling. This might indicate that actin polymerisation is no longer restricted to sites of bacterial attachment (Zhang et al., 2002). Zhang et al also showed that the pathogenicity of SipA-deficient strains in bovine infection models is attenuated (Zhang et al., 2002). SipA and SipC are homologous to invasion proteins IpaA and IpaC in *Shigella* (Kaniga et al., 1995) though they have no sequence homology to any known eukaryotic actin binding proteins (Ly and Casanova, 2007).

For the formation of the phagocytic apparatus required for bacterial internalisation branched and interconnected actin networks are required. The production of these networks is mediated by eukaryotic proteins, small GTPases of the Rho family, mainly Rac and cdc42 (Jaffe and Hall, 2005). When activated, Rac and cdc42 bind to and stimulate distinct arrays of downstream effector proteins which drive actin cytoskeleton assembly. The activation of Rac in most cells leads to formation of membrane ruffles and lamellipodia. The activation of cdc42 triggers the formation of filopodia (Jaffe and Hall, 2005). *Salmonella* produces effectors, SopE and SopE2 (*Salmonella* outer proteins E and E2) that target Rac and cdc42. These bacterially-encoded guanine-exchange factors (GEFs) activate Rac and cdc42 resulting in the formation of membrane ruffles (Hardt et al., 1998, Stender et al., 2000). SopE and

SopE2 show no sequence homology to eukaryotic GEFs but do use similar mechanisms to catalyse the nucleotide exchange to eukaryotic GEFs (Buchwald et al., 2002, Schlumberger et al., 2003).

Another strategy of *Salmonella* to induce actin rearrangements is using SopB (also known as SigD). Strains lacking SopE and SopE2 induce membrane ruffling using SopB (Zhou et al., 2001). SopB seems to lack GEF activity; instead it contains a domain related to eukaryotic phosphatidylinositol phosphatases. Phosphoinositides are important in vesicular trafficking. The modulation of phosphoinositides may facilitate *Salmonella* entry into host cells. Furthermore, SopB was found to be essential for formation and maintenance of SCVs. Actin reorganisation requires Rho family GTPases activity (Zhou et al., 2001) but as SopB has no GEF activity, SopB must activate Rho GTPases indirectly.

The Arp2/3 complex was found to be essential for efficient bacterial internalisation (Criss and Casanova, 2003). Arp2/3 is a heptameric complex involved in the formation of branched actin filament networks. In addition, Arp2/3 can initiate new assembly, introduce branching points into growing filaments, and cross link filaments to each other (Goley and Welch, 2006). The incorporation of Arp2/3 into growing filaments requires catalysation by members of the Wiskott-Aldrich syndrome family (WASP, N-WASP, Scar/WAVE1, 2 and 3), cortactin and IQGAP1 (Takenawa and Suetsugu, 2007). These are activated by members of the Rho family of GTPases. Cdc42 binds directly to WASP and N-WASP to activate them. WAVE proteins are activated indirectly by Rac. WAVE2 is thought to exist as part of a multimeric protein complex containing Abelson interacting protein 1 (Abi) and Nck-associated protein (Nap1), HSPC300 and a Rac-binding subunit PIR121 (Stradal and Scita, 2006). Rac is usually associated with membranes and thus WAVE2 complexes are recruited to sites of Rac activation by PIR121. This promotes actin assembly at or near the membrane (Stradal and Scita, 2006). Activation by *Salmonella* of the GTPases required for downstream activation of the Arp2/3 complex is another mechanism by which *Salmonella* might influence the actin cytoskeleton.

Focal adhesion complexes have an important role to play in *S. Typhimurium* entry into host cells (Shi and Casanova, 2006). Focal adhesions are a complex assembly of proteins mediating a physical link between integrins and the actin cytoskeleton. In addition, focal adhesions also transmit integrin-dependent signals to the cell interior. When cultured epithelial cells were infected with *S. Typhimurium* at the apical pole (typically devoid of integrins) the assembly of focal adhesion like structures was induced at the apical plasma membrane. It was shown using cells from genetically deficient mice, that focal adhesion kinase (FAK) and the scaffolding protein p130Cas are necessary for bacterial internalisation. The C-terminal of FAK is a proline rich domain that mediates interaction with p130Cas and has been shown to be required for bacterial internalisation (Shi and Casanova, 2006).

The inositol phosphatase activity of SopB aids the entry of bacteria into the host cell by promoting phagosome closure. This is achieved by modulating the membrane phosphoinositide levels in the vicinity of the attached bacteria. A local reduction in PtdIns(4,5)P₂ leads to dissociation of cortical actin for the nascent phagosome and an increase in membrane fluidity that allows phagosome closure (Botelho et al., 2001).

Following internalisation of *Salmonella*, the membrane of the host cell quickly returns to normal. This process is mediated in part by SptP. SptP has two distinct catalytic modules, an N-terminal RhoGAP domain and a C-terminal tyrosine phosphatase domain (Kaniga et al., 1996). The GAP domain of SptP mimics eukaryotic RhoGAPs in its overall structure and catalytic mechanism (Stebbins and Galan, 2000). SptP targets the tyrosine kinase ACK and the intermediate filament protein vimentin. ACK is a downstream effector of cdc42 and has been shown to stimulate ERK activation in *Salmonella* infection (Murli et al., 2001). Another potential target for SptP could be p130Cas which becomes transiently tyrosine phosphorylated in response to *Salmonella* infection (Shi and Casanova, 2006). It is the GTPase activity of SptP which mediates the cytoskeletal changes induced by SopE.

SopE/E2 and SptP antagonise each other's function, yet are injected simultaneously into the host cell. SopE is degraded within 30 minutes of infection by a ubiquitin/proteasome-mediated mechanism. In contrast SptP persists in the cytosol for several hours post infection (Kubori and Galan, 2003). SopB also undergoes ubiquitination in host cells but this does not lead to proteasome mediated degradation (Marcus et al., 2002).

Effector proteins; AvrA, SipA, SipB, SipC, SipD, SlrP, SopA, SopB/SigD, SopD, SopE, SopE2, SptP and SspH1 are all secreted through SPI-1 encoded T3SS (Galan, 1996). Six of these effectors have been shown to regulate the actin cytoskeleton either directly or indirectly. Furthermore, these effectors have homologues in other pathogens such as *Shigella* and *Yersinia* (Ly and Casanova, 2007). Effector proteins SopB, SopE, SopE2 and SipA have been shown to be responsible for the disruption of the structure and function of tight junctions (Boyle et al., 2006). Table 1.2 summarises the function of some of the effector proteins found in *Salmonella*.

Table 1.2. *Salmonella* virulence factors

Gene	Function	References
SipA	Binds F-actin inhibiting polymerisation - enhancing efficiency of SipC.	(Zhou et al., 1999a)
SipB	Induction of apoptosis - translocated via SPI-1 T3SS - binds & activates caspase-1, an intracellular cysteine protease also known as IL-1 β converting enzyme.	(Hersh et al., 1999)
SipC	Acts as translocase - bundles actin filaments and nucleates actin polymerisation <i>in vitro</i> , which results in cytoskeletal rearrangements <i>in vivo</i> .	(Hayward and Koronakis, 1999)
SopA	Involved in the induction of neutrophil transepithelial migration	(Wood et al., 2000)
SopB	Encoded by SPI-5, dependent on SirA, inositol phosphate phosphatase that hydrolyses phosphatidylinositol 3,4,5-triphosphate, an inhibitor of chloride secretion. Thought to mediate fluid secretion by increasing chloride secretion. Affects host cell signalling pathways that may be involved in regulation of cytokine expression such as activation of the serine-threonine kinase Akt.	(Eckmann et al., 1997, Steele-Mortimer et al., 2000)
SopD	Has additive effect to SopB in the induction of enteritis	(Jones et al., 1998)
SopE + E2	Guanine nucleotide exchange factor for cdc42, plays a role in recruitment of Arp2/3 to membrane ruffles. Required for optimal invasion of cultured epithelial cells.	(Stender et al., 2000)
SptP	GTPase activating factor from Rac1 and cdc42 and is also delivered by via SPI-1 T3SS	(Fu and Galan, 1999)
HilA	Transcriptional regulator for SPI-1, 4, 5 mediates effects of environmental factors	(Ahmer et al., 1999)
SirA	Regulates expression of HilA	(Ahmer et al., 1999)
shdA	Involved in prolonged shedding of the organism. Binds to extracellular matrix proteins particularly fibronectin.	(Kingsley et al., 2002, Kingsley et al., 2000)

1.4.3. *Salmonella* infection of M cells

M-cells are antigen sampling cells restricted to the follicle associated epithelium (FAE) (Owen, 1977, Bye et al., 1984). These cells transport antigens across mucosal epithelia to underlying lymphoid tissues where they are processed and presented in order to produce a protective immune response. M-cells are generally identified by their relatively poorly organised brush borders and basolateral lymphocyte-

containing cytoplasmic pockets. Intestinal M-cells reside in follicle associated epithelium (FAE) overlying isolated and aggregated lymphoid follicles (Jepson and Ann Clark, 1998). Some micro-organisms including *S. Typhimurium* are able to use M cells as a route of entry (Sansonetti, 2004).

Few M cell models exist to study *Salmonellae* entry. One model involves the co-culture of Caco-2 cells and Raji B cells and has been shown to contain M-like cells. Using this co-culture system it was demonstrated that *Salmonella* rapidly translocates across intact Caco-2 monolayers in a SPI-1 and SPI-2 independent manner (Martinez-Argudo and Jepson, 2008). Furthermore, Martinez-Argudo and Jepson showed that Dynamin, which is a GTPase involved in clathrin mediated endocytosis, appears to be partially involved in uptake of *Salmonella* by M cells but not macropinocytosis shown by using EIPA to inhibit macropinocytosis. They concluded that the rapid translocation of *Salmonella* appears to be a separate process from the invasion of cultured epithelial cells (Martinez-Argudo and Jepson, 2008).

It was shown that following internalisation *S. Typhimurium* bacteria were cytotoxic for M cells. Furthermore, later in infection adjacent enterocytes were invaded via the apical and basolateral sides resulting in sloughing of mucosal epithelium (Daniels et al., 1996).

1.4.4. *Salmonella* infection of macrophages

Macrophages are phagocytes and are one of the immune systems first responders. Macrophages engulf and kill bacterial pathogens and also secrete cytokines to recruit other immune cells to the site of infection.

Salmonella has evolved to be able to enter and survive within macrophages. At a low multiplicity of infection (MOI) or suboptimal expression of the SPI-1 T3SS it was shown that the proportion of apoptotic looking macrophages increases (L. Hernandez and J.E Galan unpublished observations). However, in contrast maximum expression of SPI-1 T3SS in conjunction with a medium or high MOI favours the appearance of necrotic looking macrophages (L. Hernandez and J.E Galan unpublished

observations). It has been established that the rapid form of macrophage cell death induced by *Salmonella* requires caspase-1 (Hersh et al., 1999); (Brennan and Cookson, 2000); (Järveläinen et al., 2003). However, caspase-1 deficient macrophages *in vitro* still succumb to infection after 3-4 hours (Hernandez et al., 2003). SipB has been suggested as a factor in the induction of caspase-1 dependent macrophage cell death. Immunoprecipitation experiments suggest that SipB and caspase-1 may form a complex (Chen et al., 1996a, Hersh et al., 1999). Moreover, SipB has been reported to bind several other cellular proteins (Chen et al., 1996b). More studies are required to show SipB as a definite requirement for the activation of caspase-1 dependent macrophage cell death.

It has been proposed that SipB kills macrophages by disrupting mitochondria thereby inducing autophagy and cell death (Hernandez et al., 2003). Autophagy has been linked to “Type II programmed cell death”. In this type of death, caspases are often not required for execution. The addition of pancaspase inhibitors did not prevent *Salmonella*-induced cytotoxicity of caspase-1 deficient macrophages (Hernandez et al., 2003) consistent with the “type II programmed cell death” hypothesis.

SpvB is an ADP ribosylating toxin and actin has been proposed as the main target for this activity. It has been postulated that the disruption of the actin cytoskeleton by SpvB may contribute to macrophage programmed cell death. Moreover a recent report by (Hsu et al., 2004) suggests the delayed form of macrophage programmed cell death may require stimulation of TLR-4 and the dsRNA responsive kinase PKR. Kurita et al. showed expression of SpvB in mammalian cells induced apoptosis (Kurita et al., 2003), and a deletion mutation in *spvB* abolished the ability of *S. Typhimurium* to trigger the delayed form of programmed cell death (Browne et al., 2002). SpvB may be delivered by SPI-2 T3SS but evidence reported by Gotoh, Okada et al. 2003 suggests that this is not the case.

Both the rapid and delayed form of *Salmonella*-induced programmed macrophage cell death may be due to *Salmonella*'s ability to potently stimulate the host's innate immunity outputs, leading to caspase-1 activation, as well as interfering with survival

pathways so as to tilt the balance toward cell death. Flagellin is recognised inside host cells by Ice protease activating factor (Ipaf), also known as NOD like receptor C4 (NLRC4). Ipaf activates caspase-1, which results in macrophage cell death and the release of pro-inflammatory cytokines such as IL-1 β and IL-18 (Kanneganti et al., 2007).

Macrophage infection with *Salmonella* has been reported to lead to the breakdown of Raf-1, which is a kinase central to many survival pathways (Jesenberger et al., 2000). Furthermore, the inhibition of cdc42 and Rac-1, two Rho family GTPases that are activated by SPI-1 T3SS effector proteins, was shown to reduce the cytotoxic effects of *Salmonella* on macrophages (Forsberg et al., 2003). This suggests activation of these specific GTPases by *Salmonella* may provide signals to the host cell for cell death.

It has been reported that caspase-1 deficient mice are more resistant to infection with *Salmonella* (Monack et al., 2000) and it has been proposed that this resistance is the consequence of the inability of *Salmonella* to cross the intestinal epithelial cell barrier in the absence of profuse inflammation. However, it is not clear whether reduced inflammation is a consequence of reduced apoptosis or reduced levels of IL-1 β due to lack of converting enzyme (Hernandez et al., 2004).

1.4.5. *Salmonella* infection of dendritic cells

Salmonella may also reside within dendritic cells and it has been shown in mice that they can act as vehicles for the systemic dissemination of *Salmonella* (Niess et al., 2005). *Salmonella* reside within an SCV in dendritic cells, though it differs to the SCV of macrophages. These bacteria do not proliferate but do secrete virulence factors. SPI-2 was shown to be important for the inhibition of SCVs maturing into lysosomes (Jantsch et al., 2003). Dendritic cells have been shown to sample *Salmonella* directly from the gut lumen. Dendritic cells are recruited to the site of *Salmonella* infection by enterocytes expressing CCL20 as a result of TLR-5 stimulation by bacterial flagella. Once at the site of infection dendritic cells extend dendrites between cells, without perturbing tight junction integrity, and capture

Salmonella bacteria (Maric et al., 1996, Biedzka-Sarek and El Skurnik, 2006, Niess et al., 2005).

The extent of the role of dendritic cells in salmonellosis in pigs is unknown. As *S. Typhimurium* in pigs results in an enteric infection and does not become systemic it is possible that porcine dendritic cells respond differently to *S. Typhimurium* than murine dendritic cells. However, the role of dendritic cells as professional antigen presenting cells (APCs) is still likely to be very important for bacterial clearance.

1.5. The porcine immune system

The porcine immune system, like the immune system of many mammals, might be thought of as having two branches; innate and adaptive immunity. Innate immunity largely describes physical and chemical barriers to infection such as skin, and mucus covering epithelial surfaces. In addition, innate immune systems comprise phagocytic cells and natural killer (NK) cells which also link to adaptive immunity. Adaptive immunity describes a more targeted specific response to pathogens. In addition, it produces memory, which upon the second exposure to the pathogen, generates antibodies more quickly resulting in faster clearance of the infection (Abbas and Lichtman, 2005).

1.5.1 Innate immunity

The mechanisms of innate immunity form the first line of defence to infection. The physical, cellular and biochemical defences of innate immunity are in place prior to infection and ready to respond. One of the most remarkable adaptations of the immune system is its ability to recognise self. The mechanisms of innate immunity only react to invading pathogens. The primary elements of innate immunity are; physical and chemical barriers including epithelia and mucus at the epithelial surface; phagocytes, including macrophages and neutrophils as well as natural killer cells; the complement system which consists of blood borne proteins that recognise infection using 1 of 3 pathways, and respond by promoting microbe destruction and

increasing inflammation; and cytokines that regulate and direct many of the innate immune cell responses to infection (Abbas and Lichtman, 2005).

1.5.1.1. Neutrophils

Neutrophils are also known as polymorphonuclear (PMN) leukocytes. These cells are characterised by their segmented lobular nucleus, ability to act as phagocytes, cytoplasmic granules filled with lytic enzymes including lysozyme, myeloperoxidase, elastase, cathepsins and lactoferrin, and production of reactive oxygen intermediates (ROI). Neutrophils are the most abundant white blood cells circulating the body and are the major cell type involved in acute inflammatory responses to bacterial infections (Abbas and Lichtman, 2005, Mantovani et al., 2011). Neutrophils are directed to points of infection along chemokine gradients. Molecules that activate neutrophils include interleukin (IL)-8 and tumour necrosis factor (TNF)- α . Phagocytosis of pathogens by neutrophils is mediated by different mechanisms including receptor specific and complement coated mechanisms. Once an organism has been phagocytosed, an intracellular phagosome may form around the pathogen, which then fuses with neutrophil granules to aid destruction of the ingested microbe. Neutrophils also utilise ROI to aid pathogen destruction (Kaplan and Radic, 2012).

It has been shown that porcine neutrophils respond strongly to IL-1 β and IL-8 produced by gut epithelial cells in response to *Salmonella* infection (Hyland et al., 2006).

Porcine defence peptide, cathelicidin, PR-39 has been shown to possess an array of functions. PR-39 promotes wound repair, angiogenesis and acts as a chemotactic factor for recruitment of other neutrophils. Porcine neutrophils were shown to be a major source of PR-39 (Sang and Blecha, 2009).

1.5.1.2. Monocytes and macrophages

Monocytes differentiate from progenitor cells in the bone marrow. Monocytes circulate in the blood. Once monocytes enter tissues they are referred to as

macrophages, however, in particular tissues they are known by specific names dependent on tissue type. Macrophages of the liver are referred to as Kupffer cells, in the bone, osteoclasts and in the nervous system, microglia. The primary functions of macrophages include the phagocytosis of foreign material, assistance in immune regulation, uptake of dead cells as well as debris and to promote inflammation, and the recruitment of other macrophages to sites of infection (Murray and Wynn, 2011).

Macrophages express pattern recognition receptors (PRR). PRRs recognise pathogen associated molecular patterns (PAMP) which are conserved structures present on microbes. Macrophages can be activated by ligation with PAMPs. Activation enhances both phagocytic and endocytic capabilities. In addition, once activated macrophages secrete pro-inflammatory cytokines such as Type I interferons $IFN\gamma$ and $TNF\alpha$. Furthermore, the expression of co-stimulatory molecules and SLA-II (Swine leukocyte antigen) for antigen presentation also increases, following macrophage activation (Gordon, 2003).

Macrophages are also activated by $IFN\gamma$. $CD4^+$ T cells can secrete $IFN\gamma$, activating macrophages, this activation stimulates intracellular microbicidal capabilities and the synthesis of pyrogenic cytokines $IL-1\beta$, $IL-6$ and $TNF\alpha$. Alternatively, activated macrophages are sometimes referred to as M2 macrophages. These macrophages are programmed for wound repair promoting humoral immunity and immunosuppression. These macrophages in humans and mice are often activated in the presence of $IL-4$, transforming growth factor (TGF)- β or $IL-10$ or by the uptake of apoptotic host cells or debris. In swine however, the actions of $IL-4$ are different to those in humans and mice. Study of porcine macrophages and B-cells led to the discovery that $IL-4$ blocks the secretion of immunoglobulin (Ig) and suppresses B cell proliferation in pigs, in contrast to its previously reported functions of stimulating B cell proliferation and Ig production (Murtaugh et al., 2009). In addition, $IL-4$ did not stimulate T cell proliferation. A cytokine resulting in similar effects to $IL-4$ in other species is $IL-13$. It has been reported that porcine monocytes, differentiated into dendritic cells more efficiently in the presence of $IL-13$ compared to $IL-4$, and that $IL-13$ is more evident in the peripheral lymphoid tissues of common

breeds of swine (Bautista et al., 2007). The apparent difference in function of IL-4, between humans and swine, highlight the importance for host/pathogen interaction studies in the most relevant reservoir host.

In contrast, to the macrophage's protective role, macrophages have been demonstrated to be a source of entry for porcine reproductive and respiratory syndrome virus (PRRSv) and *Salmonella*. PRRSv can even infect fully differentiated alveolar macrophages (Welch and Calvert, 2010). *Salmonellae* are able use the phagocytic nature of macrophages, using them as vehicles to move to the mesenteric lymph nodes (MLN) and for intracellular survival (Boyen et al., 2009).

1.5.1.3. Dendritic cells

Dendritic cells (DCs) have four main functions; survey the body for pathogens, activate innate defences, efficiently process exogenous antigen to activate adaptive immunity and regulate both innate and adaptive immune functions (Abbas and Lichtman, 2005). DCs are professional antigen presenting cells (APCs), and can fully prime naïve T cells (Summerfield and McCullough, 2009).

DCs can be divided into two types; conventional DCs, which describe highly efficient APCs, and plasmacytoid DCs, which secrete large amounts of type I IFNs. The DC type depends on tissue type, activation mechanism and origin. Porcine plasmacytoid DCs are the main DC population that produce IFN α and TNF α and traffic to the lymphoid tissue after activation (Summerfield and McCullough, 2009).

DCs that have not been activated are highly migratory, respond to chemokine gradients, express low surface levels of SLA-II and are highly phagocytic (Abbas and Lichtman, 2005). DCs express PRRs, and upon ligation endocytic activity is enhanced resulting in pathogen uptake and antigen presentation (Summerfield and McCullough, 2009).

DC phagosomes, unlike the phagosomes of neutrophils and macrophages, do not fuse with lysosomes to achieve a low pH and destroy microbes. As a result antigen

epitopes are preserved for antigen presentation via major histocompatibility complex (MHC), sometimes referred to as SLA in swine (Abbas and Lichtman, 2005). Activation of DCs in the absence of PRR ligation leads to a more tolerogenic phenotype and presentation of these epitopes to lymphocytes results in the release of cytokines like TGF β or IL-10 polarising T cells to a regulatory phenotype. This tolerogenic phenotype is particularly important in the mucosal tissues where DCs mediate self-tolerance and tolerance to harmless antigens.

Bimczok et al 2006 reported the presence of porcine DCs in Peyer's patches and lamina propria of the gut mucosa. In addition, some DCs have been shown to directly sample the gut lumen through intraepithelial projections that capture food antigens (Bimczok et al., 2006). The close proximity of Peyer's patches DCs to M cells was also noted, hypothesising direct antigenic transfer from M cells to DCs. Porcine DCs have also been found in the tracheal mucosa, the tonsils and the lungs (Bimczok et al., 2006, Jamin et al., 2006).

The process of antigen uptake stimulates DCs to adopt a more mature, activated form. Activated DCs express high levels of surface MHC and co-stimulatory molecules for lymphocyte activation including CD80/86 and CD40 (Abbas and Lichtman, 2005). Upon activation, surface expression of chemokine receptors is also altered enabling trafficking to different tissue types. In addition, activating cytokines are secreted resulting in the differentiation of the naïve lymphocyte into an effector phenotype. The cytokines produced are dependent upon the mechanism of DC activation (Abbas and Lichtman, 2005). The ligation of extracellular PRRs often stimulates DCs to secrete cytokines that polarise T cells into an inflammatory profile. DCs activated in the presence of factors released from tissue damage however, secrete cytokines that polarise T cells to a phenotype involved in wound healing (Summerfield and McCullough, 2009). In addition, DCs also secrete cytokines inducing isotype switching in B cells (Summerfield and McCullough, 2009).

1.5.1.4. Natural killer cells

Natural killer cells (NK) are another cell type that bridges innate and adaptive immunity. NK cells are a lymphocyte lineage but are distinct from B and T cells as they do not undergo receptor rearrangement.

In the pig NK cells are found mainly in the spleen and peripheral blood though a few have been observed in the thymus and lymph nodes (Abbas and Lichtman, 2005). NK cells make up between 2-10% of porcine peripheral blood lymphocytes with decreasing proportions with increasing age (Gerner et al., 2009). NK cells are cytolytic, resulting in lysis of the target cell. Though it is their interaction with SLA-1 that directs their response, for example recognition of SLA-1 by NK receptors (killer cell Ig-like receptor (KIR)) stimulates an inhibitory pathway in NK cells.

A unique marker to NK cells has been identified, NKp46. NKp46, recognises SLA-I, the expression of this marker subdivides NK cells into distinct states of maturation (Mair et al., 2012). NKp46⁺ NK cells were shown to produce more IFN γ after stimulation though cytolytic activity was similar. As NKp46 expression was induced on NKp46⁻ cells following exposure to IL-2, 12 and 18 it was suggested that NK cells become NKp46⁺ upon maturation (Mair et al., 2012).

A process known as antibody-dependent cell-mediated cytotoxicity (ADCC) is achieved by activation of NK cells through ligation of CD16 an FcR. Soluble Ig opsonises target cells making them recognisable to NK cells by ligation of CD16 (Abbas and Lichtman, 2005).

Activated porcine NK cells mediate cellular cytotoxicity through perforin and NK lysine; these molecules create pores in target cell membranes and induce cell death. Cytokines such as IL-2, 12, 18 and IFN γ alone or in combination with other cytokines have been shown to enhance pig NK cytotoxicity and production of IFN γ *in vitro* (Gerner et al., 2009).

1.5.2 Adaptive immunity

Adaptive immunity is so called because, the mechanisms that respond to infection increase in magnitude with successive exposures to a particular pathogen or infectious agent, i.e. this kind of immunity can adapt. The evolution of this arm of the immune system appears to have occurred with the introduction of lymphocytes during the rise of jawed fish (Agrawal et al., 1998). Lymphocytes are the predominant cell type responsible for the mechanisms of adaptive immunity. Lymphocytes display antigen receptors on their cell surface, the vast diversity of these antigen receptors is largely due to somatic gene rearrangement/recombination known as V(D)J recombination, somatic hypermutation and allelic exclusion. Somatic gene recombination describes the recombination of different gene segments, V (variable), D (diversity) and J (junction) to create the variable region of the antigen receptor (Jung et al., 2006). Somatic hypermutation describes the point at which mutations occur in germinal centre B cells. These point mutations are estimated to occur at a rate of 1 in 10^3 V gene base pairs per cell division; this is 10^3 to 10^4 times faster than other mammalian genes. This process utilises activation-induced cytosine deaminase to introduce mutations into the recombined antigenic receptor genes repairing them with DNA repair enzymes. In one clone of cells only one type of antigen receptor is expressed. Allelic exclusion silences the other allele, ensuring only one is expressed. Through these three mechanisms, T and B lymphocytes are able to provide the antigenic specificity and memory that defines adaptive immunity (Abbas and Lichtman, 2005, Knetter, 2013).

1.5.2.1. B cells

In pigs, B cells are considered the first wave of adaptive immunity as they are generated early in gestation. Pigs have ~100% productive B cell receptor (BCR) rearrangement on a single allele and no rearrangement on the second allele. The same 5 Ig isotypes found in humans and mice IgM, IgD, IgG, IgE and IgA are also found in swine. The known subclasses of these isotypes are expanding with currently six classes of IgG (Butler et al., 2006). Two forms of IgA (IgA^a and IgA^b) have been identified. Porcine B cells can be identified by their expression of IgM and CD79 α (Ig α chain). Studies of the porcine foetal thymus revealed transcripts for all 5

isotypes. The proportion of secretory Ig classes varies with age and tissue type (Butler et al., 2006).

Igs consist of heavy and light chains. Pigs express almost equal λ and κ light chains in Ig. The genes that encode the heavy chain V segment of Ig are comprised of several subgroups in humans and mice. However, swine Ig heavy variable (IGHV) genes come from a single subgroup. Approximately 99% of VDJ recombinations in swine utilise 7 of 30 IGHV genes, 2 IGHD genes and there is only one functional IGHJ gene (Butler and Wertz, 2012). The combinational diversity that can be acquired through gene rearrangement is limited in swine; resulting in only 14 possibilities compared to 9000 in humans (Butler and Wertz, 2012).

The porcine BCR repertoire achieves its diversity after somatic hypermutation is stimulated, which occurs post exposure to environmental pathogens. Before gut colonisation, pigs might be referred to as, having natural antibodies, this refers to IgM antibodies, present at birth without antigenic encounter. Studies using isolator-housed piglets showed a lack of Ig change prior to gut colonisation. Post gut colonisation, a 100-1000 fold increase in serum Ig was observed (Butler, 2009).

Antibody secreting cells known as plasma cells, are found in several porcine tissues; mucosa, lymph nodes, spleen, and the thymus (Brown and Bourne, 1976). At birth plasma cells mainly produce IgM only, but once isotype switching is induced by environmental stimuli other isotypes predominate in certain tissues. IgA-secreting cells predominate in the MLN, gut lamina propria and other mucosal tissues, while in the peripheral lymph nodes and spleen IgG secreting plasma cells predominate (Brown and Bourne, 1976).

1.5.2.2. T cells

The T cell receptor (TCR) consists of a highly variable region (V), responsible for recognising antigen, a constant region (C) and small trans-membrane and cytosolic domains. The TCR is associated with a CD3 complex consisting of 5 chain types ($\gamma\delta\epsilon\zeta\eta$) arranged into three dimers. There are two main types of TCR, the α/β

heterodimer or γ/δ heterodimer. Similar to other livestock species, the proportion of peripheral γ/δ is high at birth (~40%) and decreases with age (Chareerntantanakul and Roth, 2006). It is thought that T cell development in the pig is similar to other species, though it has not been described definitively in the pig. In other species it has been determined that thymocyte progenitors undergo TCR rearrangement and subsequent testing by cells in the thymus. Thymocytes undergo positive or negative selection dependent on recognition of “self”. Thymocytes that react strongly with self-antigens are subject to negative selection and undergo apoptosis (-ve selection). Thymocytes that recognise self-MHC receive survival signals from resident DCs, macrophages and epithelial cells (+ve selection). Mature T cells either express CD4, classified as helper (Th) or CD8 $\alpha\beta$ heterodimers classified as cytotoxic T cells (Abbas and Lichtman, 2005). In pigs the ratio of CD4-CD8+ in peripheral blood is almost double the number of CD4+ T cells. This is the opposite ratio to that found in humans (Chareerntantanakul and Roth, 2006).

Th and CTL $\alpha\beta$ cells migrate through the periphery to the spleen and lymph nodes to scan for antigens presented in the context of MHC on APCs (Abbas and Lichtman, 2005). Once an antigenic epitope is recognised through the TCR, the T cell is activated into forming an immunological synapse. This synapse using co-stimulatory and adhesion molecules to form a strong bond with the APC, this fully activates the T cell and polarises it into one effector phenotype, based on the cytokine present. The function of porcine Th cells appears to be similar to that in mice and humans (Knetter, 2013). T cells interact with SLA-II presented antigen, this stimulates activation and proliferation. The T cells main role is to secrete cytokines, resulting in enhanced anti-bactericidal capacity of macrophages (Abbas and Lichtman, 2005).

Multiple subtypes of Th cells have been identified in mice and humans including Th1, Th2, Th3, Th17 and Th22 these subtypes are based on cell differentiation requirements, the expression of transcription factors and cell function. However, only Th1, Th2 and recently Th17 phenotypes have been identified in the pig (Kiros et al 2011).

A Th1 phenotype describes T cell differentiation in the presence of IL-12 and IFN γ (Murtaugh et al., 2009). The main functions of the Th1 cell are to activate the microbicidal activity of phagocytes for destruction of intracellular pathogens, stimulate B cell class switching and stimulate naïve, activated B cells to produce Ig. A Th2 phenotype describes differentiation of T cells in the presence of IL-4 and/or IL-13, expression of the GATA-3 transcription factor, and production of IL-4, -5 and -13. The main role of a Th2 cell is to provide support for B cells in class switching and activation, optimising the humoral response (Murtaugh et al., 2009). Th17 cells differentiate in the presence of TGF β and IL-6, are stabilised by IL-23, express ROR γ T and produce IL-6, -17 and -21 (Abbas and Lichtman, 2005). The full function of these cells remains unknown, though the production of these cytokines stimulates surrounding macrophages and epithelial cells to produce chemokines which attract neutrophils and increase β -defensin expression.

Although another type of T cell has been identified in swine, regulatory T cells (T reg), these cells appear to be similar in function to other species in down regulating pro-inflammatory immune responses (Käser et al., 2008). T reg cells are identified as CD4⁺CD25⁺ and express FoxP3 and produce IL-10 and TGF β . Based on the expression of CD25 (high or low) porcine T regs can be subdivided into two groups. Only CD25 (high) cells are capable of inhibiting activated T cell proliferation. Though CD25 (high) and CD25 (low) types were shown to be capable of producing IL-10 and together, make up ~9% of the porcine T cell population.

Porcine CTLs recognise SLA-I presented antigen and respond by killing target cells, proliferating and secreting pro-inflammatory cytokines such as TNF α and IFN γ . CTLs contain perforin and carry out their lytic activity through release of this pore forming protein on target cells (Denyer et al., 2006).

In pigs, it has been shown CD4⁺ T cells, once stimulated by antigen, can permanently express a CD8 $\alpha\alpha$ homodimer, resulting in extrathymic CD4⁺CD8⁺ cells (Butler et al., 2006). CD8 is therefore not unique to CTL and as a result CD3 in combination with CD8 is used as a marker to identify CTLs. In addition, it has been

reported that these CD4+CD8+ positive cells form a CD4+ memory population. These cells can recall antigen and express markers of T cell memory such as CD29. The numbers of these cells correlate positively with animal age (Zuckermann, 1999). Expression of CD4+CD8(lo) is now used to identify Th memory cells in pigs. Furthermore, CD4+CD8(lo) up regulate production of IFN α , IL-2 and IFN γ . CD4+CD8 $\alpha\alpha$ + T cells have also been described in humans, rodents, monkeys and chickens though the proportion in swine is the greatest. At 5 months of age CD4+CD8 $\alpha\alpha$ + T cells can comprise a third of all T cells in the secondary lymphoid tissue (Zuckermann and Gaskins, 1996).

Other subtypes of T cells have been identified in mice, humans and pigs including T follicular helper cells (Tfh) and NKT cells. Tfh expression of CXCR5 results in them homing to the germinal centres of B cell follicles (Leavy, 2012). This cell type supports the activation, proliferation and differentiation of B cells in these tissues.

NKT cells are CD3+perforin+CD16+ making their phenotype similar to both NK and CTL, these cells have been identified in porcine lung and peripheral blood (Renukaradhya et al., 2011). Porcine NKT are capable of lytic activity through perforin, they recognise glycolipid antigens in the presence of CD1d, which is a molecule similar to SLA in antigen presentation. NKT can also secrete cytokines similar to Th1 or Th2 when stimulated *in vitro* (Renukaradhya et al., 2011).

Based on phenotype and tissue distribution, the γ/δ repertoire can be subdivided. CD2+CD8 $\alpha\alpha$ + and CD2+CD8 $\alpha\alpha$ - γ/δ T cells are mainly located in the spleen making up the predominant T cell population here, while CD2-CD8 γ/δ T cells are found mostly in circulation (Butler et al., 2006). Porcine CD2+ γ/δ cells are not SLA restricted and they secrete cytokines such as IL-1, IFN- α and IL-8 in response to stimulation as well as been cytolytic, though the mechanism for their antigenic recognition is under debate (Zuckermann and Gaskins, 1996, Sinkora et al., 2005, de Bruin et al., 2000). CD2+ γ/δ cells expression of CD3 separates these cells from porcine NK cells, which are CD3-. CD8- γ/δ T cells, however, may acquire CD8 $\alpha\alpha$ upon activation similar to α/β T cells as well as SLA-II (Sinkora et al., 2005). It was

reported by Takamatsu et al 2002 that acquisition of SLA-II by porcine γ/δ T cells resulted in the capability to present antigen to α/β T cells (Takamatsu et al., 2002). Antigenic stimulation within tissues appears to select and maintain the γ/δ TCR repertoire within tissues. The repertoire is highly compartmentalised within organs with a highly polyclonal repertoire identified in the spleen (Butler et al., 2006).

The thymus serves as a source for T cells and resection of the thymus in neonatal piglets greatly reduced the number of T cells. However, 3 months later T cells were found at detectable levels suggesting a second source outside the thymus is able to produce T cells. Perhaps the lymph nodes or Peyer's patches (Licence and Binns, 1995).

1.6. The immune response to *Salmonella*

The outcome of a *Salmonella* infection is dependent, in part at least, to the response of the immune system; however it also depends on other factors such as the size of bacterial inoculum, virulence of the *Salmonella* strain, and any medical treatment. In an immunologically naïve host, the primary barriers to bacterial infection, or first line of defence, are the mechanisms of the innate immune system. These include nonspecific antimicrobial factors such as neutrophils, NK cells, macrophages and dendritic cells. These cell types are described in section 1.5.1.

It is widely accepted that for clearance of intracellular bacteria, cell mediated immunity is required. Any bacterial antigens present in the cytoplasm of an infected host cell are processed via the endogenous pathway and presented to CD8+ T cells (CTLs) on MHC-I (SLA-1 in pigs). Alternatively, where bacterial antigens are present in the endosomes of infected host cells, antigens are processed via the exogenous pathway leading to presentation to CD4+ helper T cells (Th) on MHC-II (SLA-II). This results in activation of macrophages and bacterial killing via proteases and ROI. In addition, presentation of bacterial antigens on the cell surface, leads to antibodies binding, thus targeting the cell for killing by NK cells and neutrophils using ADCC.

The first cells encountered once *Salmonellae* reach the intestine are enterocytes, dendritic cells, and macrophages. The interaction of *Salmonella* with these cells induces synthesis of pro-inflammatory cytokines and chemokines, which leads to an influx of neutrophils, macrophages, and dendritic cells.

Enterocytes, DCs and macrophages express PRRs, enabling them to recognise an array of foreign molecules or PAMPs. Recognition of PAMPs activates phagocytosis of the microbe. TLRs are an example of PRRs. TLR-2, 4, 5, 9 recognise; peptidoglycan, lipopolysaccharide (LPS), flagella and bacterial DNA respectively. *Salmonella* PAMPs are recognised by the immune systems PRRs, which are present on an array of cell types. This recognition results in downstream signalling, which results in the production of an array of pro-inflammatory cytokines activating other immune cells such as macrophages, T cells and B cells.

Pro-inflammatory cytokines include interleukin (IL)-1, IL-6, IL-8, TNF α , IFN γ , monocyte chemoattractant protein-1 (MCP-1), and granulocyte macrophage colony stimulating factor (GM-CSF). It has been shown that genetic deficiencies in humans in the type I cytokine pathway (IFN γ /IL-12/IL-23) increase susceptibility to *Salmonella* and Mycobacteria (Ottenhoff et al., 2002, van de Vosse et al., 2004). Several syndromes with abnormalities in these cytokines have been identified. Interferon- γ (IFN γ) is important early in infection for the control of bacterial replication in mice (Muotiala and Mäkelä, 1990); however, IFN γ alone does not result in eradication of the bacteria (Muotiala and Mäkelä, 1993). Tumor necrosis factor alpha (TNF α) synergistically acts with IFN γ to enhance microbicidal activity and also triggers nitric oxide (NO) production (Tite et al., 1991). The potent antimicrobial activity of NO has been demonstrated against, bacteria, fungi, parasitic worms, and protozoa (Abbas and Lichtman, 2005). Neutralization of IFN γ and TNF α results in decreased killing of *Salmonella*, and increased bacterial replication respectively (Gulig et al., 1997). An inflammatory response is important for controlling infection. However, side effects of the inflammatory response may cause damage to the intestine. Indeed some of the symptoms of the host exhibited during

infection are as a result of an inflammatory immune response. Symptoms may include: fever, chills, abdominal pain, leukocytosis and diarrhoea.

The production of IFN γ from NK and T cells is induced by IL-12 from APCs. This in turn, upregulates further production of IL-12 in APCs (Coburn et al., 2007). IFN γ enhances the antimicrobial activity of macrophages, NK cells, and neutrophils, helping to clear the infection. However, protective effects against *Salmonella* infection have been reported independently of induction of IFN γ . Coburn et al. (2007) concluded that independently of induction of IFN γ , IL-12/IL-23 exerted protective effects against *Salmonella* infection. A possible IFN γ independent mechanism could be the up regulation of TNF α , GM-CSF, and IL-17 by IL-23 leading to enhanced bacterial killing and enhanced NO production in macrophages. In gut associated lymphoid tissue (GALT) and spleen infection with serovar Typhimurium has been shown to rapidly upregulate IFN γ production. Macrophages and neutrophils are the main producers of IFN γ and TNF α (Kirby et al., 2002); though NK cells also contribute to early IFN γ production that is dependent on IL-12 produced by other APCs during in *Salmonella* infection (Brigl et al., 2003).

Another component of the immune system important for *Salmonella* clearance is the complement system. After activation, in a highly regulated cascade, the complement components carry out several functions including: bacterial lysis, opsonisation leading to phagocytosis, inflammation, and immune clearance. Generally, the complement system is very effective in lysing gram-negative bacteria. However, some gram-negative bacteria have developed mechanisms for evading complement. *E. coli* and *Salmonella* possess some resistance to complement due to the structural nature of their LPS. It has been proposed that the longer LPS chains on the surface of resistant strains prevents the insertion of the membrane-attack complex into the bacterial membrane, resulting in the complex being released from the cell rather than forming a pore. The membrane-attack complex, on sensitive cells, forms a large channel through the membrane, enabling ions and small molecules to diffuse freely across the membrane. *Salmonella* have evolved other adaptations for survival in the host environment, some of which are discussed in section 1.4.

1.6.1 Porcine salmonellosis with *S. Typhimurium*

1.6.1.1. *Salmonella* shedding

Different pigs may respond differently to infection with *Salmonella*. While some may exhibit persistent shedding of the bacteria, others may shed only small numbers. Studies have been carried out in order to determine this basis for this. Experiments have identified distinct immune differences between low shedding (LS) and persistently shedding (PS) pigs. When pigs were experimentally inoculated with *S. Typhimurium*, PS pigs had longer pyrexia and increased IL-1 β , TNF α , IFN γ , and IL-12p40 at 2 days post infection compared to controls. In addition, the upregulation of STAT1, IFN β 1 and IFN γ regulation network genes was detected from blood samples. LS pigs had brief pyrexia, and *Salmonella* faecal shedding decreased more rapidly than in PS pigs. In addition, genes involved in negative immune regulation were upregulated. Endotoxin stimulation of blood from LS and PS pigs resulted in similar inflammatory profiles prior to *Salmonella* infection, though some differences in cytokine and gene expression were observed. Following 2 days infection, differences in cytokine and gene expression profiles between LS and PS were apparent. Blood from PS pigs had increased plasma IL-1 β , TNF α , IFN γ , IL-8 and IL-10. In comparison, blood from LS pigs appeared to have a diminished response to endotoxin stimulation post 2 days infection, as only plasma TNF α , IL-8 and IL-10 were increased. Furthermore, LS pigs exhibited lower IL-1 β plasma levels post infection compared with before infection. In addition, only 3 genes in LS pigs were upregulated in response to endotoxin challenge. This study by Knetter (2013) demonstrated that the immune response in pigs, which are persistent *Salmonella* shedders, differs from that of low *Salmonella* shedding pigs. PS pigs appeared to induce more of an inflammatory response than LS pigs (Knetter, 2013).

1.6.1.2. The Peyer's patch in *Salmonella* infection

The Peyer's patches (PP) in pigs appear to possess more heterogeneity in structure and function than in rodents. Swine have multiple, isolated, jejunal patches and a single, large, continuous patch in the ileum (Liebler-Tenorio and Pabst, 2006). The ileal patch differs from those in the jejunum, consisting mainly of follicles (Chu and Liu, 1984). Furthermore, the continuous ileal patch has been suggested as a site for B

cell, early differentiation and selection (Andersen et al., 1999). The majority of studies using the Peyer's patch in *Salmonella* studies of the porcine host refer to the jejunal patches.

Studies have shown that the PP in pigs serves as a portal of entry for *S. Typhimurium* (Martins et al., 2013). A recent study by Martins et al. showed that oral infection of piglets resulted in the up-regulation of several genes encoding PRRs, chemokines, DC and T-cell activation markers, Th response mediators and other immune-related molecules (Martins et al., 2013). In addition, the same study showed the upregulation of IL-1 β , CXCL2 and TNF α , pro-inflammatory genes 2 days post infection. These responses may be attributed to recognition of *Salmonella* via PRR present on macrophages and dendritic cells in the PPs. These responses result in phagocytosis of *Salmonella*, and secretion of chemokines that recruit additional inflammatory cells into the site of invasion (Broz et al., 2012). PRRs TLR-2, 4, 5, 8 and NOD2 were shown to be upregulated during *Salmonella* infection of PP. The inflammation as a result of the activation of caspase 1, in turn resulting in IL-1 β production, is considered a key mechanism in *Salmonella* pathogenesis at the mucosal level in mice, where it was shown that IL-1 β and IL-18 were important for resistance to systemic infection (Raupach et al., 2006). A study employing the use of porcine gut loops however, did not detect upregulation of IL-1 β and chemokines encoding genes in PP upon *S. Typhimurium* infection (Meurens et al., 2009). This discrepancy was attributed to differences in experimental approaches by Martins et al. The laser-capture micro-dissection technique employed by Martins et al. was not used by Meurens et al. This resulted in some PP areas not being analysed, such as dome and interfollicular zones; this might affect the response observed. For example, if the increase in IL-1 β is localised to a very small area, an increase may not have been detected with the inclusion of other areas. In addition, the two studies used different bacterial strains and one study used oral infection, while the other applied the bacteria directly to the intestine. *Salmonellae* undergo changes during passage to the gut as described in section 1.4. Thus administration of *Salmonella* directly to the gut may affect bacterial pathogenicity and the subsequent immune response. Furthermore, the timing of sample collection differed between studies. Martins et al.

sampled 2 days post infection while Meurens sampled after 24 hours. There were several experimental differences between studies, these may explain the observed differences in IL-1 β levels, between the PP micro-dissection (Martins et al., 2013) and the whole PP studies (Meurens et al., 2009).

Generally, the results from Martins et al. indicated similarity to the murine typhoid model reviewed by Tam et al. Innate immune mechanisms are effectively induced in the porcine PPs following oral infection with *S. Typhimurium* (Tam et al., 2008). As described in section 1.5.1 these mechanisms are important for bacterial clearance but could also trigger the second line of defence, adaptive immunity. The genes coding for molecules involved in DC activation (CD80, CD83, CD40, IL-12p40, IL-23p19 and CCR7) and T-cell mediated processes (IFN γ , CD40L, CD28, CCL19 and IL-21) were found to be up-regulated in infected PP (Martins et al., 2013). High mRNA levels were observed for IFN γ and IL-12p40, though IL-18 was not up-regulated. IL-18 has been shown to act synergistically with IL-12 in the generation of IFN γ and cell-mediated immunity. However, in a mouse study it was demonstrated that IL-18 was relevant for resistance to systemic infection but not during the intestinal phase of infection (Raupach et al., 2006). This may also be true for swine. The down-regulation or absence of Th2 (IL-4 and IL-13) expression was also observed (Martins et al., 2013).

1.6.1.3. Protection from *Salmonella* challenge and vaccination

The initial response to *Salmonella* infection requires the production of inflammatory cytokines, leading to infiltration of activated phagocytes into the tissues. However, to establish a longer lasting immunity, the mechanisms of the adaptive immune system are required. The functions of T- and B-cells result in clearance of *Salmonella* from infected tissues and resistance to re-infection requires T-cells, and inflammatory cytokines such as IFN γ , TNF α and IL-12 as well as opsonising antibody. Seroconversion is often considered a measure of immunity, and following exposure or vaccination with *Salmonella*, antibody responses to LPS and protein determinants have been detected in several species, including mice (Carter and Collins, 1974) and pigs (Lumsden and Wilkie, 1992). However, seroconversion and/or the presence of

T-cell memory do not always appear to correlate with resistance to re-infection, though the reasons for this are not fully understood (Mastroeni et al., 2001).

The use of a *Salmonella* vaccine could provide a useful tool for reducing salmonellosis in both humans and livestock species. An effective vaccine would provide resistance to *Salmonella* challenge resulting in few or no symptoms. The use of live attenuated vaccines is serotype specific, though in some cases does provide a small amount of cross protection (Schwarz et al., 2011). Immunisation studies of mice have shown that during a secondary sub-lethal infection bacterial growth was controlled in the reticuloendothelial system but required the presence of both CD4+ and CD8+ T-cells and inflammatory cytokines such as IFN γ , TNF α and IL-12 (Tite et al., 1991, Mastroeni et al., 2001, Everest et al., 1998).

It has been shown that low levels of protection against *Salmonella* infection in mice can be induced by immunisation with porins, flagella or polysaccharide fractions (e.g. LPS, O-antigen), though to achieve high levels of protection against virulent salmonellae, immunity to both immuno-dominant LPS O-polysaccharide determinants and also non-serovar specific determinants (presumably proteins) will be required (Kuusi et al., 1979, Strindelius et al., 2004, Watson et al., 1992).

1.7. Flagella

Flagella are a major bacterial surface factor and act primarily as motility organelles. In *Salmonella*, flagella appear to originate from random points around the bacterial surface (peritrichous) and vary in number usually with between 5 and 10 per cell. The flagellar filaments have a consistent diameter of approximately 20nm but vary in length from 5 to 15 μ m (Berg and Anderson, 1973, Silverman and Simon, 1974, Macnab, 1996).

1.7.1. Flagellar Structure

Bacterial flagella consist of; a basal body connecting the flagella to the bacterial cell; a hook providing a link between the filament and the basal body; and the filament that rotates to provide forward propulsion (Figure 1.2). The basal body consists of a rod and several rings (Figure 1.2). Some of the rings make up the flagella motor, which is divided into 2 major parts, the stator (motA and motB) that remains stationary, and rotor (C ring, MS ring and rod) that rotates. The remaining rings L & P located in the outer membrane are stationary (Figure 1.2) (Macnab, 1996).

Salmonella flagella consist mainly of one of two proteins, the filament protein (FliC (phase 1) or FljB (phase 2)). The length of the filament is a cylindrical structure made up of overall around 20,000 to 30,000 flagellin (FliC or FljB) subunits (Kondoh and Hotani, 1974). The filament is helical with ~11 subunits per two turns (O'Brien and Bennett, 1972). The filament can be either left or right handed. However the 'normal' form which is more stable under physiological conditions is the left handed form (Macnab and Ornston, 1977). The motor contains a switch which allows the filament to be rotated in either anti-clockwise or clockwise directions (Berg, 1974, Silverman and Simon, 1974). When the left handed form is rotated in an anti-clockwise direction a helical wave travels from the proximal to distal end of the filament, pushing the cell forward. Several filaments with a left handed structure form a bundle to propel the cell. This mode of motion is referred to as "run" (Anderson, 1974, Macnab, 1977, Turner et al., 2000).

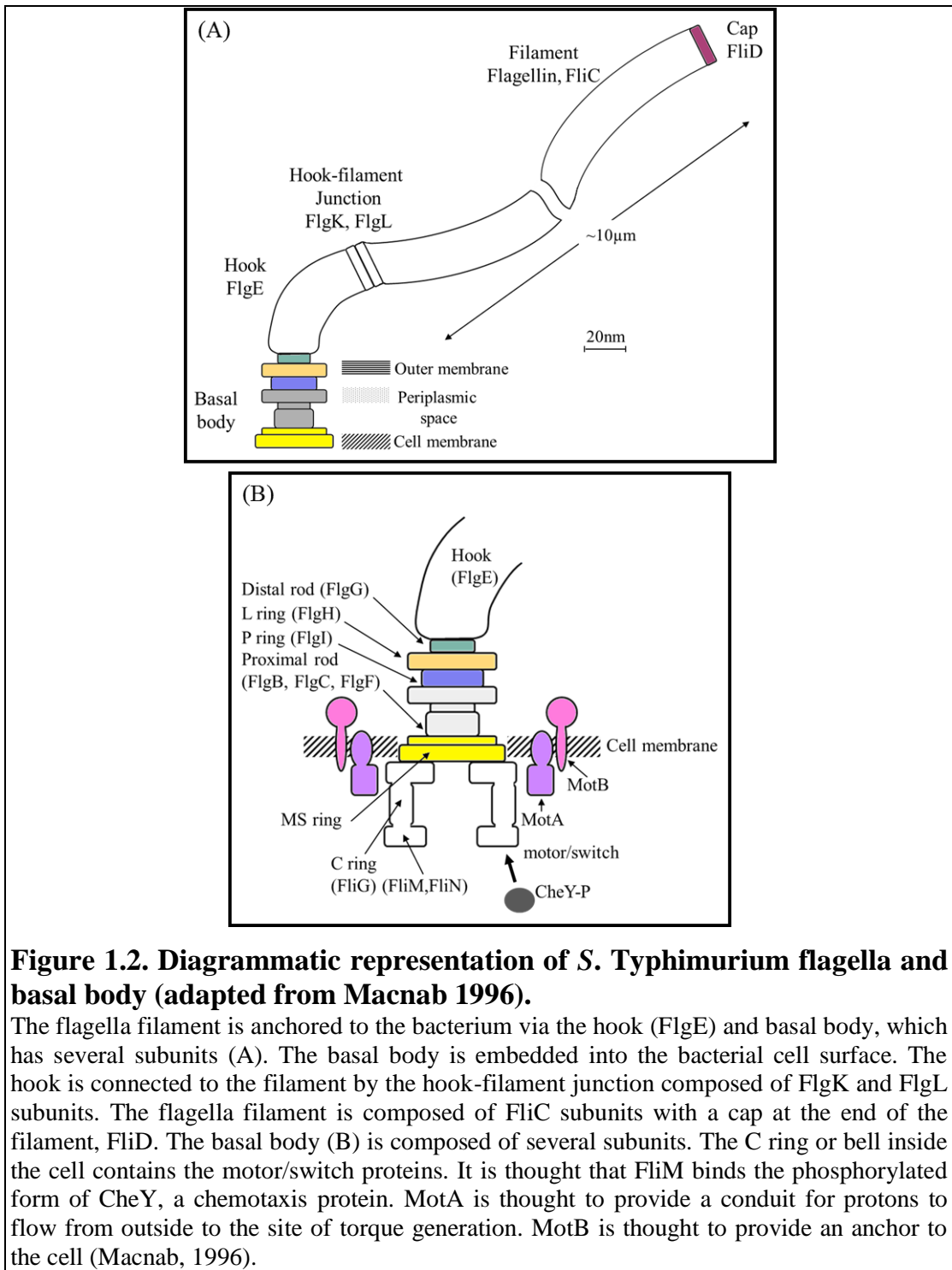


Figure 1.2. Diagrammatic representation of *S. Typhimurium* flagella and basal body (adapted from Macnab 1996).

The flagella filament is anchored to the bacterium via the hook (FlgE) and basal body, which has several subunits (A). The basal body is embedded into the bacterial cell surface. The hook is connected to the filament by the hook-filament junction composed of FlgK and FlgL subunits. The flagella filament is composed of FliC subunits with a cap at the end of the filament, FliD. The basal body (B) is composed of several subunits. The C ring or bell inside the cell contains the motor/switch proteins. It is thought that FliM binds the phosphorylated form of CheY, a chemotaxis protein. MotA is thought to provide a conduit for protons to flow from outside to the site of torque generation. MotB is thought to provide an anchor to the cell (Macnab, 1996).

1.7.2. Flagellar Energetics

The energy for flagella rotation is not from adenosine tri-phosphate (ATP). Instead, the energy is produced using a proton gradient, which produces proton motive force (Manson et al., 1977, Larsen et al., 1974). MotA and MotB proteins form a complex and are part of the stator part of the motor (Figure 1.2). Mutants lacking MotA and MotB are non-motile and both proteins have been shown to be involved in proton conduction. MotA appears to be important for delivery of protons across the membrane and the utilisation of those protons in generating torque. MotB also appears to be important for the proton channel as it forms a complex with MotA. MotB also acts as an anchor with its interaction with the peptidoglycan layer.

The current model for how torque is generated by MotA and MotB suggests that the association and dissociation of the proton to the aspartic acid residue of MotB results in conformational changes which produce rotation (Berg, 2003).

1.7.3. Motility

Salmonella move rapidly in liquid media with their flagella rotating at hundreds of revolutions per second (Kudo et al., 1990, Ryu et al., 2000). The swimming of *Salmonella* has two modes referred to as “run” and “tumble”. The “run” mode, which usually lasts a few seconds, is a bundle of filaments rotating in an anti-clockwise direction. The “tumble” mode results from a quick change in direction of the motor to a clockwise direction (Macnab and Ornston, 1977, Turner et al., 2000). This change in direction of rotation causes the bundle of filaments to un-bundle and become un-coordinated. These separated filaments generate forces that change the orientation of the cell. A tumble lasts for only a fraction of a second re-orienting the cell. This process is important for bacterial taxis (Yonekura et al., 2003).

In addition to swimming, some hyperflagellated bacterial species are able to swarm. Swarming describes movement through a liquid film over a solid substrate such as an agar plate (Harshey, 1994).

1.7.4. Flagellar regulation

The production of flagella is energetically costly. The requirement for a motile phenotype and flagella production in stirred culture is presumably low where nutrients and air are distributed by stirring. Production of flagella results in a growth disadvantage that is estimated to be ~2% (Macnab, 1996). Consequently, in stirred culture non-flagellate mutants will outgrow wild type (WT) cells. Furthermore, when grown in stirred culture non-flagellate mutants spontaneously occur in ~10 days and overtake the motile population (Macnab, 1996).

Flagella are costly to produce, therefore their production is regulated. Indeed flagellar assembly has been shown to be dependent on growth phase. When cells were taken from a stationary phase culture and added to fresh media, maximum flagellation and hence motility was not reached until late log phase (Macnab, 1996).

1.7.5. Flagellar gene hierarchy

A hierarchy of flagella genes are responsible for assembly of new flagellar organelles. In *Salmonella* the flagellar regulon consists of 17 operons, divided into classes 1, 2 and 3. At the top of the hierarchy is the master regulator class 1 gene, *FlhDC*. FlhD and FlhC proteins consist of predominately alpha-helical structures with FlhC containing a unique zinc-binding domain. FlhD and FlhC form FlhD₄C₂, a hexameric complex (Campos et al., 2001, Wang et al., 2006). Expression of FlhDC is required for expression of class 2 flagellar genes (Figure 3.2) (Kutsukake et al., 1990). The class 2 genes include FliA (σ^{28}) which is required for transcription of many of the class 3 genes, although some class 3 genes can be expressed independently of FliA, and it has been shown that FliA mutants assemble all three of the hook-associated proteins (Homma et al., 1984). In addition, *flgM* & *flgN* genes can also be expressed independently of FliA by read through of the transcript from *flgA*, a class 2 promoter (Gillen and Hughes, 1993, Kutsukake, 1994).

FlgM is an anti-FliA (σ^{28}) factor. FlgM negatively regulates FliA by binding it. Upon completion of the basal body-hook structure the flagellar protein export apparatus switches substrate specificity and transports FlgM out of the cell. Inhibition of class

3 genes is then alleviated and transcription initiation by FliA dependent class 3 flagellar genes can then proceed (Figure 1.3) (Hughes et al., 1993).

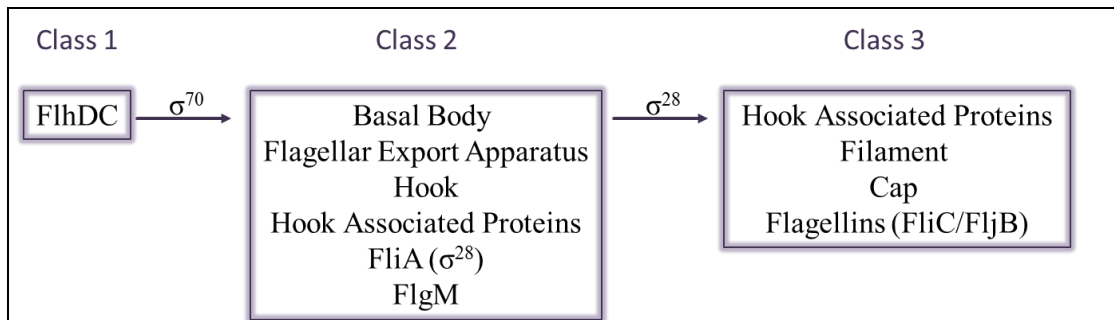


Figure 1.3. The flagellar master regulator (adapted from Smith and Hoover 2009).

The figure shows how the expression of the flagellar master regulon is required for the expression of subsequent flagellar genes. The sigma (σ) factors shown are required for transcription initiation. FliA (σ^{28}) is negatively regulated by FlgM which binds it. Before transcription initiation by FliA of the flagellins (right) FlgM is removed from the cell via the flagellum specific pathway, alleviating negative regulation of FliA (Smith and Hoover, 2009).

1.7.6. Flagellar master regulator, FlhD₄C₂

Flagellar master regulator FlhDC is required for expression of flagellar class 2 genes and therefore is a target for many flagellar regulatory factors. The master regulator, FlhDC is controlled by several regulators including those linked to environmental conditions such as temperature, pH and osmolarity.

Heat shock proteins DnaK, DnaJ and GrpE respond to temperature and have been shown to regulate *flhDC* expression (Shi et al., 1992). An increase in the osmolarity of the surrounding medium results in down regulation of flagella expression dependent on OmpR, a transfactor of osmoregulation. An increase in medium osmolarity results in phosphorylation of OmpR which negatively regulates flagellar expression (Shin and Park, 1995). Global regulatory proteins such as histone-like nucleoid-structuring protein (H-NS) and AMP-catabolite activator protein (CAP) also affect flagellar expression. Flagella expression is inhibited under acidic conditions and this regulation is dependent on H-NS which represses expression of *flhDC* (Soutourina et al., 2002, Soutourina et al., 1999). Flagellar synthesis has been shown to be sensitive to the availability of carbon sources, mediated by cyclic AMP-

catabolite activator protein (CAP) that activates *flhDC* expression (Soutourina et al., 1999, Kutsukake, 1997). In addition, YdiV, an ELA-like protein also appears to connect flagella expression with availability of nutrients, negatively regulating flagellar class 2 expression in low nutrient conditions (Takaya et al., 2012). The FlhD₄C₂ complex can be degraded by the ATP-dependent protease ClpXP thus preventing its activity (Claret and Hughes, 2000, Tomoyasu et al., 2002). This ClpXP degradation of FlhD₄C₂ is accelerated in the presence of YdiV (Takaya et al., 2012). Additional mechanisms of FlhD₄C₂ regulation and stability involve FliT, FliZ and CsrA. FliT interacts with the FlhD₄C₂ complex and prevents its activity, thus down regulating expression of flagellar class 2 genes (Yamamoto and Kutsukake, 2006) (Figure 1.4). FliZ, a DNA-binding regulatory protein, is involved in regulation of flagellar assembly via a feedback loop involving YdiV. FliZ is a positive regulator for class 2 genes (Wada et al., 2011) (Figure 1.4). CsrA is a global RNA-binding protein and increases the stability of *flhDC* mRNA (Wei et al., 2001).

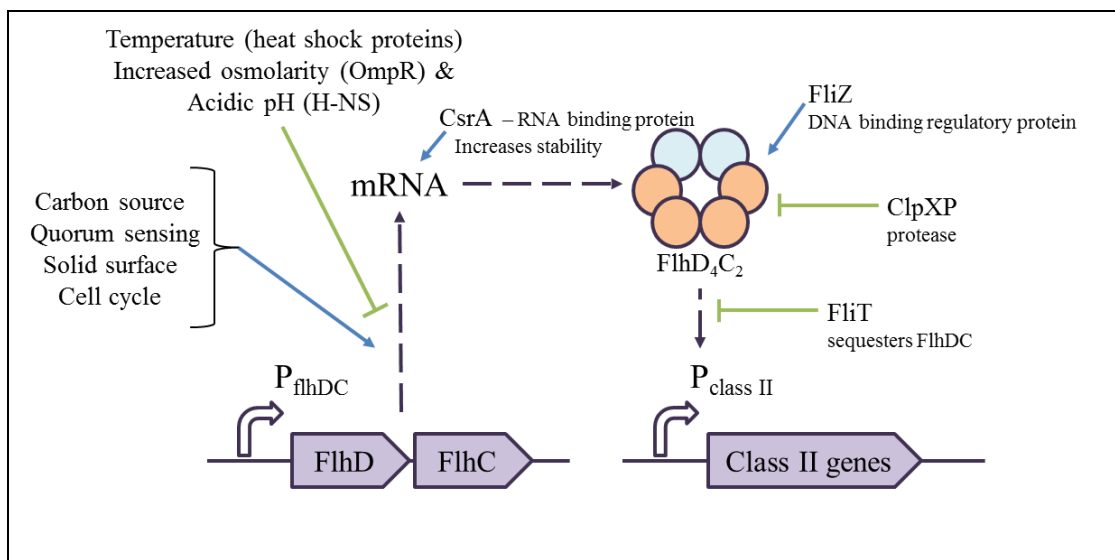


Figure 1.4. Regulation of FlhDC expression (adapted from Smith and Hoover 2009).

Multiple factors are responsible for expression of FlhDC, the flagellar master regulon. These factors include temperature and pH as shown in the figure. The arrows indicate a positive effect on expression while the flat ended arrows indicate a negative regulation effect (Smith and Hoover, 2009, Soutourina and Bertin, 2003).

1.7.7. Chaperones

The order of gene and protein expression is mainly controlled by the flagellar gene classes. However, the number of subunits and order of export must also be controlled if flagellar assembly is to proceed correctly.

The status of flagellar assembly is monitored by chaperones such as FlgN, FliT and FliS. FlgN binds with hook-associated proteins FlgK and FlgL, FliT the filament cap and FliS flagellin. These chaperones protect their substrates from degradation, and prevent their aggregation prior to secretion via the flagella specific export pathway. In addition to their roles as chaperone proteins, FlgN and FliT have another regulatory role. Free FlgN promotes translation of FlgM and FliT negatively regulates class 2 promoters (Bennett and Hughes, 2000, Kutsukake et al., 1999).

1.7.8. Hook length

The flagellar filament length in *Salmonella* can vary from 5 to 15 μ m, however the hook length is more regulated at ~55nm. The hook length is controlled by the protein FliK. FliK mutants have a polyhook phenotype; this describes bacteria that have longer than normal hooks. Once assembly of the hook basal body complex is complete the flagella export apparatus undergoes a conformational change. The secretion substrates then change to filament type substrates. This change directly involves FlhB and FliK. FlhB is membrane bound and functions as an export switch. After completion of the hook FlhB undergoes autocleavage resulting in electrostatic and conformational changes. These changes may affect interactions with other export apparatus proteins. FliK acts as a molecular ruler to control the length of the flagellar hook. If the hook is very short the binding domain of FliK cannot interact with the C-terminal of FlhB resulting in FliK secretion from the cell. When flagellar hook length reaches ~55nm the binding domain of FliK interacts with the C-terminal of FlhB, this interaction facilitates the export substrate switch (Anderson et al., 2010).

1.7.9. Co-regulation

Some other virulence genes are co-regulated with motility genes. Genes *barA* and *sirA* have been shown to decrease expression of motility genes in *S. Typhimurium*. BarA and SirA form a two component sensor kinase and response regulator resulting in increased expression of virulence genes. Teplitski, Goodier et al showed that SirA repressed *flhDC*. In addition, it was shown that BarA phosphorylates SirA. This then indirectly reduces flagellar expression by binding the *csrB* promoter, which influences flagellar expression (Teplitski et al., 2003). Ellermeier and Slauch also describe co-regulation of invasion genes with motility genes by RtsA and RtsB regulatory proteins. RtsA appears to be involved in regulation of *Salmonella* invasion genes while RtsB binds the *flhDC* promoter and down-regulates its expression.

1.7.10. Flagellar phase variation

Salmonella enterica has the ability to switch between two alternate, antigenic forms of its flagellin filament protein, expressing either FliC or FljB. This switching (flagellar phase variation) is achieved by stochastic inversion of a promoter, the *hin* switch, that produces both FljB flagellin and an inhibitor (FljA) of FliC flagellin formation. When the *fljB-fljA* operon is expressed, only FljB flagellar filaments are produced; when the operon is not transcribed, the gene for FliC flagellin (*fliC*) is released from inhibition and FliC flagellar filaments are formed (Macnab, 1996).

The biological relevance of these two types of flagella filament protein is still not understood. It has been postulated that the presence of a second phase type of flagella may offer an advantage to the bacteria by avoiding recognition by the immune system. However, studies have shown that both FliC and FljB flagella activate TLR-5 mediated NF- κ B signalling (Simon and Samuel, 2007b).

In the mouse model of typhoid fever Ikeda et al showed a selective advantage for FliC flagella. *S. Typhimurium* expressing FliC flagella appeared to grow or survive better than FljB expressing strains in the spleens of infected mice. In bovine loops and epithelial cells there appeared to be no such advantage (Ikeda et al., 2001). These

results may suggest a role for phase variation in the typhoid type of infection but perhaps no such requirement in gastroenteritis.

1.8. Flagella as an adhesin

Previous studies on flagella from several bacterial species including *Campylobacter*, *Pseudomonas*, and *Escherichia coli* have shown that flagella are important as an adhesin (De Bentzmann et al., 1996, Newell et al., 1985, Mahajan et al., 2009).

Salmonella enterica subtype enterica serovar Enteritidis flagella have been shown to be important for bacterial binding to chick gut explants (Allen-Vercoe and Woodward, 1999). Furthermore, Dibb-Fuller et al. showed that *S. Enteritidis* flagella mutants adherence to human intestinal epithelial cell lines INT-407 and Caco-2 was reduced in comparison to the WT (Dibb-Fuller et al., 1999).

1.9. Hypothesis and aims

Flagella are one of the pathogen associated molecular pattern (PAMP) proteins present on the surface *Salmonella*. Recognition of flagella by TLR-5 results in a pro-inflammatory response. Flagella play a key role during host-pathogen interaction by making the bacteria motile. This is important for the invasion of host cells and the survival of the pathogen. We hypothesised that the deletion of *fliC* and *fljB* would affect the adherence and invasion *Salmonella* Typhimurium (SL1344). This hypothesis was investigated using *in vitro* infection models including *ex vivo* colonic explants. A vaccine trial in pigs was carried out to investigate the immunogenicity and potential of these purified flagella proteins to act as part of a multivalent *Salmonella* vaccine in pigs. Pigs were monitored for clinical signs, *Salmonella* shedding rate, tissue colonisation and the specific antibody responses.

2. Establishment and characterisation of porcine primary colonic cell culture

2. 1. Introduction

The principle site of colonisation by *Salmonella* in mice are the lymphoid follicles of Peyer's patches in the ileum (Carter and Collins, 1974, Hohmann et al., 1978). Following this discovery in mice, it has been shown in livestock species that *Salmonella* invade the cells of the ileum including the cells of the Peyer's patch and surrounding epithelium (Santos et al., 2002, Reed et al., 1986, Bolton et al., 1999). The epithelium overlying the Peyer's patches is distinct from the surrounding epithelium, as it expresses M-cells and no goblet cells (Wray and Wray, 2000). The epithelium surrounding Peyer's patches consists mainly of enterocytes and these cells form the majority of the cell types in the intestine.

Much of the current knowledge of *Salmonellae* interactions with specific host cells has come from work using cell lines (Wray and Wray, 2000). Cell lines are typically derived from host cells that have lost the ability to regulate normal cell growth and division. These cells can conveniently continue to grow and divide, almost indefinitely. The Caco-2 cell lineage, which is a widely used *in vitro* model of human intestinal epithelium, is derived from a human adenocarcinoma (Fogh and Trempe, 1975). Despite their immortalisation, Caco-2 cells have been shown to resemble enterocytes and are capable of forming polarized monolayers (Chantret et al., 1988). Caco-2 cells have been an important tool in the study of the epithelial cell interactions of *Salmonellae* (Finlay and Falkow, 1990, Ogushi et al., 2001, Coconnier et al., 2000).

A number of well-characterised intestinal epithelial cell lines have been developed from other, non-human host species. In particular, epithelial cell lines have been successfully derived from different regions of the porcine gastrointestinal tract. IPEC-J2 cells are derived from porcine jejunum, while IPI-2I cells are derived from the porcine ileum. IPEC-J2 and IPI-2I cell lines have both been shown to resemble enterocytes, and form tight junctions and monolayers, using microscopy techniques (Mariani et al., 2009, Schierack et al., 2006, Kaeffer et al., 1993). These cell lines have been used to study the response of porcine intestinal epithelial cells to

Salmonella LPS (Arce et al., 2008), and the production of porcine beta defensins in response to *Salmonella* infection (Veldhuizen et al., 2006).

The use of cell lines is not without its disadvantages. In order to become continuously growing, the majority of cell lines have undergone some sort of cell-cycle changes and selection. Furthermore, where cells have been passaged many times, cell characteristics can be lost. In high passage number lineages of caco-2 cells, a reduced density of microvilli is readily observed (Elvidge. J, unpublished). The use of caco-2 cells has been reviewed by Sambury *et al.* this review highlights some of the issues with this cell model, including the use of different clonal lines and sub-types within caco-2 lineages used across different labs, potentially making study comparisons difficult (Sambury et al., 2005). Furthermore, cell lineages such as caco-2 generally contain cells of only one type. Together, cellular changes as a result of continuous growth and lack of cell diversity may mean that *in vitro* cell line monolayers are unlikely to express the full complement of *in vivo* cellular receptors. If this were the case, they could not fully reflect the conditions present in an intact intestinal epithelium.

One alternative to the use of cell lines is primary cell culture. Primary intestinal cell cultures can be derived from culturing isolated intestinal crypts. A cell culture model developed from porcine intestinal crypts would have some advantages over immortalised cell lines. Cultured crypts (primary cells) have the potential to produce a monolayer of cells consisting of more than one cell type, as seen *in vivo*. Cell types including epithelial cells, and M-like cells have been demonstrated to grow in crypt cultures (Miyazawa et al., 2010). Furthermore, the cells in primary cultures have not been immortalised or passaged many times, perhaps providing a closer representation of an intact intestinal epithelium.

Primary cell cultures shown to resemble the native tissue could provide relevant systems in which to study host-pathogen interactions. Primary intestinal cell cultures from the bovine intestine have been developed and characterised to this end (Miyazawa et al., 2010, Mahajan et al., 2009). These cultures were used to study the

role of flagella in *Escherichia coli* O157:H7 adherence to the bovine terminal rectum (Mahajan et al., 2009). This knowledge was applied in the development of an *E. coli* O157:H7 flagella-based vaccine in the bovine reservoir (McNeilly et al., 2008, McNeilly et al., 2010). Therefore a relevant primary cell culture model may provide new insights into how *Salmonella* interacts with the porcine intestinal epithelium, with potential applications for its control within this reservoir species.

Intestinal epithelia are comprised of several cell types, including absorptive enterocytes, enteroendocrine cells, goblet cells, Paneth cells and M-cells (Appleton et al., 1980). Stem cells are present at the base of intestinal crypts (Figure 2.1) and function to self-renew and replenish the cells of the intestine. These stem cells divide, and with each division they displace the cells near the top of the crypt outwards, to form the intestinal surface (Appleton et al., 1980).

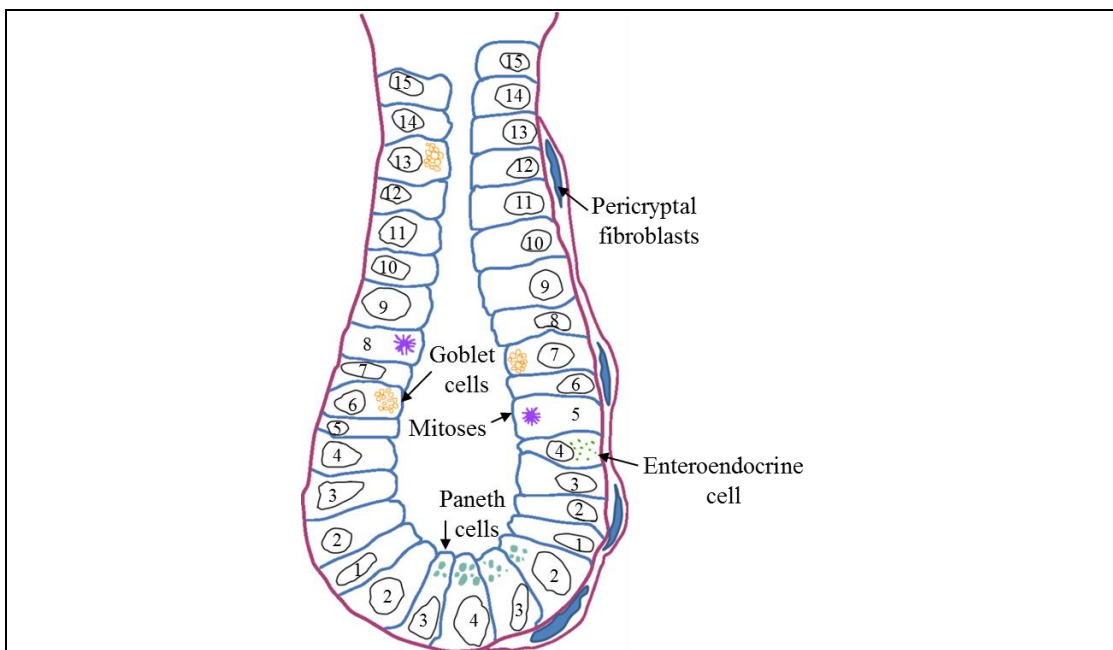


Figure 2.1. Schematic representation of a small intestinal crypt (adapted from Appleton, Sunter et al. 1980).

Location of the different cell types in the small intestinal crypt. Cells are labelled from 1 to 15. Stem cells are present at position 1. Cell division above position 10 is uncommon. The majority of cells are displaced upward from position 1 (Appleton et al., 1980).

2.2. Aims

- To develop a method for the purification and culture of porcine intestinal crypts from the colon of adult pigs presented for slaughter.
- To characterise this *in vitro* porcine primary colonic cell culture model.
- To investigate *S. Typhimurium* interactions with porcine primary intestinal cells.

Achieving these aims will establish a new *in vitro* infection model for the study of porcine intestinal pathogens, specifically *S. Typhimurium*.

2.3 Materials and methods

2.3.1. Bacterial growth conditions

Strains (Table 2.1) were grown at 37°C in Luria-Bertani (LB) broth (Invitrogen) with shaking (200 rpm), and statically on LB agar (37°C, 18h).

2.3.2. Bacterial strains

Table 2.1. *S. Typhimurium* strains used in this study.

Strain	Characteristics	Source & Reference
<i>S. Typhimurium</i> VacT (CMG 39)	Attenuated, resistant to naladixin. Derived from Maskan strain	Lohmann animal health
<i>S. Typhimurium</i> Maskan (CMG 38)	WT	Lohmann animal health
<i>S. Typhimurium</i> (CMG 35)	WT (SL1344)	Institute of animal health

2.3.3. Acetic acid treatment of *ex vivo* porcine ileum

Porcine ileum tissue was obtained from a local abattoir and transported on ice to the laboratory. The ileum was opened longitudinally and rinsed with water. The ileum was placed into 2% acetic acid (Sigma) overnight at 4°C then rinsed with water before photographs were taken by Norrie Russell.

2.3.4. Isolation of porcine colonic epithelium

A local abattoir generously provided porcine colonic tissue from adult pigs which were presented for slaughter. The tissues were obtained following the abattoirs procedures for removing hair, by treatment with hot water. Tissues were transported on ice to the laboratory. The sections of tissue obtained from the abattoir were assessed visually to determine the type of tissue. The presence of Peyer's patches in the ileum/jejunum, the size of the intestinal section and appearance of the mucosal surface were all used to confirm tissue type. Sections of intestine were opened longitudinally and rinsed with water. Sections ~20cm in length were placed into sterile pots of washing media (Hanks Balanced Salt Solution (HBSS) containing

100U/ml penicillin, 30µg/ml streptomycin (Invitrogen), 25µg/ml gentamicin (Sigma) and 2.5µg/ml amphotericin B (Fungizone, Invitrogen)).

2.3.5. Isolation of porcine colonic crypts

The mucosal epithelium was scraped gently with a glass slide to remove excess mucus. This was followed by firmer scrapping of the mucosal epithelium to remove epithelial cells from the connective tissue beneath. Scrapings were collected, shaken in wash media, and then centrifuged at 300×g for 2min. The supernatant was discarded. This washing was repeated until the supernatant was clear. The final pelleted tissue was digested in ~1:1 in digestion media (Dulbecco's Minimum Essential Medium (DMEM, Sigma), 1% (v/v) FBS (Sigma), 100U/ml penicillin, 30µg/ml streptomycin, 25µg/ml gentamicin with 75U/ml collagenase (Sigma C2674) and 20µg/ml dispase I (Roche)). Tissue scrapings were digested for 40min at 37°C, 200rpm. Post-digestion, a small aliquot of the digested tissue was observed under the light microscope to assess the integrity of the crypts. The digested tissue was washed ~1:2 in wash media by gentle mixing. The suspension was then centrifuged at 300×g for 2min to separate intact crypts from other cellular debris. This washing process was repeated until the supernatant was clear. Once isolated, the crypts were re-suspended in culture media (described in section 2.3.6).

2.3.6. Porcine primary colonic cell culture

Crypts were cultured on Type IV collagen (Porcogen) coated plates or coverslips. For collagen coating, 200µg/ml acidified collagen was added to each well of a 24 well plate and left to air dry (laminar flow hood) for 16h at RT. Crypts were diluted in culture media to give ~1600/ml. Isolated crypts (~800 crypts) were added to each well of a 24-well tissue culture plate (Corning, with or without coverslips), or into a 12-well trans-well plate (Corning). The crypts were incubated for 48h at 37°C in 5% CO₂, in primary culture medium which had a final composition of 5% (v/v) FBS, 100U/ml penicillin, 30µg/ml streptomycin (Invitrogen), and 2.5µg/ml amphotericin B (Invitrogen) in DMEM/HAMS F-12 (BioWhittaker). After 48h, cellular debris was gently removed and new media added. For maintenance of the cells, every 48h half

the media was removed from the top of the well and replaced with an equal volume of fresh media.

2.3.7. Trans-epithelial electrical resistance (TEER)

Trans-epithelial electrical resistance (TEER) was measured to confirm cell monolayer integrity. Cells were seeded onto 3.0µm pore size transwell-inserts (Corning). TEER was measured at several time points (World Precision Instruments). The resistance value obtained was calculated using the following equation:

$$\Omega/\text{cm}^2 = \frac{(\text{cell well TEER} - \text{blank well TEER})}{\text{well area size (cm}^2\text{)}}$$

(Miyazawa et al., 2010)

2.3.8. Paracellular permeability using FITC-Dextran

To determine paracellular permeability 20kDa FITC-dextran (Sigma) was applied to the apical surface of the cell monolayer grown on 3.0µm pore, trans-well plates (Corning). FITC-dextran was diluted into DMEM with 10% (v/v) FBS (Sigma) to a final concentration of 1.0 mg/ml. After 3h incubation, both apical and basal media were collected and fluorescence intensities measured at 528 nm, with a sensitivity setting of 35 (BioTek, Synergy HT)(Miyazawa et al., 2010).

2.3.9. Immunofluorescence staining

Cells were grown on glass coverslips until confluent. Cells were washed with phosphate buffered saline (PBS) and fixed with 2% (w/v) paraformaldehyde (PFA, Sigma) for 20min at RT, then permeabilised with 0.2% (v/v) Triton X-100 (Sigma) for 2min, RT. The cells were then gently washed with PBS three times and stained with a pan-cytokeratin which recognises cytokeratins; 1, 5, 6, and 8 mouse IgG antibody (1:100, Sigma), or cytokeratin-18 mouse IgG antibody (1:100, clone CK5, Sigma,) for 16h at 4°C. The cells were then washed again with PBS three times. The antibody was detected with anti-mouse FITC-conjugated rabbit IgG antibody (Sigma, 1:100). Finally, the cells were washed with PBS before being fixed onto a glass slide with fluorescence mounting medium (Vectashield containing DAPI, Vector

Labs). The slides were stored at 4°C in darkness until viewed under the confocal microscope.

2.3.10. Confocal microscopy

Imaging was carried out at the IMPACT facility run by Trudi Gillespie in the Centre for Integrative Physiology at the University of Edinburgh. The images were captured using a Zeiss Axiovert confocal system using Argon and Helium/Neon lasers and a Titanium:Sapphire multi-photon laser. Z-slices were imaged at an optimum distance calculated by Zeiss software. Projections of the stacked images were produced using AxioVision and the final images were presented using GIMP2.6.11.

2.3.11. Ultra-structural studies

Sections of intestinal tissue (1cm × 1cm) were cut and placed into a 24-well tissue culture plate (Corning). *S. Typhimurium* (SL1344) suspended in MEM HEPES (Sigma) were added to the sections of intestinal tissue, 10⁷ bacteria/section (100µl) and incubated at 37°C in 5% CO₂ for 30, 60 and 90min. The sections were fixed with 3% (w/v) glutaraldehyde in 100 mM sodium cacodylate pH 7.4 and stored at 4°C.

For ultra-structural studies of porcine intestinal primary cell cultures, isolated crypts were seeded onto collagen coated 13mm Thermanox coverslips, and confluent cells were used. The cells were washed with PBS once and *S. Typhimurium* (Maskan) suspended in MEM HEPES (Sigma) were added to the coverslips (10⁶ bacteria/well) and incubated at 37°C in 5% CO₂ for 30, 60 and 90min. The cells were fixed with 3% (w/v) glutaraldehyde in 100mM sodium cacodylate at pH 7.4 and stored at 4°C.

2.3.12. Scanning electron microscopy (SEM)

Processing and imaging of samples for scanning electron microscopy (SEM) was carried out at the Electron Microscopy facility run by Steven Mitchell at the University of Edinburgh. Samples were fixed in 3% (w/v) glutaraldehyde in 100mM sodium cacodylate at pH 7.4 for at least 24h at 4°C before being transported to the Electron Microscopy facility. Samples were subsequently washed three times, (10min each) with 100mM sodium cacodylate, then post-fixed in 1% (w/v) osmium

tetroxide in 100mM sodium cacodylate for 45min. Samples were then washed again (three times for 10min each) with 100mM sodium cacodylate and dehydrated in graded acetones (10min each), followed by another two changes (10min) in analar acetone. Dehydrated samples were then critical point dried in a Polaron E 3000 series II drying apparatus, mounted on aluminium stubs, coated in an Emscope SC 500 sputter coater with a 10nm thick layer of gold palladium, and viewed in a Hitachi S-4700 scanning electron microscope. The images were presented using GIMP2.6.11.

2.3.13. *S. Typhimurium* adherence and invasion of porcine primary colonic cells

A freshly grown agar plate culture of *Salmonella* (VacT) was used to inoculate 10ml of LB. Bacteria were grown in a shaking incubator at 200 rpm at 37°C for 18h. Bacteria were diluted and grown to an OD₆₀₀ of 0.4, pelleted and re-suspended in the same volume of MEM HEPES (Lonza). Intestinal colonic cells were washed with MEM HEPES before 50µl of bacterial suspension was added. Cultured cells were incubated at 37°C in 5% CO₂ for 15, 30, 60, or 120min. For quantification of intracellular bacteria, gentamicin was added to the wells (100µg/ml, 600µl/well) for 20min to kill extracellular bacteria. After bacterial challenge the cells were washed with PBS (Sigma) and treated with 0.5% (v/v) triton X-100 (Sigma) (250µl per well). The cells were mechanically removed from the bottom of the wells using a scraper. The collected suspension was serially diluted and plated onto LB agar (Invitrogen) plates, which were incubated at 37°C, 18h. Bacterial colonies were counted, with binding and invasion counts expressed as colony forming units per ml (CFU/ml) (van Asten et al., 2004, McCormick et al., 1993, Lee and Falkow, 1990).

2.4. Results

Specific areas of the intestine are comprised of specialised cell populations. The ileum contains patches distinctly different to the surrounding epithelium. These patches are known as Peyer's patches and contain the majority of M-cells in the ileum. Figure 2.2 shows sections of ileum tissue with and without treatment with acetic acid. The Peyer's patch is labelled with an arrow. A section of porcine colon is shown in Figure 2.2(C). The colon has no detectable Peyer's patches and the epithelial layer consists mainly of enterocytes. However, isolated lymphoid follicles are found throughout the porcine colon, with an increased frequency in the central colonic flexure (Liebler-Tenorio and Pabst, 2006). The Peyer's patches and isolated lymphoid follicles of the colon form the follicle associated epithelium (FAE) of the intestine. The FAE comprise specialised cell populations, the FAE overlies a sub-epithelial dome, containing M-cells, macrophages, dendritic cells, and B and T cells.

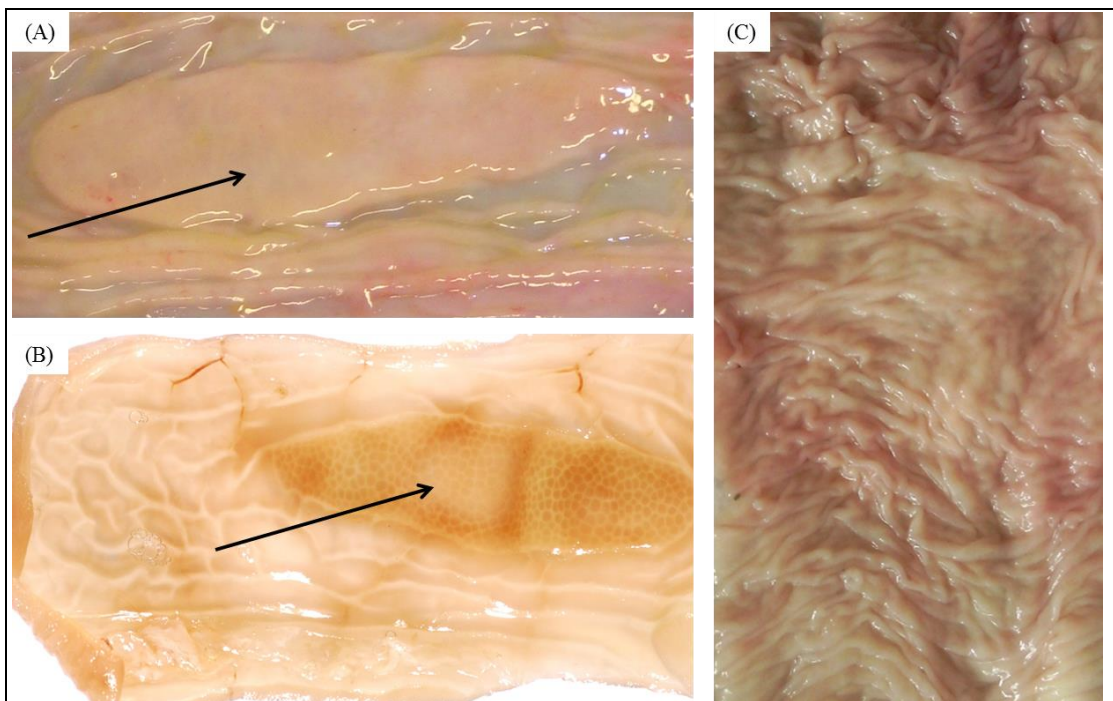


Figure 2.2 Peyer's patches of the porcine intestine.

Sections of Ileum tissue without (A) and with (B) acetic acid treatment (methods section 2.3.3). Peyer's patches are labelled with arrows. (C) A section from porcine colon. There are no visible Peyer's patches. Photograph (B) taken by Norrie Russell.

2.4.1. Development of porcine colonic primary cell culture

Intact intestinal crypts were isolated from porcine colonic tissue. The previously published method for isolating and culturing bovine terminal rectal crypts was used as a starting point (Mahajan, Currie et al. 2009), though many modifications had to be made to optimise both the isolation and culture of porcine colonic crypts.

The isolation of crypts from both the ileum and jejunum were also attempted. However, these crypts proved more difficult to isolate from cell debris and also proved more difficult to grow in culture. It was for these reasons that the culture of colonic crypts was chosen for further studies. The colon contains isolated lymphoid follicles in addition, to many enterocytes. It was expected that a colonic cell culture would contain mainly enterocytes with the possibility that some M-cells might be present.

The method for isolation of bovine rectal crypts describes digestion for a period of 120min. It was found that this incubation period was too long for isolation of intact porcine colonic crypts. After 120min the integrity of crypts had started to fail due to the digestive enzymes present. Incubation times ranging from 30-120min were attempted to optimise the number of intact porcine crypts present in the media. It was found that a digestion period of 40min with collagenase and Dispase I enzymes yielded a high number of intact colonic crypts.

Once digested, the tissue/crypt suspension was washed 5-7 times with HBSS as described in the bovine terminal rectal crypt isolation method (Mahajan et al., 2009). However, the large number of crypts present in the porcine digest meant that less than half of the digested tissue was used for further culture. This was diluted 1:4 with wash media (HBSS containing 100U/ml penicillin, 30µg/ml streptomycin (Invitrogen), 25µg/ml gentamicin (Sigma) and 2.5µg/ml amphotericin B (Fungizone, Invitrogen)). The digest was washed by centrifuging (300×g, 2min). The cellular debris generated during digestion was smaller than the crypts and remained in suspension, whilst the crypts formed a loose pellet at the bottom of the tube.

To culture the isolated crypts, several different compositions of primary cell culture media were used. The first primary cell culture medium used was the bovine terminal rectal crypt culture media described in Mahajan *et al.*, 2005. This media consisted of DMEM (Sigma) with FBS (2% (v/v), Sigma), gentamicin (25µg/ml, Sigma), penicillin (100U/ml, Invitrogen), streptomycin (30µg/ml, Invitrogen), L-glutamine (2mM, Invitrogen), epidermal growth factor (10ng/ml, Sigma), and insulin (0.25U/ml, Sigma). Following the protocol laid out in Mahajan *et al.*, after 48h the media was changed from primary culture medium to feeding medium (MEM D-valine (Sigma) containing 5% (v/v) FBS, gentamicin (25µg/ml, Sigma), penicillin (100U/ml, Invitrogen), streptomycin (30µg/ml, Invitrogen), L-glutamine (2mM, Invitrogen), epidermal growth factor (10ng/ml, Sigma), and insulin (0.25U/ml, Sigma). It was found that porcine crypts did not differentiate well following this protocol, as confluent cell growth was not achieved. Therefore several modifications were made to optimise crypt differentiation. Firstly, half the porcine colonic cultures were kept in primary culture media for one week as an alternative to changing to feeding medium. However, changing the type of medium made little or no difference to the confluent growth of the cells. Neither primary culture medium nor feeding medium grown cells gave confluent growth. In addition, cultures incubated with 7.5% (v/v) FBS compared to 5% (v/v) FBS appeared to have no growth advantage, and neither set of cells reached confluence.

To see if the addition of porcine serum encouraged crypt differentiation, porcine serum and FBS-based feeding medium were also directly compared. To obtain porcine serum, whole blood was obtained from a local abattoir, allowed to clot and was then centrifuged (4°C, 1500×g, 10min) to obtain serum. This serum was then heat inactivated (56°C, 1h). The origin of the serum appeared to make little or no difference to rate of growth of the cells. These cells were differentiated from the same batch of purified crypts and when cultured in bovine or porcine serum, they appeared to grow at the same rate. As heat-inactivated FBS is readily available to purchase, and because FBS is less likely to contain specific antibodies, which might

interfere with pathogen experiments on the cells, it was decided to proceed with FBS as a growth supplement rather than porcine serum from adult pigs.

After trying several different combinations of supplements in DMEM and MEM D-valine with little success, a different base medium was tried; DMEM/Hams F-12 (BioWhittaker). DMEM/Hams F-12 is the base media used to grow IPEC-J2 cells, a porcine intestinal epithelial cell line (Schierack et al., 2006) derived from the jejunum of an un-suckled neonatal piglet, maintained in continuous culture. It is also the base media for IPI-2I cells, a transformed (SV40) porcine intestinal epithelial cell line derived from the ileum of an adult boar (Kaeffer et al., 1993, Arce et al., 2008). Two compositions of DMEM/Hams F-12 as a base medium were tried. IPEC-J2 based media; 5% (v/v) FBS (Sigma), 100U/ml penicillin, 30µg/ml streptomycin (Invitrogen) and IPI-2I based media; 10% (v/v) FBS (Sigma), 100U/ml penicillin, 30 µg/ml streptomycin (Invitrogen) and 2mM L-glutamine. Porcine crypts appeared grow equally well in both types of porcine media. It was decided that the IPEC-J2 based media would be used to culture the crypts as 5% (v/v) FBS seemed sufficient. After successive fungal contaminations of crypt suspensions, the addition of amphotericin B was tried to see if it impacted on the growth of the crypts. Crypts grew equally well or better with the addition of amphotericin B, which significantly reduced the number of fungus-contaminated wells. The final composition of primary cell culture media consisted of 5% (v/v) FBS (Sigma), 100U/ml penicillin, 30µg/ml streptomycin (Invitrogen) and 2.5µg/ml amphotericin B (Fungizone, Invitrogen) in DMEM/HAMS F-12 media (BioWhittaker). A summary of some of the different media compositions which were used are shown in Table 2.2.

Table 2.2. Determination of appropriate porcine primary cell culture media

Base media	Supplements	Adaptations	Description/findings
DMEM (Sigma)	FBS 2% (v/v) (Sigma), gentamicin (25µg/ml, Sigma), penicillin(100U/ml, Invitrogen), streptomycin (30µg/ml, Invitrogen), L-glutamine (2 mM, Invitrogen), epidermal growth factor (10ng/ml, Sigma), and insulin (0.25U/ml, Sigma)	Cells were grown in this media for longer than the original protocol stated, for 1 week	Porcine colonic crypts adhered but did not differentiate very well in this culture media, despite duration of incubation
MEM D-valine (Sigma)	FBS 5% (v/v) (Sigma), gentamicin (25µg/ml, Sigma), penicillin(100U/ml, Invitrogen), streptomycin (30µg/ml, Invitrogen), L-glutamine (2mM, Invitrogen), epidermal growth factor (10ng/ml, Sigma), and insulin (0.25U/ml, Sigma)	FBS 5% (v/v) (Sigma) increased to FBS 7.5% (v/v) (Sigma) 5% (v/v) FBS was replaced with 5% (v/v) adult porcine serum	Porcine colonic crypts adhered but did not differentiate very well in this culture media. The increase in FBS concentration did not increase crypt differentiation.
DMEM/HAMS F-12 (BioWhittaker)	10% FBS (v/v) (Sigma), 100U/ml penicillin, 30µg/ml streptomycin (Invitrogen) and 2mM L-glutamine		Porcine colonic crypts adhered and differentiated well in this culture media
DMEM/HAMS F-12 (BioWhittaker)	5% FBS (v/v) (Sigma), 100U/ml penicillin, 30µg/ml streptomycin (Invitrogen)	Addition of 2.5µg/ml amphotericin B (Fungizone, Invitrogen)	Porcine colonic crypts adhered and differentiated well in this culture media, with and without addition of amphotericin B

To obtain a single layer of confluent porcine primary cells, an optimal number of crypts needed to be added to each well. Too many crypts per well resulted in the crypts not adhering to the collagen coating, too few crypts resulted in non-confluent growth. Approximately 500-1000 crypts were added to each well of 24-well tissue culture plates coated with collagen Type IV. It was found that 800-1000 crypts per well (of a 24-well plate) were required in order to produce confluent cell layers. This was dependent on the integrity of the crypts isolated. Where crypts were of poor integrity, 1000 crypts/well were required for confluent cell growth. Due to the delicate nature of the crypts, breaking of some crypts during the repeated washing periods was unavoidable. However, the repeated washing was proved necessary, as when large amounts of cellular debris were present crypts were prevented from adhering to the collagen coating. Images of isolated crypts and cultured cells are shown in Figure 2.3.

Initially, bovine type IV collagen (Purecol) was used to coat the tissue culture plates prior to addition of crypts. Porcine type IV collagen (Porcogen) was also used, to see if this would better encourage porcine crypts to adhere and differentiate. These crypts were grown with FBS feeding medium (using DEM and DMEM/F12 as a base). There appeared to be no obvious advantage to using porcine collagen over bovine collagen, as it did not appear to increase crypt adherence or differentiation. However, it was decided to keep using porcine collagen in order to keep inter-species variation to a minimum.

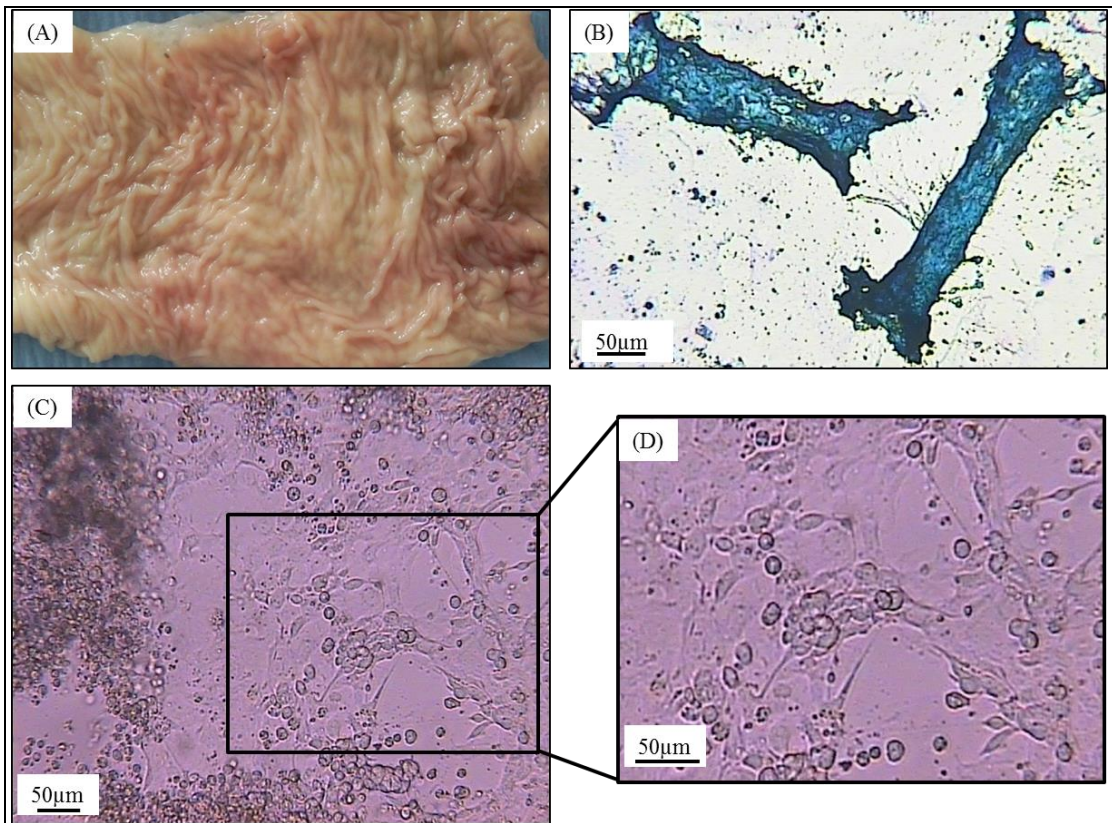


Figure 2.3. Establishment of colonic primary cell culture.

Mucosal scrapings from porcine colon (A) were digested, using 75U/ml collagenase (Sigma) and 20µg/ml dispase I (Roche), to release intact crypts (B). Crypts cleared of tissue and cellular debris were seeded onto Type IV collagen coated plates. Cells following 2 days of culture of crypts at 37°C, 5% CO₂ are shown in (C) and (D).

2.4.2. Porcine primary intestinal cells form confluent monolayers

Porcine colonic primary cells were cultured and appeared to form confluent monolayers, as observed by light microscopy (Figure 2.3). The monolayer integrity was then investigated by measuring trans-epithelial electrical resistance (TEER) over the course of the 20 days of primary cell culture and by measuring the paracellular permeability of the cells by adding FITC-dextran. Figure 2.4(A) shows that the TEER of the cultured cells increased over time. Results in figure 2.4(A) are with primary colonic cells grown in trans-well plates, but a similar pattern of TEER results were observed when these cells were grown in 12-well plates (data not shown). Figure 2.4(B) indicates that there was limited paracellular permeability, as post incubation with FITC-dextran, the level of detectable FITC-dextran was minimal, and any FITC-dextran detected was restricted to the apical surface. This suggests that the cells form tight junctions and that there were few gaps between cells to let the dextran through to the basal surface. These assays confirmed the formation of confluent monolayers by isolated porcine colonic crypts.

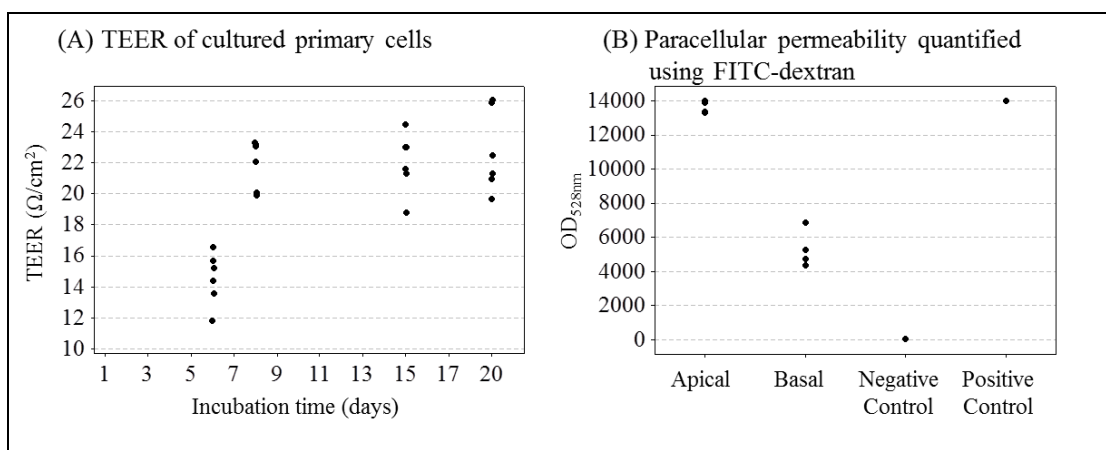


Figure 2.4. Porcine primary intestinal cells form confluent monolayers.

(A) Trans-epithelial resistance (TEER) of primary colonic cultures was measured over time to establish cell monolayer integrity (methods section 2.3.7). Cells were seeded onto 3.0 μm pore size transwell-inserts (Corning). TEER was measured at several time points and each time point shown represents six technical replicates from one plate. (B) Paracellular permeability determination using FITC-labelled dextran (methods section 2.3.8). These absorbance results show four technical replicates.

2.4.3. Porcine primary colonic cells express epithelial specific markers

A primary cell culture developed from colonic crypts could potentially contain a number of cell types. To determine if epithelial cells were the predominant cell type present in the colonic cell culture, the culture was phenotypically characterised using a marker for epithelial cells (Sun et al., 1983). Antibodies to intermediate filament protein cytokeratins 1, 5, 6, and 8 were used to immuno-label epithelial cells. Figure 2.5 shows immuno-fluorescent images of porcine colonic cultured cells labelled with cytokeratin antibodies (green). This figure shows that the majority of cells present were stained by the cytokeratin antibodies (green). The small number of cells not stained by the cytokeratin antibodies are visible as nuclei (blue) alone (Figure 2.5(C)).

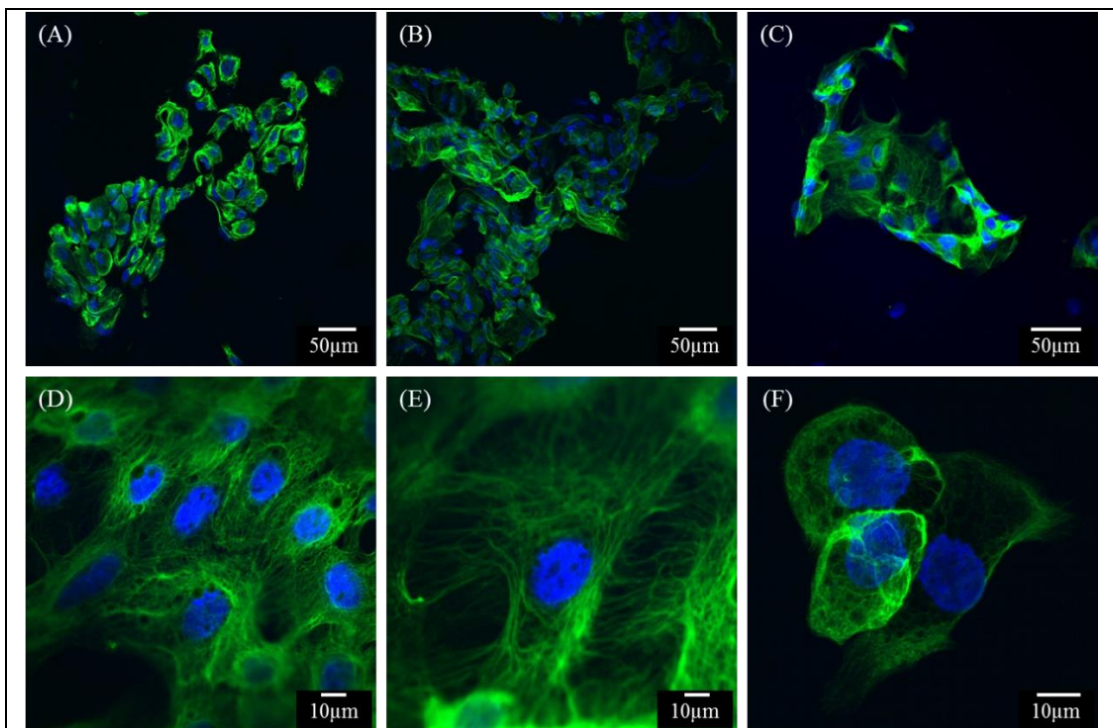


Figure 2.5. Characterisation of porcine primary colonic cells for epithelial-specific cell markers.

(A-E) Immunofluorescence of porcine primary colonic cells with epithelial-specific mouse pan-cytokeratin antibodies (green, methods section 2.3.9). Cell nuclei were stained with DAPI (blue). The majority of the cells cultured appeared to express cytokeratins (A, B, C) and the stained intermediate filaments can be seen clearly in (D, E, F). Images were acquired using Zeiss axiovert microscope and presented using GIMP2.6.11 software.

2.4.4. Ultra-structural characterisation of porcine colonic explants and primary colonic cells using scanning electron microscopy

To establish the presence of microvilli on the porcine primary colonic cultured cells, the cells were imaged using scanning electron microscopy (Figure 2.6). The epithelial surface of the porcine primary colonic cells is covered with dense microvilli. These microvilli were comparable to microvilli seen on other cultured cells and intestinal tissues (Figure 2.7). In addition, to establish if porcine colonic explants could be used as an *ex vivo* model for *Salmonella* infection, explants were imaged using scanning electron microscopy to confirm retention of cell morphology. Figure 2.7 shows that the morphology of the tissue appeared to be retained 1h post-excision from surrounding tissue, if placed in MEM-HEPES at 37°C, 5% CO₂.

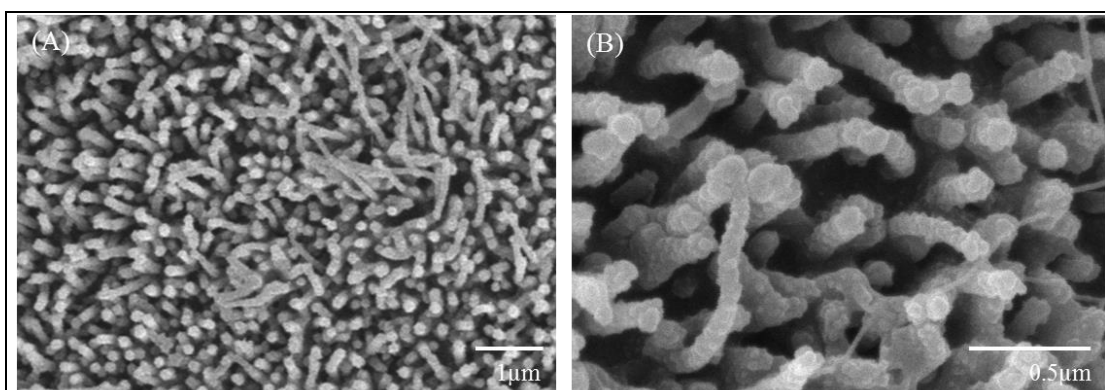


Figure 2.6. Ultra-structural characterisation of primary colonic cells.

(A-B) Scanning electron microscopy of primary porcine colonic cells (methods section 2.3.11). Cells were grown to $\geq 80\%$ confluency then fixed with 3% (w/v) glutaraldehyde in 100 mM sodium cacodylate pH 7.4 and processed for scanning electron microscopy (Steven Mitchell). Images were taken using Hitachi S-4700 scanning electron microscope and presented using GIMP2.6.11 software.

2.4.5. Porcine primary cell culture contains a sub-population of cells, M-cells

As the primary cell culture was developed from colonic crypts, it could potentially contain a number of cell types including M-cells. To determine if M-cells were present in the colonic cell culture, the monolayers were immuno-phenotypically characterised using a marker for porcine M-cells. It was demonstrated by Gebert *et al.* that the cytoskeleton of porcine M-cells differed from surrounding enterocytes. Porcine M-cells were marked by cytokeratin-18 antibodies where the surrounding enterocytes were not. These cells were confirmed to be M-cells by uptake of fluorescein labelled yeast particles (Gebert et al., 1994). Antibodies to intermediate filament protein cytokeratin-18 were used to immuno-label M-cells (Figure 2.7).

Porcine primary colonic cells were imaged using scanning electron microscopy to determine if M-like cells were present in cultures, as M-cells can be characterised by their lack or shortening of microvilli (Owen and Jones, 1974). M-like cells do appear to be present in the primary colonic cell culture (Figure 2.7 & 2.8). Furthermore, *Salmonella* have been shown to enter M-cells (Jones et al., 1994) and *Salmonella* can be seen interacting with these M-like cells of these porcine colonic cultures (Figure 2.8).

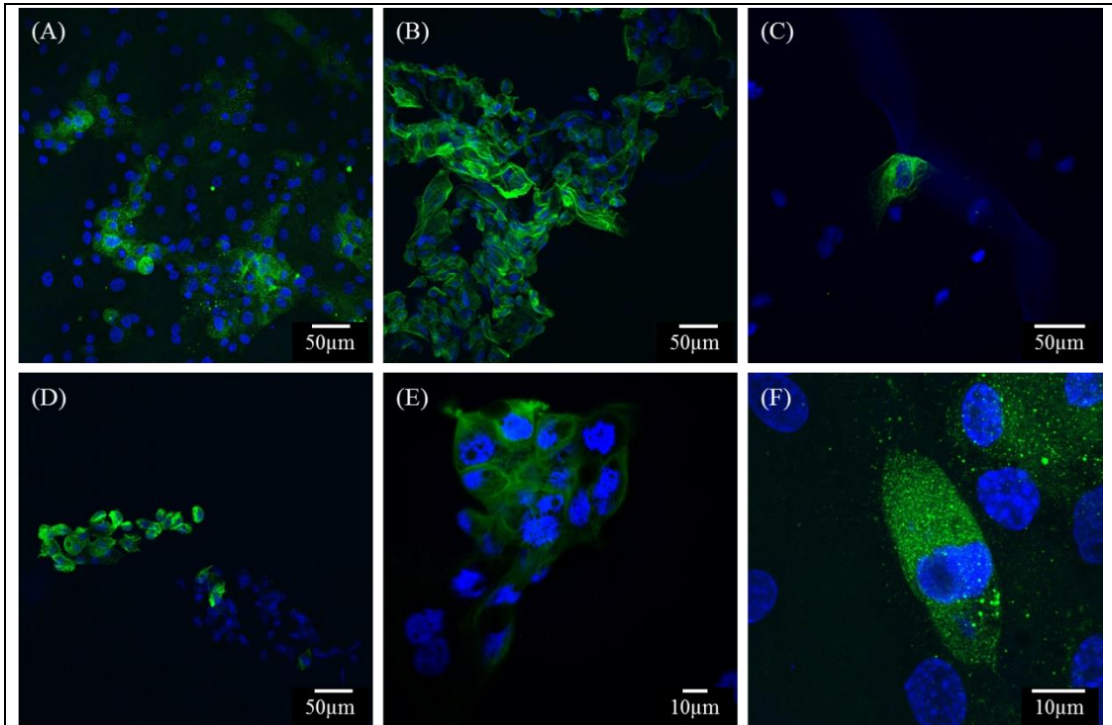


Figure 2.7. Porcine primary cell culture contains M-like cells.

(A-D) Immunofluorescence to determine if cultured cells from porcine colonic crypts contained M-cells an immune-marker for M-cells in pigs, cytokeratin-18 (methods section 2.3.9). Cells were immuno-labelled mouse cytokeratin-18 antibodies (green). Cell nuclei were stained with DAPI (blue). Images were acquired using Zeiss axiovert microscope and presented using GIMP2.6.11 software.

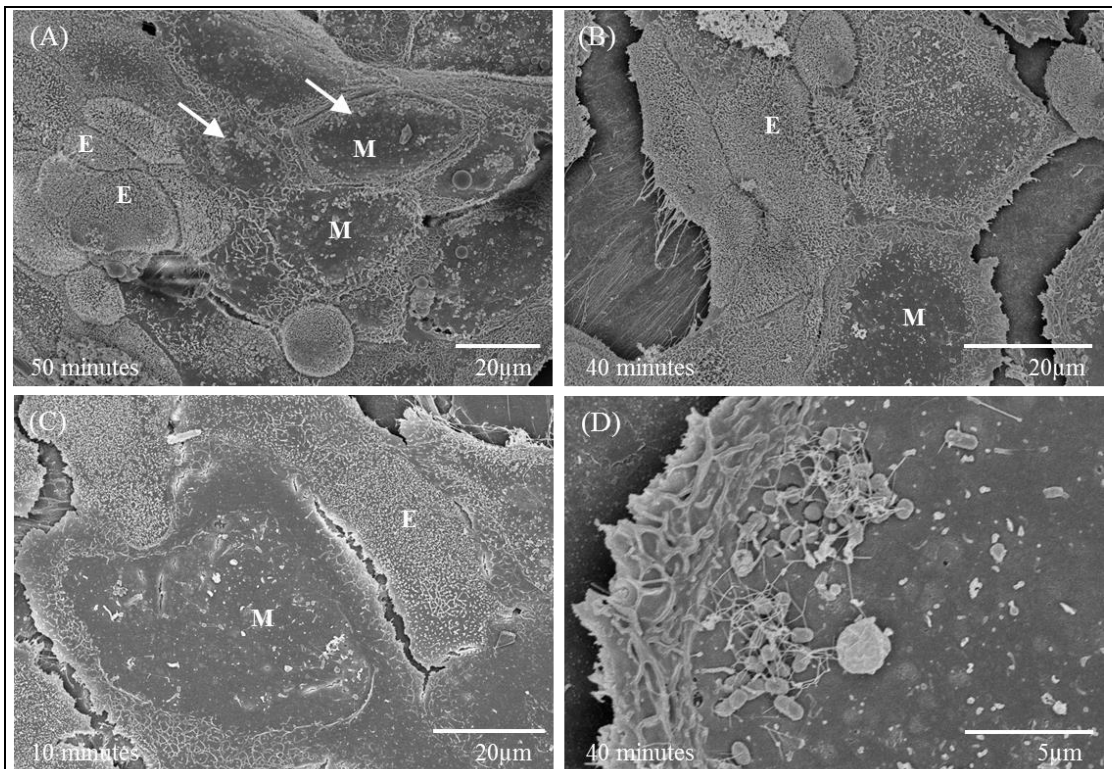


Figure 2.8. Ultra-structural characterisation of porcine primary colonic M-cells by scanning electron microscopy.

In contrast to enterocytes (E) with well pronounced microvilli, a subset of cells in colonic cultures expressed sparse and stunted microvilli typical of M-cells (M). *S. Typhimurium* primarily interacted with cells resembling M-cells indicated by arrows. For scanning electron microscopy (methods section 2.3.12), cells were grown to $\geq 80\%$ confluency then challenged with *S. Typhimurium* (Maskan WT) bacteria at a multiplicity of infection of 10-20 bacteria per cell for the times indicated. Images were acquired using Hitachi S-4700 scanning electron microscope and presented using GIMP2.6.11 software.

2.4.6. Ultra-structural characterisation of *S. Typhimurium* interaction with porcine colonic explants

Salmonella induces cellular changes, such as membrane ruffling, which is typically associated with its invasion of epithelial cells. To ascertain if membrane ruffling could be observed on porcine colonic explants, sections of porcine colonic tissue were challenged with *S. Typhimurium* (SL1344) then imaged using scanning electron microscopy. Figure 2.9 shows actin re-organisation very likely induced by *Salmonella* bacteria on the porcine colonic explants (Figure 2.9(B)).

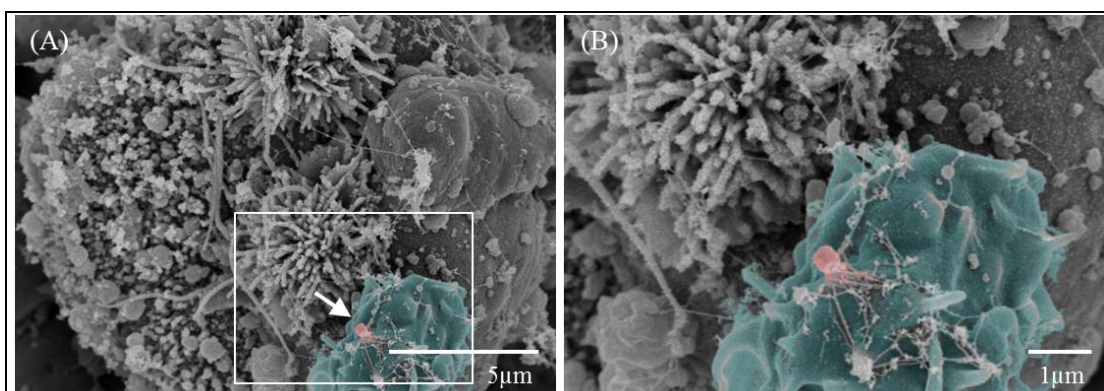


Figure 2.9. Ultra-structural characterisation of *S. Typhimurium* interaction with porcine colonic explants.

Scanning electron microscopy reveals epithelial surfaces of the porcine colonic explants are covered with microvilli and an *S. Typhimurium* bacterium can be seen on the surface (A), coloured red. It appears that actin re-organisation is occurring in the cell where the bacterium can be seen on the surface (B), coloured blue. To ascertain if membrane ruffling could be observed on porcine colonic explants, sections of porcine colonic tissue were challenged with *S. Typhimurium* (SL1344) for 90 min (methods section 2.3.12). Images were acquired using Hitachi S-4700 scanning electron microscope then coloured and presented using GIMP2.6.11 software.

2.4.7. *S. Typhimurium* interaction with porcine primary colonic cells

To verify that similar *S. Typhimurium* interactions occur with porcine colonic primary cells compared to previously published models of infection, primary colonic cells were challenged with *S. Typhimurium* and imaged using scanning electron microscopy (Figure 2.10) and confocal microscopy (Figure 2.11). Figure 2.10 shows *S. Typhimurium* entering cells after different infection periods (40, 50 and 90min). Figure 2.11 shows confocal microscopy images of intracellular *S. Typhimurium*. Orthogonal z-slices reveal that *S. Typhimurium* (red) become intracellular within 30min, as they appear in the same plane on the image as the cytokeratin-stained intermediate filaments (green). These observations suggest that porcine primary colonic cells are invaded by *S. Typhimurium* bacteria in a similar way to that previously demonstrated in published models of *Salmonella* infection (Galán and Zhou, 2000).

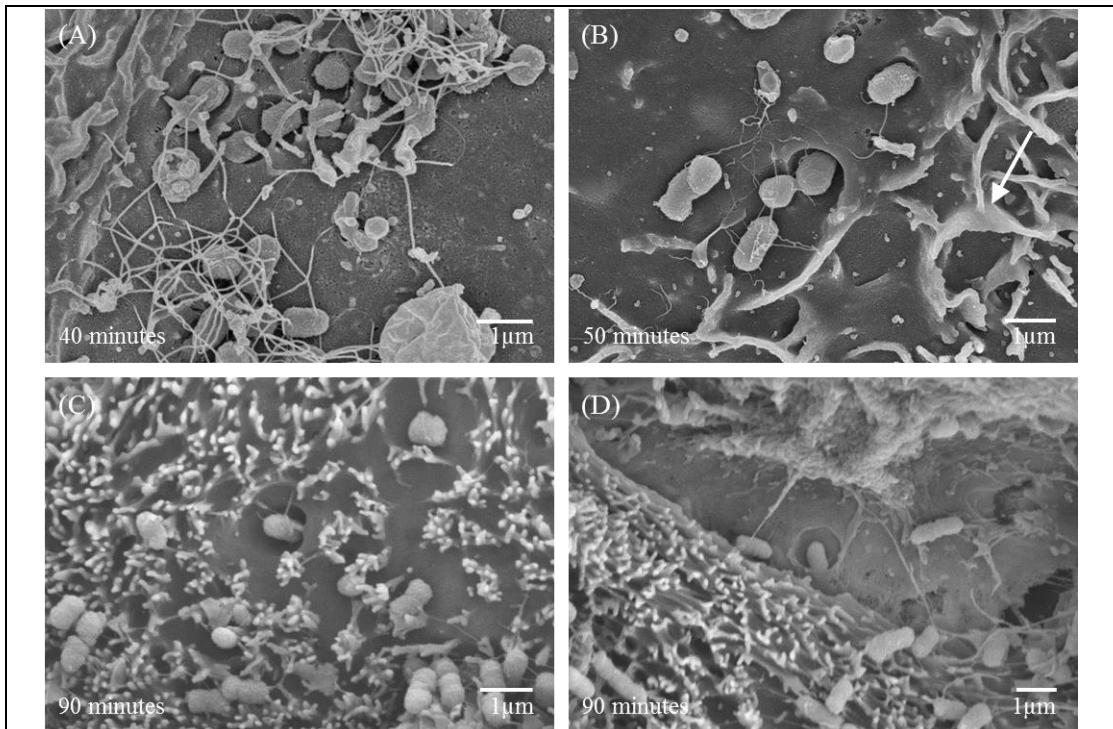
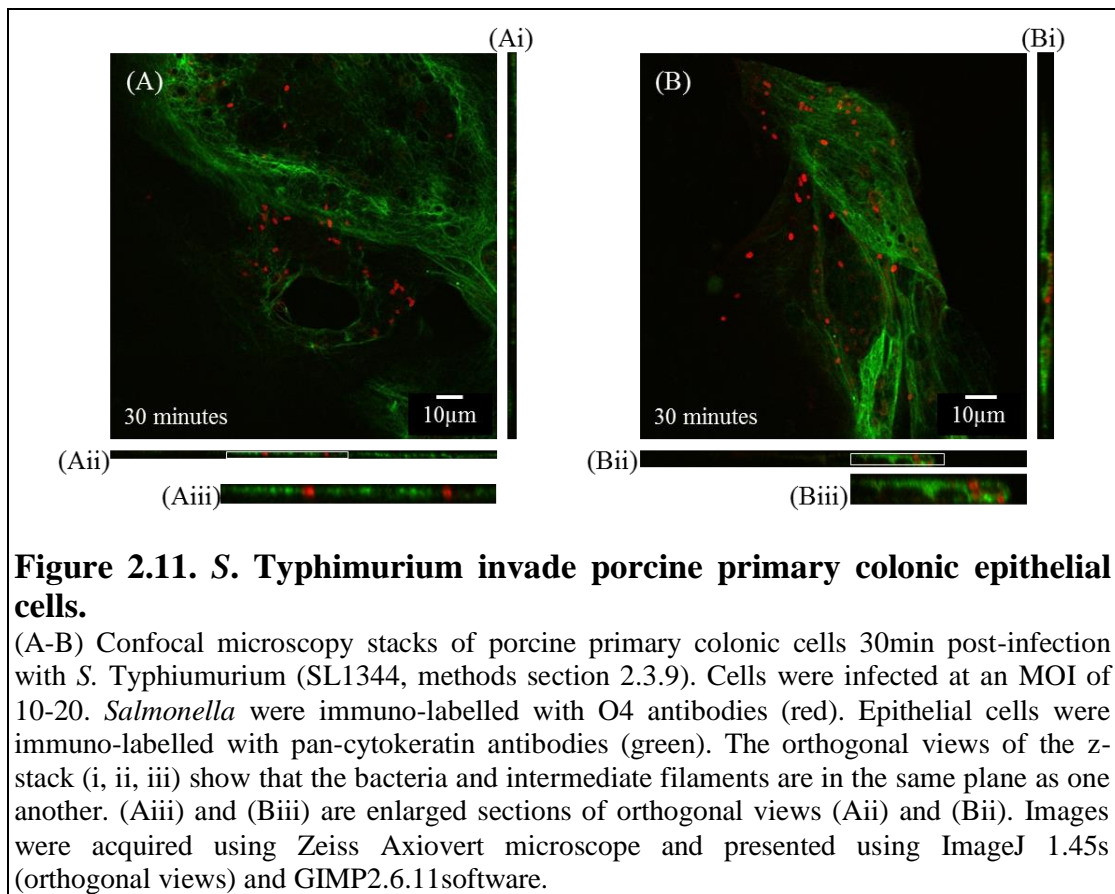


Figure 2.10. *S. Typhimurium* invade porcine primary colonic cells.

(A-D) Scanning electron microscopy of *S. Typhimurium* (SL1344) interactions with porcine primary colonic cells after bacterial challenge for the times indicated (methods section 2.3.12). (B) The bacterium appears to be entering a cell with fewer microvilli and it was postulated that this cell may be an M-cell. Membrane ruffling was also observed and is indicated by an arrow. For scanning electron microscopy, crypts were seeded onto collagen coated Thermanox coverslips. The images were acquired using Hitachi S-4700 scanning electron microscope and presented using GIMP2.6.11 software.



2.4.8. *S. Typhimurium* adherence and invasion of porcine primary colonic cells

Binding assays were conducted to determine if *S. Typhimurium* were able to adhere to and invade the porcine colonic cells. At each of the time points tested, sufficient *S. Typhimurium* were recovered to count colonies on LB agar plates without enrichment (Figure 2.12).

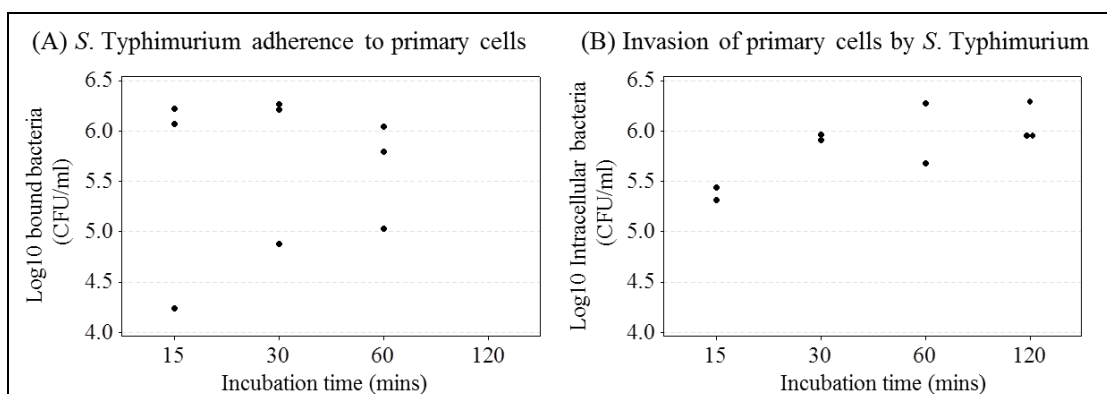


Figure 2.12. *S. Typhimurium* adherence and invasion of porcine primary colonic cells.

(A) Total adherence to porcine primary colonic cells by *S. Typhimurium* (VacT) after times indicated (methods section 2.3.13). (B) Intracellular *S. Typhimurium* (VacT) after challenge of porcine primary colonic cells for times indicated (methods section 2.3.13). Cells were challenged at a multiplicity of infection of 10-20 and adherent and intracellular (post-gentamicin treatment) bacteria were quantified by plating out serial dilutions onto LB plates, expressed as colony forming units per ml (CFU/ml). Results shown are from a minimum of two independent experiments where each experiment had a minimum of three replicates.

2.5. Discussion

A primary cell culture model developed from porcine intestinal crypts has some advantages over immortalised cell lines. Cultured crypts (primary cells) have the potential to produce a monolayer of cells consisting of more than one cell type. This makes primary cell culture more similar to the environment in the host than immortalised cell lines might be.

The primary aim for this study was to develop a porcine primary cell culture from crypts isolated from porcine intestine. A protocol for isolating and culturing porcine intestinal cells from crypts was developed and these cells were then characterised. The cells were observed to form intact monolayers by light microscopy, TEER and were shown to exhibit low permeability to FITC-labelled dextran (Figure 2.4).

Microscopy confirmed that the majority of cells present in the culture were of epithelial origin, expressing cytokeratins as shown by immunofluorescence (Figure 2.5), and microvilli as shown by scanning electron microscopy (Figure 2.6). In addition, it was shown that there was an M-like subset of cells within the primary cell culture. These cells were observed to express cytokeratin-18, a marker for M cells in pigs (Gebert et al., 1994) as shown in Figure 2.7. Figure 2.8 demonstrates a subset of cells with sparse microvilli, which is a classic characteristic of antigen sampling M cells. This observation together with cytokeratin-18 staining indicates that these primary cell cultures contain M cells.

There are other possible types of characterisation that could be undertaken with this *in vitro* infection model, such as expression of various genetic markers by QPCR, which has not yet been undertaken. However, an *in vitro* porcine primary cell culture model method has been developed, and the cell model is sufficiently characterised for the cells to be used for further *in vitro* infection studies.

In addition to the porcine colonic epithelial cell culture model, an *ex vivo* model was developed using thin sections of porcine colon. The colonic explants were shown to retain morphology for at least 90min post-excision and incubation with bacteria.

Furthermore, scanning electron micrographs shown in Figure 2.9 show *S. Typhimurium* interacting with the cell surface and what appears to be actin re-organisation at the cell surface. *Salmonella* induction of actin cytoskeleton rearrangements is a well-documented feature of this organism's infection of enterocytes. This observation suggests that *ex vivo* porcine colonic explants could be one appropriate model for study of *S. Typhimurium* interactions with porcine intestine.

While the aim of this study was realised by the development and characterisation of this porcine primary colonic cell culture model, there were several problems with its use as a routine infection model. Obtaining porcine colonic tissue from the abattoir on a regular basis became impractical due to the reduction in number of pigs being slaughtered and a change in management. High-throughput studies were not possible to undertake, due to the time taken for working cell monolayers to develop (2-3 weeks), and the high susceptibility of primary cells to fungal infection. Due to time constraints, it was decided to reserve the porcine intestinal model for specific assays to answer specific questions and use the intestinal epithelial cell line IPEC-J2 on a routine basis in order to increase output.

**3. *Salmonella* Typhimurium Flagella Filament
Proteins FliC and FljB Play a Role in Bacterial
Adherence to Porcine Intestinal Epithelium**

3.1 Introduction

The primary function of bacterial flagella is to provide motility. However, in increasing numbers of bacterial species it has been shown that motility is not the sole purpose of flagella. The long flagellar filament has been implicated as an adhesin in some bacterial species.

The flagella of *Pseudomonas aeruginosa* have been shown to bind asialoGM1 and GM1, which are expressed on the apical surface of respiratory epithelial cells (De Bentzmann et al., 1996, Feldman et al., 1998). Feldman *et al.* revealed that *P. aeruginosa* flagella were important in the initial stages of infection of pneumonia in the neonatal mouse model. The model demonstrated that only 25% of the mice infected with a *fliC* mutant developed pneumonia, compared with 80% of mice infected by the wild-type strain. This difference was partially attributed to the absence of bacterial motility in the mutant. However, it was postulated that the long flagellar appendage could be used by the organism as a tether to the epithelial surface (Feldman et al., 1998). Furthermore, *P. aeruginosa* flagella have also been shown to bind Muc1, which is abundantly expressed by respiratory epithelial cells (Lillehoj et al., 2002).

Newell *et al.* in 1985 hypothesised that the flagella of *Campylobacter jejuni* bound human epithelial cells. They demonstrated that the flagellated non-motile variant bound to INT 407 and HeLa human epithelial cell lines more efficiently than the aflagellate variant or the wild-type. This may suggest low affinity binding interactions by the flagellum that result in increased binding in the absence of motility shearing forces (Newell et al., 1985). Other studies have shown a role for *C. jejuni* flagella in invasion of gut epithelia, specifically by flagellin FlaA not FlaB (Wassenaar et al., 1991, Grant et al., 1993).

Tasteyre *et al.* demonstrated a role for *Clostridium difficile* flagella in bacterial adherence to the cecum in mice. The numbers of recovered non-flagellated bacteria from mouse cecum were 10-fold lower than the flagellated strain. In addition, purified *C. difficile* flagellar protein FliD was shown to bind to Vero cells (Tasteyre et al., 2001).

Flagella from *Escherichia coli* strains have been implicated in a role for binding epithelial cells. Girón *et al.* observed enteropathogenic *E. coli* (EPEC) flagella binding to HeLa epithelial cells. Furthermore, bacterial binding was reduced in the presence of specific anti-flagella antibodies. Additionally, Girón *et al.* demonstrated that the presence of epithelial cell factors in the growth media up-regulated flagella expression and motility (Girón *et al.*, 2002). Roy *et al.* (Roy *et al.*, 2009) characterised an interaction between enterotoxogenic *E. coli* (ETEC) flagella and EtpA, an adhesin component of a two-partner secretion (TPS) system secreted by ETEC. They went on to demonstrate that this interaction was important for bacterial adherence to Caco-2 epithelial cells (Roy *et al.*, 2009). La Ragione investigated the role of fimbriae and flagella in the role of avian colibacillosis and showed *E. coli* strain O78:K80 flagella were important for adherence to HT2916E, a mucus secreting cell line, but not to Hep-2 cells that do not produce mucus. (La Ragione *et al.*, 2000). Another example of *E. coli* flagella playing a role in host-cell adherence is enterohemorrhagic *E. coli* (EHEC) serotype O157:H7. Mahajan *et al.* showed that H7 flagella mediate initial adherence of EHEC O157:H7 to cattle terminal rectal epithelial cells (Mahajan *et al.*, 2009).

S. Enteritidis flagella have been shown to be important for bacterial binding to chick gut explants (Allen-Vercoe and Woodward, 1999). In addition, Dibb-Fuller *et al.* showed reduced binding by *S. Enteritidis* flagella mutants to human intestinal epithelial cell lines INT-407 and Caco-2 compared to the wild-type strain (Dibb-Fuller *et al.*, 1999). Flagellated *S. Typhimurium* have also been shown to bind cholesterol-coated surfaces *in vitro* (Crawford *et al.*, 2010).

In summary, flagella play a key role during host-pathogen interactions for a number of bacterial species, by mediating motility and initial attachment to colonisation sites. This is important for establishing adherence to and invasion of host cells, and therefore survival and persistence of the pathogen. The specific mechanisms for initial adherence to host epithelia by *S. Typhimurium* have not yet been determined. With the above in mind, it was hypothesised that FliC and FljB may play a role in adherence to the porcine intestinal epithelium.

3.2. Aims

- To determine the specific contributions of FliC and FljB flagella on *S. Typhimurium* binding and invasion in *in vitro* and *ex vivo* porcine intestinal epithelia infection models, and compare this to a human intestinal cell line.
- To assess the physical binding capabilities of FliC and FljB to porcine intestinal epithelial cell lines and colonic explants
- To investigate the role of motility in flagella-dependent porcine intestinal epithelial cell binding of *S. Typhimurium*.
- To use microscopy to investigate *S. Typhimurium* flagella interactions with *in vitro* and *ex vivo* porcine intestinal epithelia.

Achieving the above aims will establish whether there is a role for FliC and/or FljB flagella during *S. Typhimurium* adherence and invasion of porcine intestinal epithelium.

3.3. Materials and methods

3.3.1. Bacterial strains

Table 3.1 Bacterial strains

Strain	Characteristics	Source & Reference
SL1344 (CMG 82)	WT - Expresses FliC and FljB flagella	Angus Best (Arques et al., 2009)
SL1344 Δ <i>fliC</i> (CMG 83)	λ red removal of <i>fliC</i> gene - Expresses FljB flagella	Angus Best (Arques et al., 2009)
SL1344 Δ <i>fljB</i> (CMG 84)	λ red removal of <i>fljB</i> gene - Expresses FliC flagella	Angus Best (Arques et al., 2009)
SL1344 Δ <i>fliC</i> & Δ <i>fljB</i> (CMG 85)	λ red removal of <i>fliC</i> & <i>fljB</i> genes - No flagella filament expression	Angus Best (Arques et al., 2009)

3.3.2. Media and growth conditions

Strains (Table 3.1) were grown at 37°C in LB broth (Invitrogen) or in 0.3M NaCl LB broth with and without shaking (200rpm), or on LB or motility agar (section 3.2.3). Assay-specific media and growth conditions are detailed in their relevant sections.

3.3.3. Motility and flagellar phase switch assay:

Motility agar consisted of 1% (w/v) Tryptone, 0.5% (w/v) NaCl and 0.3% (w/v) granulated agar (Ikeda et al., 2001). One colony from a freshly streaked plate was inoculated into the centre of the motility agar using a sterile needle. Where required, FliC antiserum diluted 1:600, or FljB antiserum diluted 1:600 (*Salmonella* Hi, Mast, and *Salmonella* H2, Difco), were added to the agar just prior to pouring. Plates were incubated 18h at 37°C. The halo of growth was measured from the point of inoculation.

3.3.4. Analysis of flagellin proteins:

Flagellar filament proteins were purified by mechanical shearing of the bacteria with a homogenizer (IKA, T10). *S. Typhimurium* SL1344 single flagella mutant strains were grown statically for 18h at 37°C in 500ml LB. Bacteria were collected by centrifugation (5,000×g 4°C, 15min) then suspended in an equal volume of PBS (Sigma) and collected again before being suspended in 20ml PBS. The suspension

was homogenized on ice, speed 6 for 60s. Bacterial cells were removed by centrifugation (5,000×g 4°C, 15min). The remaining suspension was further centrifuged (16,300×g 4°C, 10min) to ensure complete removal of bacteria. To pellet flagella, the supernatant was centrifuged (145,000×g 4°C, 90min) and the pellet was suspended in 500µl PBS (Ikeda et al., 2001). Flagella suspensions were stored at -20°C.

3.3.4.1. Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE)

The flagellin suspensions were diluted in Laemmli sample buffer (4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (v/v) bromphenol blue and 125mM Tris-HCl, pH 6.8) then heated to 95-100°C for 5min. Flagellin suspensions were then loaded onto a 4% (v/v) polyacrylamide stacking gel and subsequently run through 12%, (v/v) polyacrylamide, 1% SDS (w/v), 1mm thick, resolving gel. The gel was run in 25mM Tris, 192mM glycine, 0.1% (w/v) SDS at 200V for 60min. To stain the protein in the resolving gel, gelcode blue safe (Thermo Scientific) stain was used. Gels were incubated for a minimum of 1h in protein stain with gentle rocking, at room temperature (RT), before de-staining in distilled water, with gentle rocking at 4°C for 16h (Laemmli, 1970). Gel images were acquired using an image scanner (Epson).

3.3.4.2. Western blotting

Flagella proteins in 12% (v/v) SDS-PAGE gels were Western-blotted onto nitrocellulose (Amersham, GE Healthcare) using a semi-dry method. Using a Trans-blot semi-dry transfer cell (Bio-Rad), the semi-dry method was carried out at RT in Schaeffer-Nielsen buffer (48mM Tris, 39mM glycine, 20% (v/v) methanol, 0.04% (v/v) SDS, pH 9.2), and transferred at 15V for 30min.

Table 3.2 Antibodies used in Western blotting and ELISAs

Antibody/stain	Dilution (unless otherwise stated)	Isotype and Species	Supplier
Anti-FliC	1:500	IgG Rabbit	Mast Assure
Anti-FljB	1:500	IgG Rabbit	Difco
Anti-O4 LPS	1:500	IgG Rabbit	Mast Assure
Anti-Rabbit IgG-HRP	1:1000	IgG Goat	BD

Blots were blocked in 5% (w/v) skimmed milk powder (Marvel), 0.1% (v/v) Tween20 (Sigma) in Dulbecco A PBS (PBST) at 4°C for 16h. Primary antibodies, either anti-FliC or anti-FljB monoclonal rabbit IgG (Table 3.2), were diluted in 5% (w/v) skimmed milk powder, 0.1% (v/v) PBST and incubated with blots for >1h at RT, with gentle rocking. Blots then were washed three times in 0.1% (v/v) PBST at 15min intervals with gentle rocking at RT. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibodies (Table 3.2) were diluted in 5% (w/v) skimmed milk powder, 0.1% (v/v) PBST and were incubated with blots for at least 1h at RT with gentle rocking. Following incubation, blots were washed as above.

Blots were developed using an enhanced chemi-luminescence reagent (ECL), specifically Pico-West SuperSignal ECL (Thermo Scientific). The subsequent signal from the blots was captured using GBOX iChemiXT from Syngene and GeneSnap (Syngene) software. Blots captured on the GBOX, were placed on an acetate sheet and covered with a thin layer of ECL reagent. The settings used were a focus of 180 at a distance of 285mm, with the highest quality capture time (Towbin et al., 1979).

3.3.5. Maintenance of cell lines

The porcine intestinal epithelial cell line IPEC-J2, courtesy of Dr Peter Schierack, was maintained as described by Schierack *et al.* 2006. In brief, cells were maintained in DMEM/F-12 (Lonza); 100U/ml penicillin, 30µg/ml streptomycin (Invitrogen); 5% (v/v) foetal bovine serum (Sigma) at 37°C, in 5% CO₂. The medium was changed every 48h. The human intestinal epithelial cell line Caco-2 was maintained in DMEM containing 100U/ml penicillin, 30 µg/ml streptomycin (Invitrogen); 5% (v/v) foetal bovine serum (Sigma) at 37°C, 5% CO₂. The medium was changed every 48h.

3.3.6. Adherence and invasion assay

A freshly grown agar plate culture of *Salmonella* was used to inoculate 5ml of 0.3M NaCl LB (1% (w/v) Tryptone (BD bacto), 0.5% (w/v) Yeast Extract (Sigma) and 1.75% (w/v) NaCl). Bacteria were grown with low aeration in a shaking incubator at 200rpm at 37°C for 18h. Bacteria were then pelleted and re-suspended to an OD₆₀₀ of ~0.3 in MEM HEPES (Lonza). This suspension was serially diluted and plated onto

LB agar (Invitrogen) plates to determine numbers of bacteria added to the cells. Intestinal epithelial cells (IPEC-J2 & Caco-2) were washed with MEM HEPES before 50µl of bacterial suspension was added. Cultured cells were incubated at 37°C in 5% CO₂ for 15 (10min on Caco-2), or 180min. For quantification of intracellular bacteria, gentamicin was added to the wells (100µg/ml, 600µl/well) for 20min to kill extracellular bacteria. After bacterial challenge the cells were washed with PBS (Sigma) and treated with 0.5% (v/v) triton X-100 (Sigma) (250µl per well). The cells were mechanically removed from the bottom of the wells using a scraper. The collected suspension was serially diluted and plated onto LB agar (Invitrogen) plates which were incubated at 37°C, 18h. Bacterial colonies were counted, with binding and invasion counts expressed as a percentage of the number of bacteria added to the cells (van Asten et al., 2004, McCormick et al., 1993, Lee and Falkow, 1990).

Statistical analysis was carried out in Minitab 16. Data were assessed for normality using a Kolmogorov-Smirnov test ($P > 0.05$). Analyses of differences between bacterial strains were carried out on the number of bacteria recovered from each experiment, assessed by using general linear models (GLMs) to look at overall differences. If overall differences were obtained ($P < 0.05$), standard post-hoc Tukey pair-wise comparisons were undertaken to establish differences between bacterial strains. Due to the variation in the number of bacteria added to the cells, the number of bacteria added was included in the analysis as a co-variate.

3.3.6.1. Adherence and invasion assays with centrifugation

In an attempt to assess the role of motility in *S. Typhimurium* flagella binding to porcine epithelia, adherence and invasion assays were carried following centrifugation of bacteria onto the cells.

The bacteria and IPEC-J2 cells were grown, washed and infected as described in 3.2.6. The only difference in protocol was the additional step prior to incubation of the bacteria with the IPEC-J2 cells. The 24-well plates were centrifuged at 300×g for 5min. Incubation, treatment of the cells post infection and analysis of the results was carried out as described in 3.2.6.

3.3.7. In-cell enzyme linked immuno-sorbent assay (ELISA)

3.3.7.1. Flagella adherence Assay of cultured epithelial cells

S. Typhimurium flagella adherence to intestinal epithelial cells was measured using an in-cell enzyme linked immuno-sorbent assay (ELISA). IPEC-J2 and Caco-2 cells were grown to 80-90% confluence in 24-well tissue culture plates (Corning). Purified flagella proteins from *S. Typhimurium* SL1344 were added to the cells (1 µg/well); plates were centrifuged (300×g, 15min) and incubated for 3h (37°C, 5% CO₂, 80% humidity). Cells were then washed three times with PBS, fixed with 2% (w/v) PFA for 15min at RT then permeabilised with 0.1% (v/v) Triton X-100 for 1min. Cells were then washed three times with PBS, before been treated with 1% (v/v) H₂O₂ in methanol to quench endogenous peroxidase activity for 20min, RT. Cells were then washed as above before been blocked with 3% (w/v) bovine serum albumin (BSA, fraction V, Sigma) in PBS at 4°C for at least 16h. After blocking, flagella-specific antibodies (Table 3.2, but anti-FliC rabbit IgG was used at a 1:250 dilution, and anti-FljB IgG rabbit was used at a 1:500 dilution), were added diluted in 3% (w/v) BSA in PBS and incubated with cells for 1h, RT. Cells were then washed as before and HRP-conjugated secondary antibodies (Table 3.2) were added diluted in 3% (w/v) BSA in PBS, incubated for 1h at RT and washed as before. Binding was detected using SuperSignal West Pico ECL reagents (Thermo Scientific) using GBOX iChemiXT running GeneSnap (Syngene) the focus setting was 57 and the distance was set at 575mm. Densitometry was performed on inverted images using GeneTools software (Syngene) with a 75 spot radius. To indicate how confluent the cells were; cells were washed with PBS and 0.1% (v/v) crystal violet stain (Sigma) was added, incubated at 4°C for 30min then washed three times with PBS. 20% (v/v) acetone in ethanol (both Fisher Scientific) was added, incubated for 30min at RT and the absorbance was measured at 595nm using Synergy HT plate reader (BioTek).

Analysis of densitometry results used the following formula:

$$\text{Relative protein binding} = \frac{\text{Densitometry result} - \text{No primary antibody control}}{\text{Crystal Violet result} - \text{No protein added control}}$$

3.3.8. Adherence inhibition assay

To investigate the role of flagella during interaction of *S. Typhimurium* with intestinal epithelium, adherence inhibition assays were performed using both porcine intestinal epithelial cells (IPEC-J2) and *ex-vivo* colonic tissue explants. Flagella specific antisera (Table 3.2) were added to *S. Typhimurium* culture prior to addition to porcine intestinal cells or *ex vivo* colonic tissue. Adherence of *Salmonella* was measured indirectly using *Salmonella* lipopolysaccharide (LPS) antibodies (anti-O4, Table 3.2).

3.3.8.1. Adherence inhibition assay of IPEC-J2 cells

IPEC-J2 cells were grown to confluence in 24-well tissue culture plates (Corning). *Salmonella* were sub-cultured from a streaked LB agar plate into 5ml of 0.3M NaCl LB and cultured with low aeration at 37°C, 200rpm for 18h. Cultures were centrifuged at 4,000×g for 5min and pellets re-suspended in MEM HEPES (Lonza) to an OD₆₀₀ of 0.3. FliC and FljB specific rabbit IgG (Table 3.2) were added to separate bacterial cultures at 1:10 dilution for 30min, with gentle rocking at RT prior to addition to the cells. Bacteria were added to cells at an MOI of 10-20 and incubated for 3h at 37°C 5% CO₂ 80 % humidity post centrifugation at 300×g for 5min. Cells were then washed three times with PBS, fixed with 2% (w/v) PFA for 15min at RT then permeabilised with 0.1% (v/v) Triton X-100 for 1min. Cells were then washed three times with PBS, before been treated with 1% (v/v) H₂O₂ in methanol to quench endogenous peroxidase activity at RT for 20min. Cells were then washed as above before been blocked with 3% (w/v) BSA in PBS at 4°C for at least 16h. After blocking, anti-O4 rabbit IgG (Table 3.2) was diluted in 3% (w/v) BSA in PBS and incubated with cells for 1h at RT. Cells were washed as before then anti-rabbit IgG-HRP (Table 3.2) was added, diluted in 3% (w/v) BSA in PBS, incubated for 1h at RT and washed as before. Binding was detected using SuperSignal West Pico ECL reagents (Thermo Fisher) using GBOX iChemiXT running GeneSnap (Syngene) the focus setting was 50 and the distance was set at 625mm. Densitometry was performed on inverted images using GeneTools software (Syngene) with a 65 spot radius. To indicate how confluent the cells were; cells were washed with PBS

and 0.1% (v/v) crystal violet stain (Sigma) was added, incubated at 4°C for 30min then washed three times with PBS. 20% (v/v) acetone in ethanol (both Fisher Scientific) was added, incubated for 30min at RT and the absorbance was measured at 595nm using Synergy HT plate reader (BioTek).

Results from in-cell binding assays are expressed as relative binding, using the densitometry results and the following formula:

$$\text{Relative protein binding} = \frac{\text{Densitometry result} - \text{No primary antibody control}}{\text{Crystal Violet result} - \text{No protein added control}}$$

Statistical analysis was carried out in Minitab 16. Data were assessed for normality using a Kolmogorov-Smirnov test ($p > 0.05$). Analysis of differences in antibody treated and non-treated bacteria were assessed first by using GLMs to look at overall differences, where p-values of < 0.05 were taken as significant. If overall differences were obtained, standard post-hoc Tukey pair-wise comparisons were undertaken to establish differences between antibody treatments.

3.3.8.2. Adherence inhibition assay of porcine colonic explants

Porcine colonic tissue was collected from Wishaw abattoir and transported to Roslin on ice. The tissue was washed with water to remove faecal matter, and soaked in HBSS containing 100U/ml penicillin, 30µg/ml streptomycin (Invitrogen), 25µg/ml gentamicin (Sigma) and 2.5µg/ml Amphotericin B (Fungizone, Invitrogen)). Mucus was removed and circular explants taken using an 8mm diameter biopsy punch (Stiefel Laboratories). For infection of the explants, *Salmonella* were grown as with IPEC-J2 cell infections, section 3.2.8.1. Explants were challenged with 10^5 bacteria/explant then centrifuged ($300 \times g$, 5min) before been incubated at 37°C, 5% CO₂, 80% humidity for 1h. Explants were then washed three times with PBS, fixed with 2% (w/v) PFA for 15min at RT then permeabilised with 0.1% (v/v) Triton X-100 for 1min. Cells were then washed three times with PBS, before been treated with 1% (v/v) H₂O₂ in methanol to quench endogenous peroxidase activity at RT for 20min. Cells were then washed as above before been blocked with 3% (w/v) BSA in PBS at 4°C for at least 16h. After blocking, anti-O4 rabbit IgG (Table 3.2) was added

to 3% (w/v) BSA in PBS and incubated with cells for 1h at RT. Cells were washed as before then anti-rabbit IgG-HRP (Table 3.2) was added in 3% (w/v) BSA in PBS, incubated for 1h at RT and washed as before. Binding was detected using SuperSignal West Pico ECL reagents (Thermo Fisher) using GBOX iChemixT running GeneSnap (Syngene) the focus setting was 50 and the distance was set at 625mm. Densitometry was performed on inverted images using GeneTools software (Syngene) with a 65 spot radius. To indicate how confluent the cells were; cells were washed with PBS and 0.1% (v/v) crystal violet stain (Sigma) was added, incubated at 4°C for 30min then washed three times with PBS. 20% (v/v) acetone in ethanol was added, incubated for 30min at RT and the absorbance was measured at 595nm using Synergy HT plate reader (BioTek).

Results from in-cell binding assays are expressed as relative binding, using the densitometry results and the following formula:

$$\text{Relative protein binding} = \frac{\text{Densitometry result} - \text{No primary antibody control}}{\text{Crystal Violet result} - \text{No protein added control}}$$

Statistical analysis was carried out in Minitab 16. Data were assessed for normality using a Kolmogorov-Smirnov test ($p > 0.05$). Analysis of differences in antibody treated and non-treated bacteria were assessed first by using GLMs to look at overall differences, where p-values of < 0.05 were taken as significant. If overall differences were obtained, standard post-hoc Tukey pair-wise comparisons were undertaken to establish differences between antibody treatments.

3.3.9. Microscopy

3.3.9.1 Immunofluorescence staining

IPEC-J2 cells were grown to confluence on glass coverslips in 24 well tissue culture plates (Corning). *Salmonella* were sub-cultured from a streaked LB agar plate into 5 ml of 0.3M NaCl LB and cultured with low aeration at 37°C, 200rpm for 18h. Cultures were centrifuged at 4000×g for 5min and pellets re-suspended in MEM HEPES (Lonza) to an OD₆₀₀ of 0.3. Cells were challenged with *Salmonella* for 10 or 20min. Post-challenge, cells were washed three times with PBS. Cells were then

fixed with 2% (w/v) PFA (Sigma) (20min, RT). Cells were then permeabilised with 0.2% (v/v) Triton X-100 (Sigma) (2min, RT). The cells were then gently washed with PBS three times and stained with primary antibodies; anti-O4, anti-FliC or anti-FljB (see Table 3.3 for details) 16h at 4°C. The cells were then washed with PBS three times. The primary antibody was detected with FITC-labelled, anti-rabbit IgG antibody (Table 3.3), incubated for 1h RT then washed as before and further stained with Texas-red labelled phalloidin and then DAPI (see Table 3.3 for details) for 1h each with PBS washing in-between. Finally the cells were washed with PBS before been fixed onto a glass slide with florescence mounting medium (Vectashield, Vector Labs). The slides were stored at 4°C in darkness until viewed under the confocal microscope.

Table 3.3 Microscopy antibody and staining information

Antibody/stain	Dilution (in PBS)	Isotype and Species	Supplier
Anti-FliC	1:100	IgG Rabbit	Mast Assure
Anti-FljB	1:500	IgG Rabbit	Difco
Anti-O4	1:500	IgG Rabbit	Mast Assure
Anti-Rabbit IgG-FITC	1:500	IgG Goat	R&D
Phalloidin-Texas red	1:500		Invitrogen
DAPI	1:1000		Merck

3.3.9.2. Confocal microscopy

Imaging was carried out at the IMPACT facility run by Trudi Gillespie in the Centre for Integrative Physiology at the University of Edinburgh. The images were captured using a Zeiss Axiovert confocal system using Argon and Helium/Neon lasers and a Titanium:Sapphire multi-photon laser. Z-slices were imaged at an optimum distance calculated by Zeiss software. Projections of the stacked images were produced using AxioVision and the final images were presented using GIMP2.6.11.

3.3.9.3. Ultra-structural studies

For ultra-structural studies, isolated crypts were seeded onto collagen coated 13mm Thermanox coverslips. The cells were used when they reached confluence. The cells were washed with PBS once and *S. Typhimurium* (either Maskan obtained from Lohmann Animal Health or SL1344 from Institute of Animal Health) suspended in

MEM HEPES (Sigma) and 10^4 bacteria/well were added to the cells and incubated at 37°C in 5% CO₂ for 30, 60 and 90min. The cells were fixed with 3% (w/v) glutaraldehyde in 100 mM sodium cacodylate pH 7.4 and stored at 4°C.

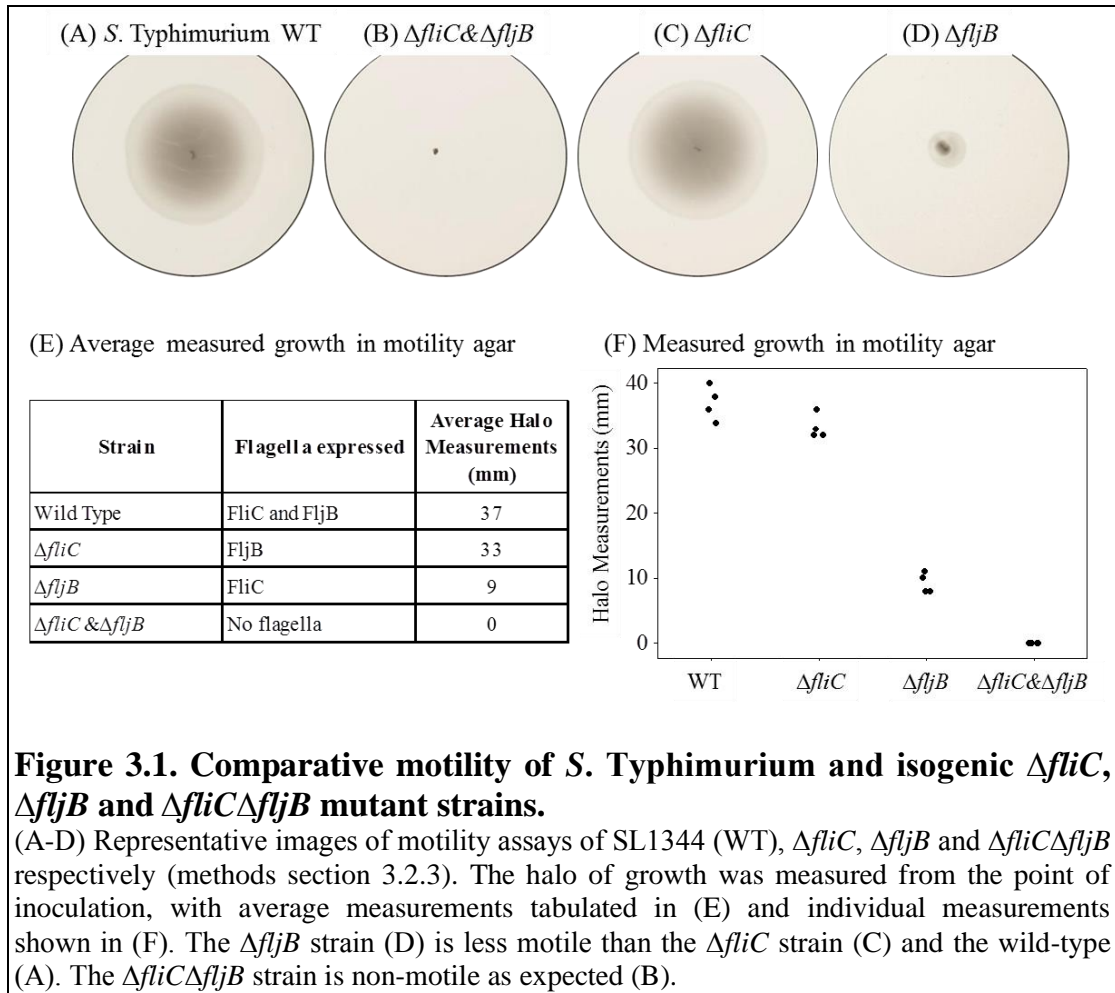
3.3.9.4. Scanning electron microscopy (SEM)

Processing and imaging of samples for scanning electron microscopy was carried out at the Electron Microscopy facility run by Steven Mitchell at the University of Edinburgh. Samples were processed as follows; fixed in 3% (w/v) glutaraldehyde in 100mM sodium cacodylate at pH 7.4 for 24h; washed in three 10min changes of 100mM sodium cacodylate; post-fixed in 1% osmium tetroxide in 100mM sodium cacodylate for 45min; washed in three 10min changes of 100mM sodium cacodylate; dehydrated in graded acetones for 10min each, followed by another two 10min changes in analar acetone. Dehydrated samples were then critical point dried in a Polaron E 3000 series II drying apparatus, mounted on aluminium stubs, coated in an Emscope SC 500 sputter coater with a 10nm thick layer of gold palladium, and viewed in a Hitachi S-4700 scanning electron microscope. The images were presented using GIMP2.6.11.

3.4. Results

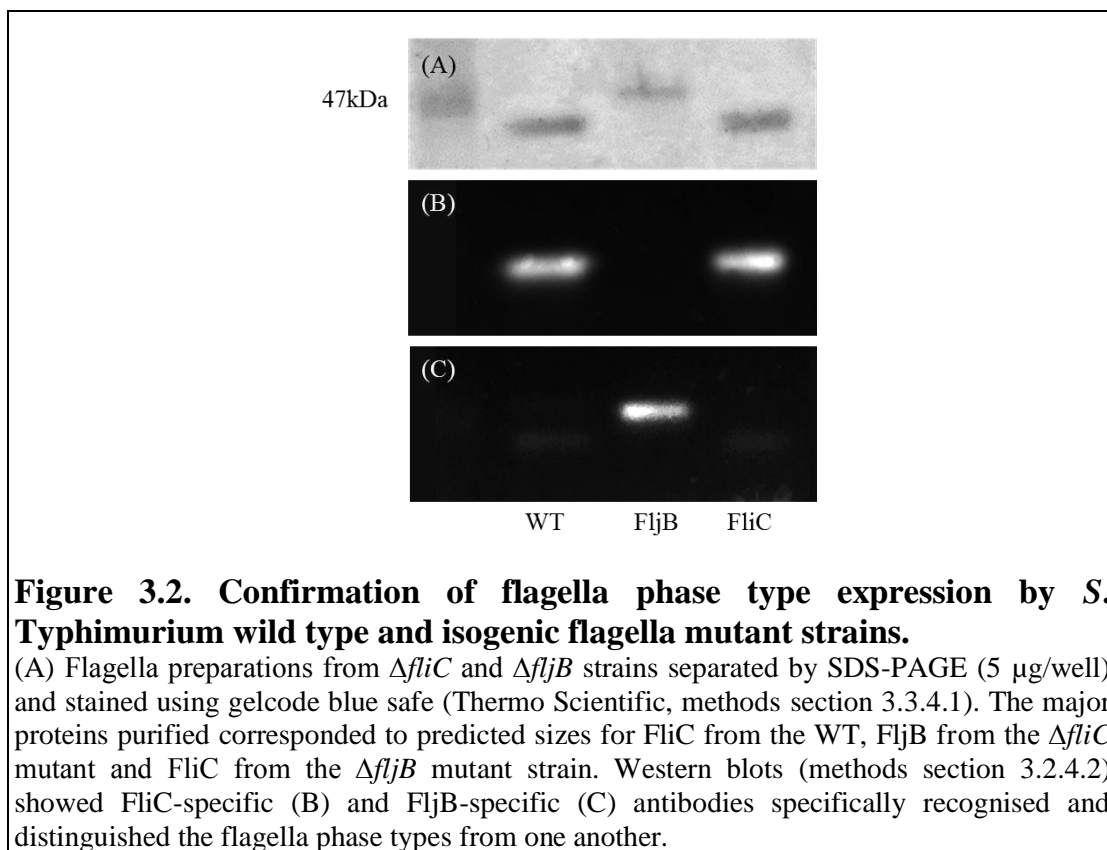
3.4.1. Comparative motility of *S. Typhimurium* SL1344 wildtype and isogenic $\Delta fliC$ and $\Delta fljB$ mutant strains.

To characterise the flagella expression of wild-type *S. Typhimurium* SL1344 (WT) (to see if it is capable of expressing FliC or FljB flagella filament proteins) and its isogenic mutant strains $\Delta fliC$, $\Delta fljB$ and $\Delta fliC\Delta fljB$, motility assays were completed. Figure 3.1 demonstrates that all strains except $\Delta fliC\Delta fljB$ were motile. The $\Delta fljB$ strain appears to be a lot less motile than the WT. A possible explanation is that although unable to express *fljB*, the strain may still express *fljA*, which represses expression of *fliC* (Macnab, 1996). The *hin* promoter switch changes orientation to result in the expression of either *fliC* or *fljB/fljA* genes. While the *fljB* gene has been removed, resulting in the loss of expression of *fljB*, the *fljA* gene remains and may be expressed when *hin* is in the *fljB/fljA* orientation. If the $\Delta fljB$ strain is expressing *fljA*, *fliC* will not be expressed, resulting in low levels of motility due to reduced expression of flagella filament proteins.



3.4.2. Flagella filament protein isolates

SDS-PAGE analysis of flagella preparations showed that one major protein approximately 47kDa in size was purified in each case (Figure 3.2). Western blot analysis of purified flagella filament proteins from mutant *Salmonella* strains showed that FliC and FljB flagella filament proteins could be distinguished using specific monoclonal antibodies (Figure 3.2).



3.4.3. *S. Typhimurium* capacity to switch flagella type.

S. Typhimurium is capable of switching flagella phase type. To determine if a flagella phase switch could be induced, motility assays using *S. Typhimurium* strain VacT were carried out, with and without the addition of flagella-specific antisera. Addition of flagella-specific antibodies reduced motility (Figure 3.3(A-C)). In the absence of flagella-specific antibodies, VacT was shown to express FliC flagella (Figure 3.3(D-F)). Serological analysis of flagella proteins isolated from flagella-specific IgG-treated bacteria by Western blot showed FljB expression in the presence of anti-FliC rabbit IgG (Figure 3.3(F)) and FliC expression in the presence of anti-FljB rabbit IgG (Figure 3.3(E)). This indicates that a flagella phase switch occurs in the presence of flagella antibodies.

These preliminary findings suggest that *Salmonella* may be able to “sense” the mechanical restriction of one flagella phase type and switch expression to the other. However, more research is required to support this theory. Unfortunately the time constraints of this project prevented further study of this hypothesis.

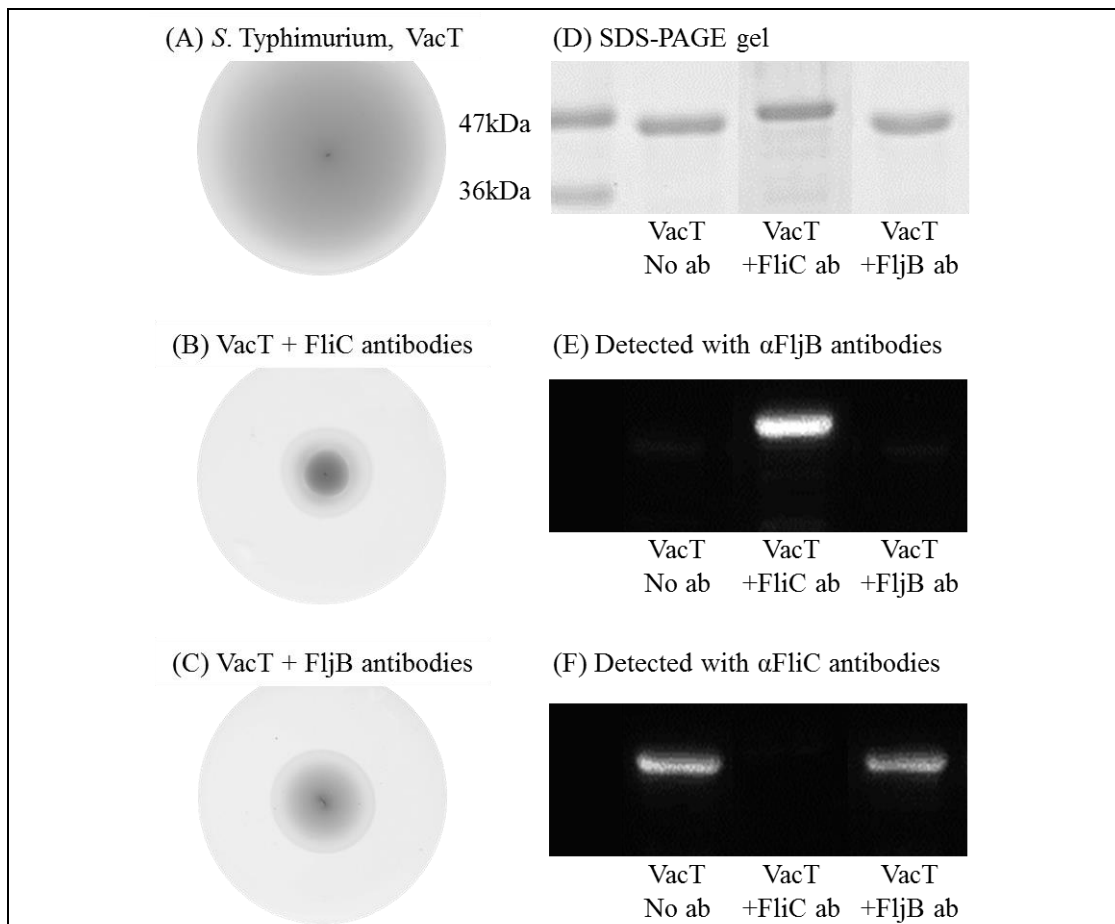


Figure 3.3. *S. Typhimurium* switch flagella phase types in response to antibody recognition.

Motility assays (methods section 3.3.3) of VacT without flagella-specific IgG (A), with FliC-specific rabbit IgG (B) and FljB-specific rabbit IgG (C). These images are representative of 4 plates for each strain. Flagella antibodies were used at a 1:600 dilution and mixed into the agar. Flagella protein preparations from differential antibody treatments of VacT separated by SDS-PAGE; one major band was isolated from each of the flagella preparations (D). Western blots of (A) with anti-FljB rabbit IgG (E) and anti-FliC rabbit IgG (F) primary antibodies show that when flagella specific IgG were added to *S. Typhimurium* VacT the opposite flagella phase type was expressed.

3.4.4. Flagella expression associated with porcine intestinal epithelial cells

To examine flagella expression on porcine intestinal epithelial cells, SEM and confocal microscopy techniques were employed. Figure 3.4 confirms that *S. Typhimurium* can express flagella during interactions with porcine intestinal epithelial cells.

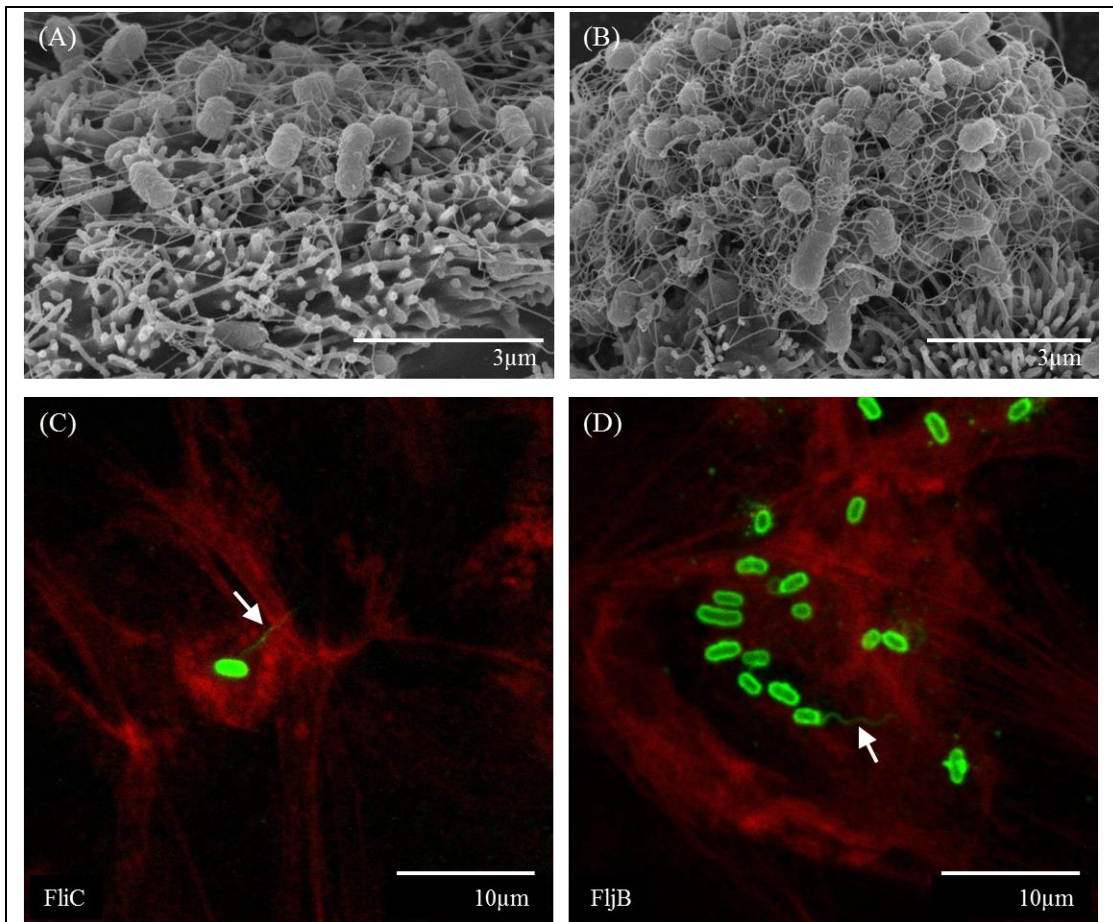


Figure 3.4. Flagellar expression associated with porcine intestinal epithelial cells.

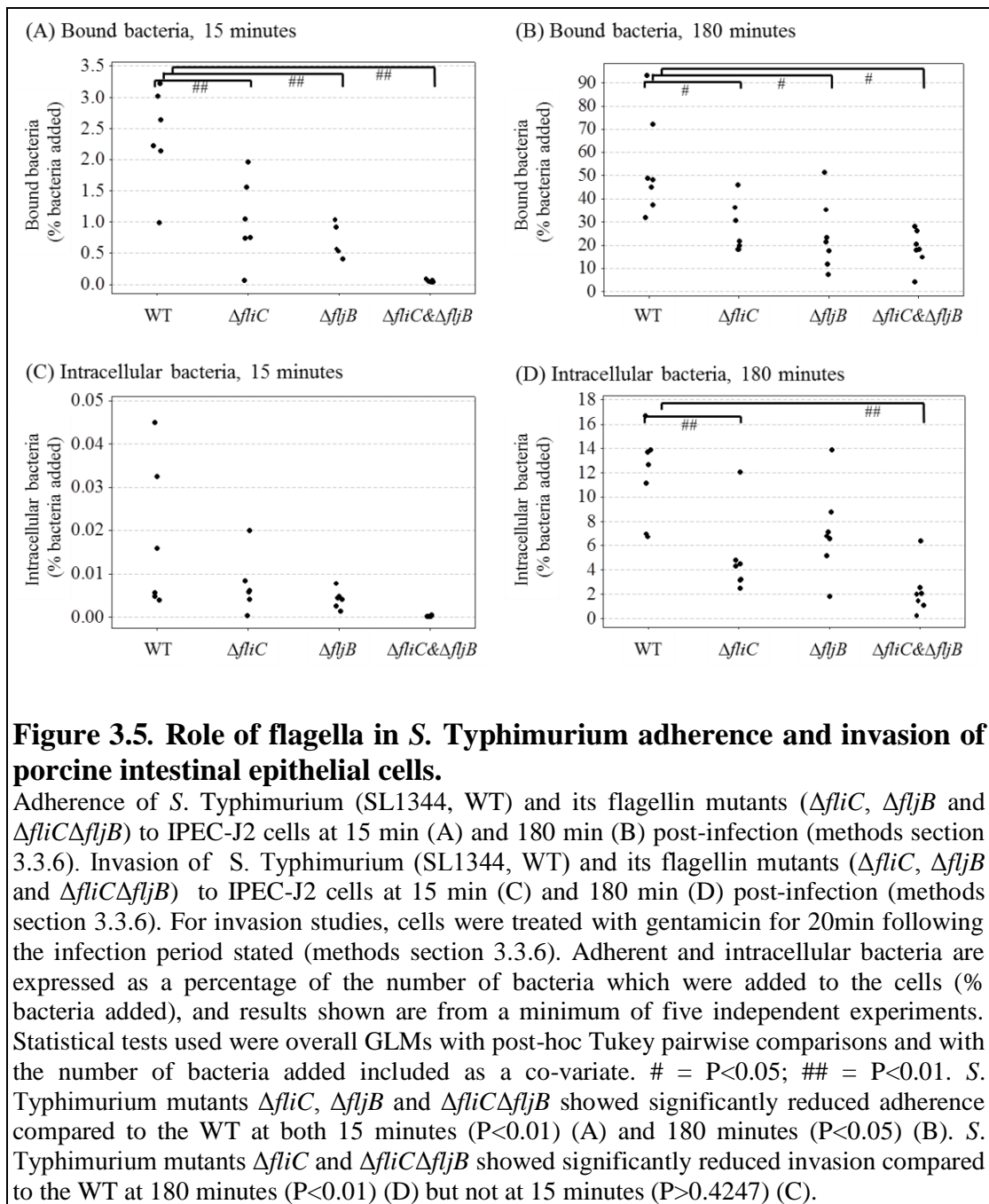
(A-B) SEM micrographs of *S. Typhimurium* (SL1344) interacting with porcine primary colonic epithelial cells, 90min post-infection. *S. Typhimurium* bacteria are expressing flagella-like filaments which can be seen interacting with the epithelial cell surface and other bacteria. Images were taken using an Hitachi S-4700 scanning electron microscope (methods section 3.2.9.4). (C-D) Confocal microscopy projections of *S. Typhimurium* (SL1344) interacting with the porcine intestinal epithelial cell line IPEC-J2, expressing FliC (C) and FljB (D) flagella (arrows), 10min post-infection. Images were taken using a Zeiss LSM5 Pascal confocal microscope (methods section 3.3.9.2).

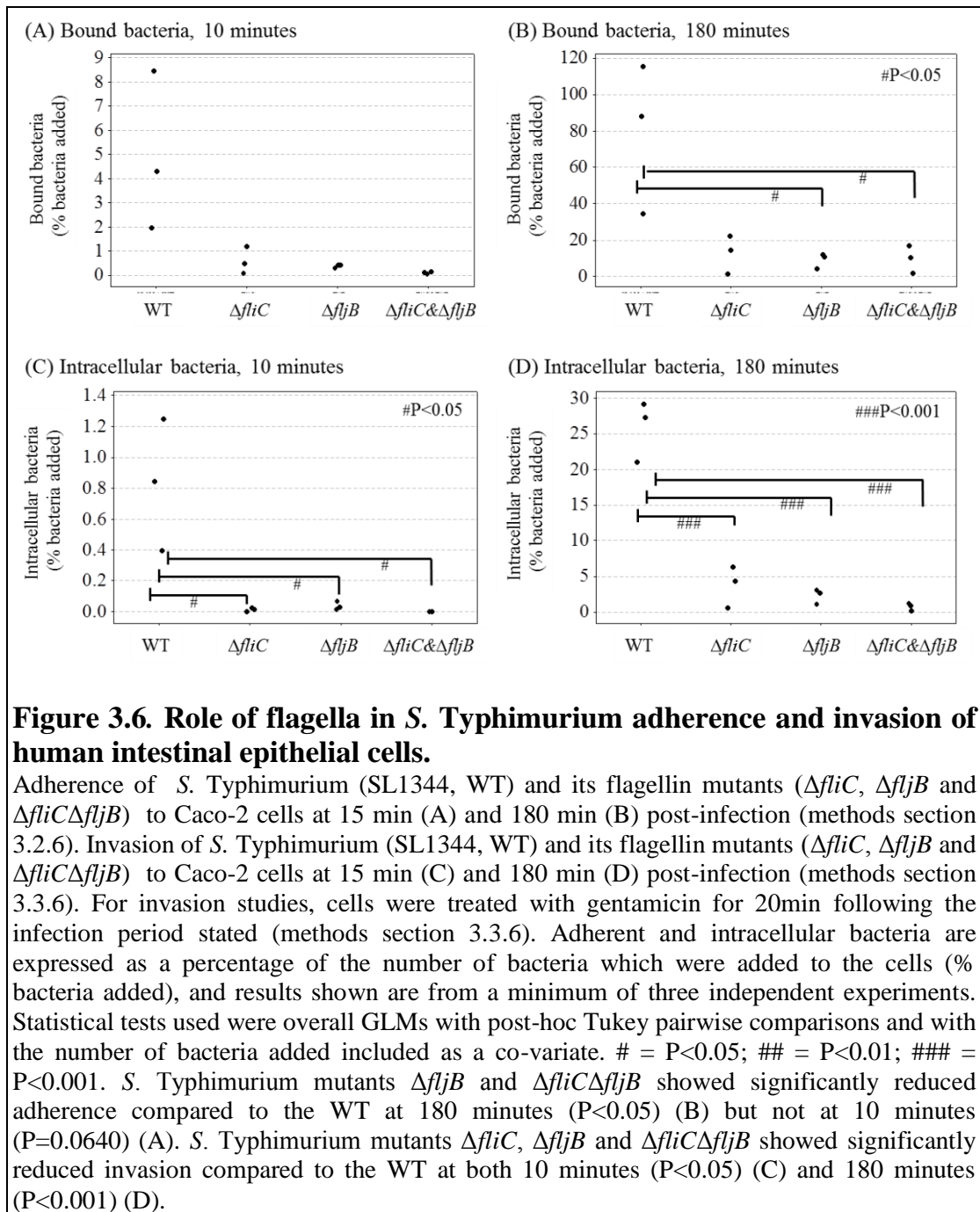
3.4.5. *S. Typhimurium* flagellin mutants exhibited diminished adherence to porcine and human intestinal epithelial cells

To establish if these flagella filament proteins are important for adherence and/or invasion of porcine (Figure 3.5) and human (Figure 3.6) intestinal epithelial cells, adherence and invasion assays were performed. Where *S. Typhimurium* was unable to express either FliC and FljB flagellins ($\Delta fliC$ & $\Delta fljB$), binding to and invasion of porcine intestinal epithelial cells after 180min of infection was reduced when compared to the wild-type ($P < 0.05$, Figure 3.5(B) and 3.5(D)). The same phenotype was also observed with human intestinal epithelial cells, indicating this was not a host-species specific phenomenon (Figure 3.6(B) and 3.6(D)).

Binding and invasion of porcine and human intestinal epithelial cells by motile *S. Typhimurium* single mutant strains $\Delta fliC$ and $\Delta fljB$ was also tested. Both single mutants showed reduced binding to porcine and human intestinal epithelial cells when compared to wild-type. The phenotype was most apparent on human intestinal epithelial cells, but even on porcine intestinal epithelial cells there was a significant difference at 15 min ($\Delta fliC$ $P = 0.0018$, $\Delta fljB$ $P = 0.0016$) and 180 min ($\Delta fliC$ $P = 0.0150$, $\Delta fljB$ $P = 0.0102$) infection periods.

Invasion of both porcine and human intestinal epithelial cell lines by *S. Typhimurium* was also affected by expression of flagella filament proteins. Intracellular bacterial numbers of $\Delta fliC$ (porcine, $P = 0.0046$, human, $P < 0.001$) and $\Delta fliC$ & $\Delta fljB$ (porcine, $P = 0.0013$, human, $P < 0.001$) were significantly reduced after 180 min infection compared to the wild-type. As $\Delta fliC$ and WT strains were very similar in motility tests (Figure 3.1), flagellar motility is unlikely to be the sole explanation for the reduced adherence of the mutant strains compared to the wild-type.





To assess the contribution of flagellar motility in the binding and invasion of porcine epithelial cells shown in Figure 3.5, additional binding and invasion assays were conducted. To reduce the effect of differences in motility, bacteria were centrifuged onto epithelial monolayers (Figure 3.7). There was no statistical difference between the wild-type and flagella mutant strains in these binding and invasion assays ($P>0.083$). However, differences in the number of intracellular bacteria at 180min between $\Delta fliC$ and wild-type strains did not reach statistical significance ($P=0.083$). This suggests that a subtle difference may exist between strains that may have been detected had there been less variation between experiments. The $\Delta fliC$ strain is as motile as the wild-type but has a reduced binding phenotype, suggesting that motility is not the only factor involved. While motility plays a very important role in infection of cells, the reduced binding phenotype of the $\Delta fliC$ strain suggests that flagella may play an additional role in infection of cells. However, the variation between $\Delta fljB$ and wild-type or $\Delta fliC\Delta fljB$ and wild-type strains in the number of adherent and intracellular bacteria (Figure 3.5) could be attributed to reduced motility, as the $\Delta fljB$ strain and the $\Delta fliC\Delta fljB$ were less motile than the wild-type (Figure 3.1), and when centrifuged there was no statistical difference between strains (Figure 3.7). However, centrifugation could have negated the requirement for surface factors important for initial adherence, as the bacteria were forced onto the cells. An alternative experiment to show that lack of motility was not the sole explanation for the lower bacterial numbers of the mutant strains would be to use a motility mutant which still expresses flagella, (mot^-). Unfortunately there was insufficient time to make mot^- strains for this study.

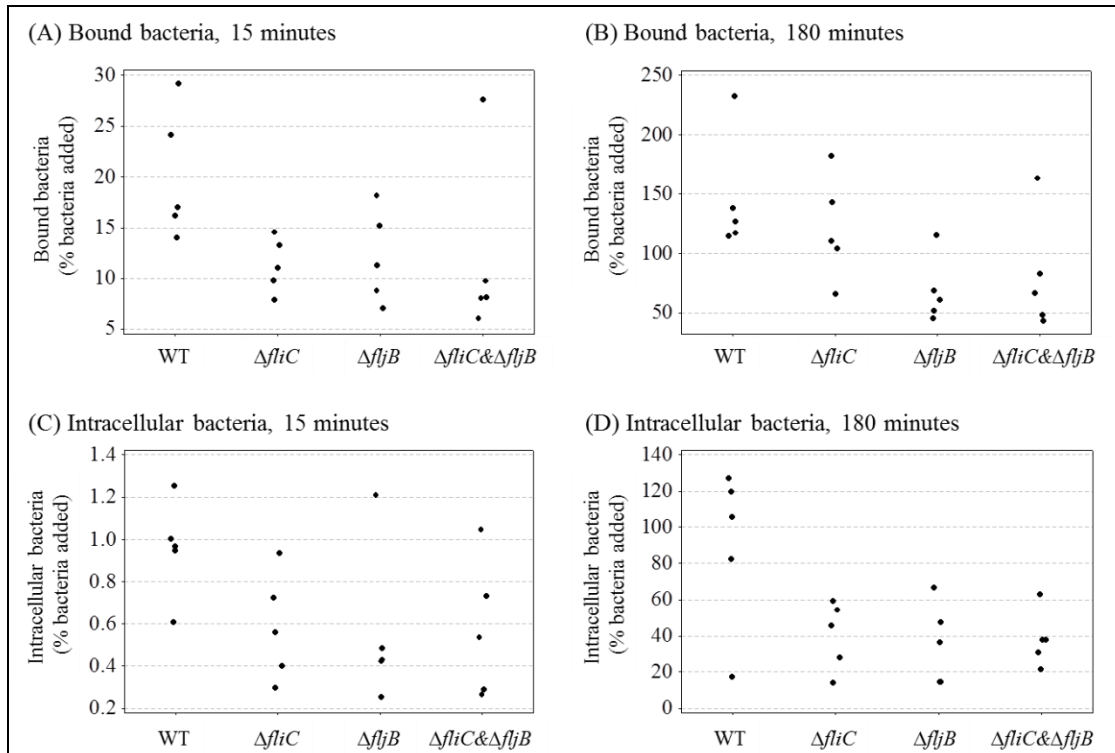
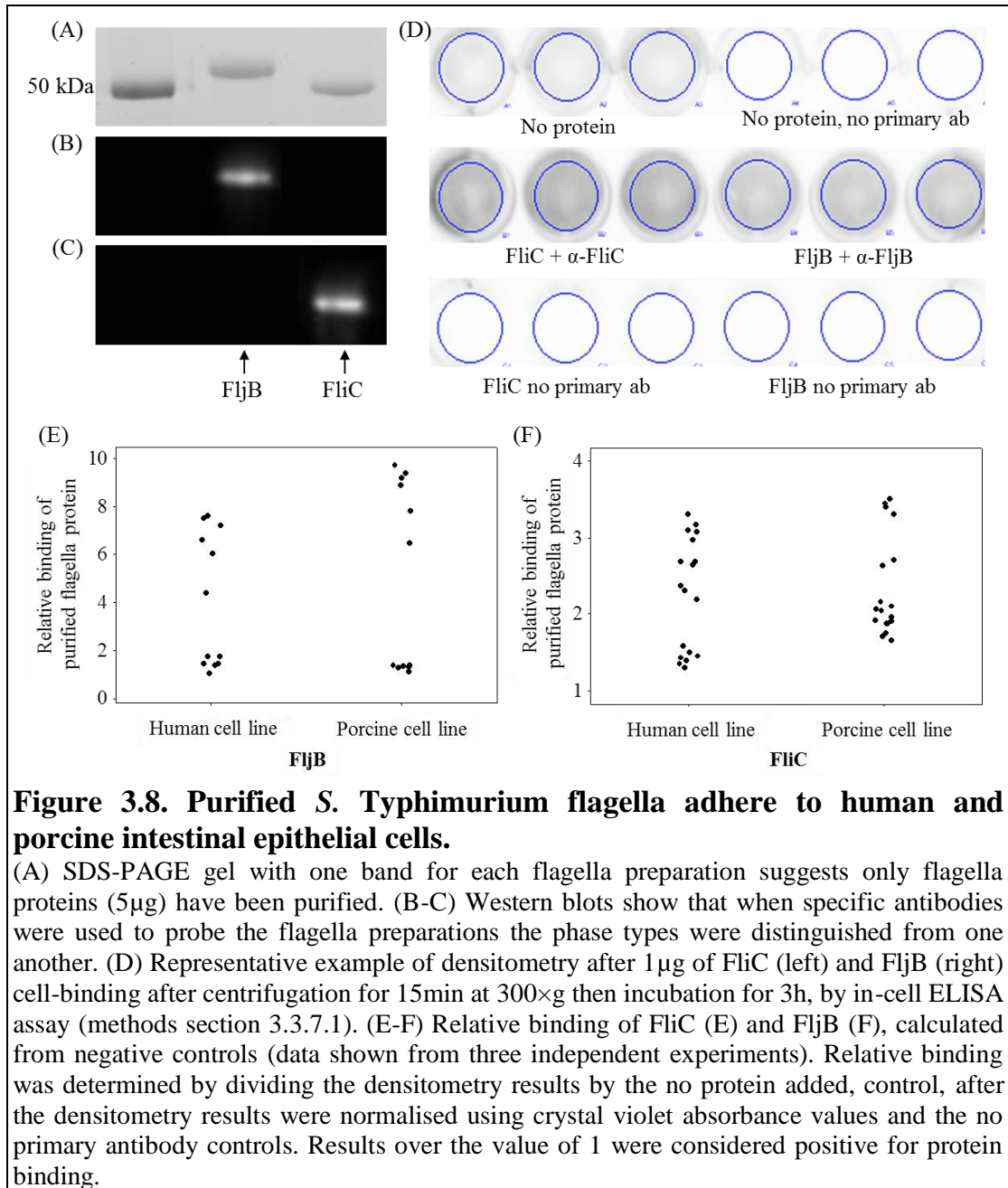


Figure 3.7. Role of motility during *S. Typhimurium* adherence and invasion of porcine intestinal epithelial cells.

Adherence after gentle centrifugation of *S. Typhimurium* (SL1344, WT) and its flagellin mutants ($\Delta fliC$, $\Delta fljB$ and $\Delta fliC\Delta fljB$) onto IPEC-J2 cells at 15min (A) and 180min (B) post-infection (methods section 3.3.6.1). Invasion after gentle centrifugation of *S. Typhimurium* (SL1344, WT) and its flagellin mutants ($\Delta fliC$, $\Delta fljB$ and $\Delta fliC\Delta fljB$) onto IPEC-J2 cells at 15min (C) and 180min (D) post-infection (methods section 3.3.6.1). For invasion studies, cells were treated with gentamicin for 20min following the infection period stated (methods section 3.3.6). Gentle centrifugation of bacteria onto IPEC-J2 cells was undertaken according to methods section 3.3.6.1. Adherent and intracellular bacteria are expressed as a percentage of the number of bacteria which were added to the cells (% bacteria added), and results shown are from a minimum of five independent experiments. Statistical tests used were overall GLMs with post-hoc Tukey pairwise comparisons and with the number of bacteria added included as a co-variate. No statistical difference was shown between strains at the time points tested ($P>0.0833$).

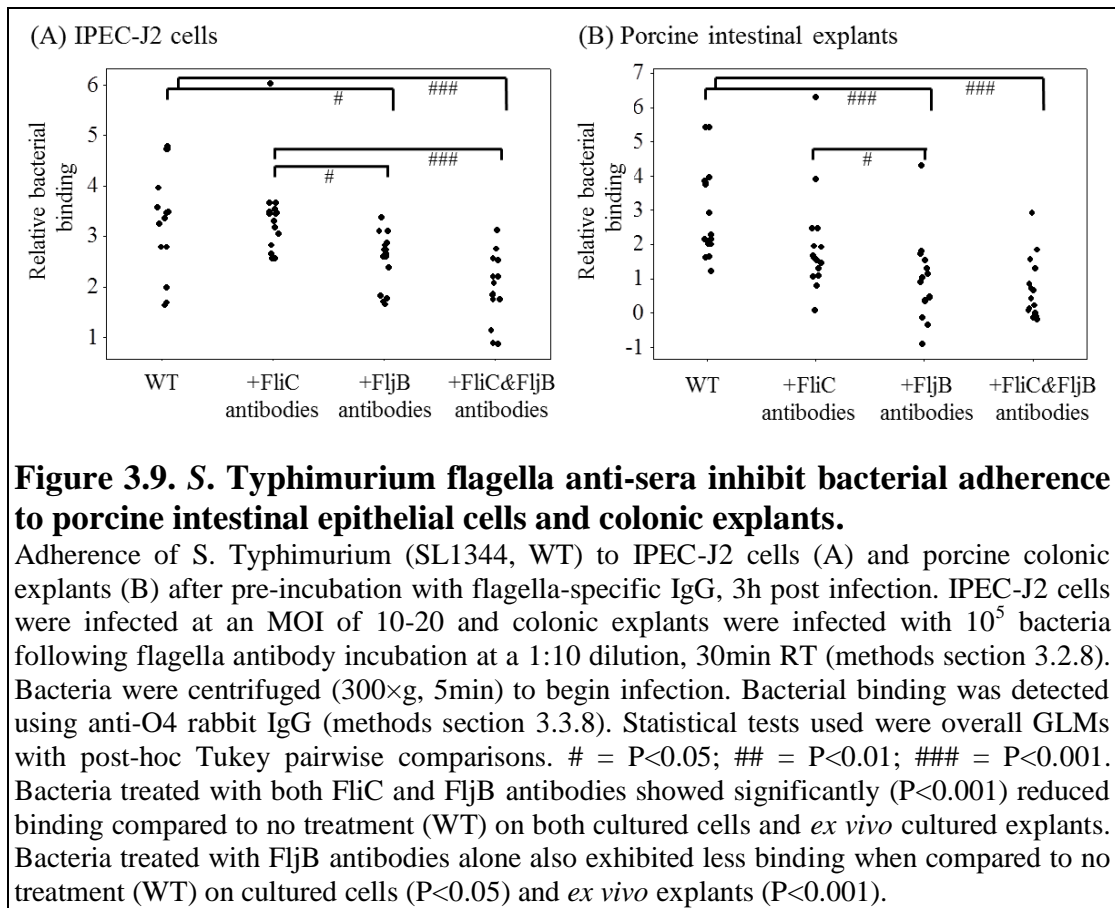
3.4.6. Purified *S. Typhimurium* flagella adhered to porcine intestinal epithelial cells

To determine if flagella filament proteins FliC and FljB adhered to human and porcine intestinal epithelial cells purified flagella filament proteins were used in adherence assays. Figure 3.8(E-F) shows the relative protein binding of purified flagella filament proteins to both human and porcine intestinal epithelial cells (IPEC-J2 & Caco-2 cell monolayers respectively). These findings indicate that flagella may play a role in *S. Typhimurium* adherence to intestinal cells during infection.



3.4.7. Flagella antisera inhibited *S. Typhimurium* binding to porcine intestinal epithelial cells and porcine intestine

To determine if the binding of flagella to porcine intestinal epithelial cells could be inhibited by the addition of specific antibodies, adherence assays were performed using flagella-specific IgG and *S. Typhimurium* wild-type SL1344. The adherence assays shown in Figure 3.9 were conducted on both IPEC-J2 cells and on intestinal explants from porcine colon obtained from a local abattoir. The adherence assays showed that the addition of both FliC and FljB flagella-specific IgG together reduced bacterial binding ($P < 0.001$). Though bacterial binding appeared to be reduced in the presence of FliC-specific IgG, this was not statistically significant ($P > 0.05$). The addition of FljB-specific IgG significantly reduced bacterial binding, though this was less pronounced ($P < 0.05$) compared to addition of both FliC- and FljB-specific IgG ($P < 0.001$). It was hypothesised that FliC filaments were still able to bind to host cells following addition of FljB-specific IgG alone.



3.4.8. *S. Typhimurium* flagella interact with the cell surface of porcine intestinal cells

To investigate this potential flagellar interaction with porcine intestinal epithelial cells more closely, further imaging was undertaken. Intestinal explants and porcine primary intestinal cells were challenged with *S. Typhimurium* (Maskan) and imaged using SEM. Figure 3.10 shows that *S. Typhimurium* bacteria could be seen bound to the cultured cells, expressing flagella-like surface structures. These flagella-like structures appear to penetrate the cell membrane at the time points as early as 30min (Figure 3.10(E-G)) and 10min (Figure 3.10(A-D)). The flagella appear to weave through the cell membrane and can be seen clearly where they join the bacteria, fade, and become clear again. The flagella do not just stay on the cell surface. The images show that flagella appear to physically tether the bacteria to the host cell surface, potentially by intercalation into the host cell membrane.

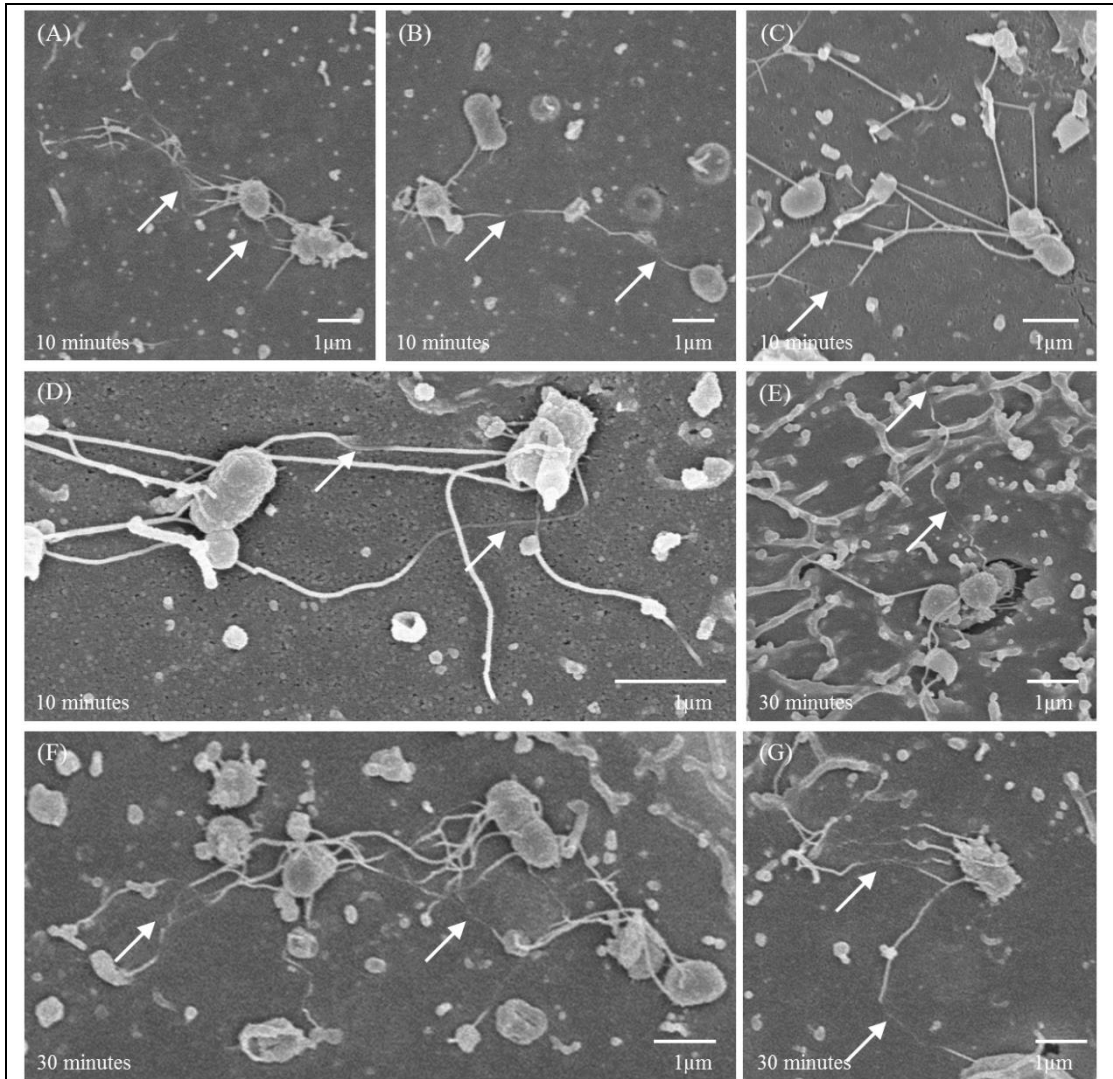


Figure 3.10. Flagella expression during *S. Typhimurium* interaction with primary porcine colonic epithelial cells.

SEM micrographs of *S. Typhimurium* (Maskan) flagella interacting with primary porcine intestinal epithelial cell surfaces 10min (A-D) and 30min (E-G) post-infection (methods section 3.3.9.4). These images show *Salmonella* bacteria expressing flagella like structures penetrating the host cell surface.

3.4.9. *S. Typhimurium* expressed flagella during attachment to porcine intestinal epithelial cells

To determine if *S. Typhimurium* flagella penetrate the cell surface to become intracellular, confocal microscopy techniques were employed. Figure 3.11 shows porcine intestinal epithelial cell line (IPEC-J2) infected with *S. Typhimurium*. Flagella specific antibodies were used to stain flagella filaments (green) which can be seen inside porcine intestinal epithelial cells (Figure 3.11(C)). Furthermore, flagella filaments (green) appear to be interacting with actin filaments (red). Figure 3.14(D-F) shows in yellow where flagella and actin filaments co-localise with one another.

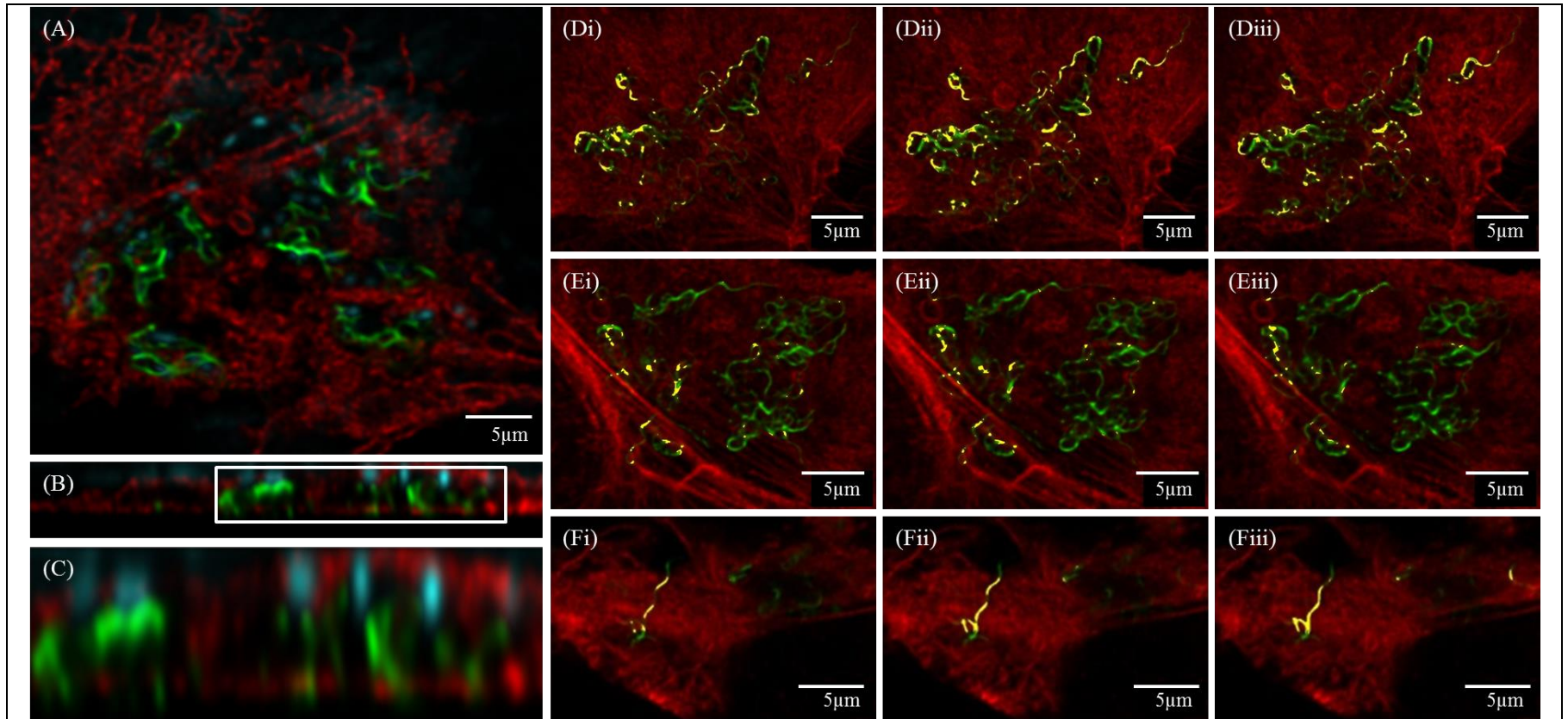


Figure 3.11 *S. Typhimurium* flagella co-localise with actin filaments inside porcine intestinal epithelial cells.

Confocal microscopy of *S. Typhimurium* (SL1344) interactions with IPEC-J2 cells 20min post-infection at MOI of 10-20 (methods section 3.3.9.2). DAPI (blue) was used to stain the bacterial DNA, phalloidin texas red (red) was used to stain the actin filaments and flagella antibodies (green) were used to stain the flagella filaments (methods section 3.3.9.1). (A) An example z-plane where the bacteria, actin and flagella can all be seen. (B) Side view of the z-stack, showing the flagella inside the cell, and an enlarged side view of the z-stack (C). The bacterial DNA appears to be at the top of the cell and the flagella appear to be inside the cell. (D-F) Co-localisation of actin and flagella (yellow) using the ImageJ co-localisation plug-in. Images (i,ii and iii) are a series of z-slices showing co-localisation of actin and flagella through the stack.

3.5. Discussion

Previous studies on flagella from several bacterial species including *C. jejuni*, *P. aeruginosa*, and *E. coli* have shown its importance as an adhesin (De Bentzmann et al., 1996, Newell et al., 1985, Mahajan et al., 2009). This current study suggests that *S. Typhimurium* flagella have a role in adherence to host epithelia, specifically the porcine intestinal epithelium.

Bacterial binding assays (Figures 3.5 and 3.6) with flagella mutant strains indicated that flagella were important for adherence in both porcine and human intestinal epithelial cells. Bacterial adherence by flagella mutant strain, $\Delta fliC\&\Delta fljB$, was significantly reduced at 180min compared to the wild-type on both cell types. In addition, invasion of both porcine and human intestinal epithelial cell lines by *S. Typhimurium* was affected by flagella filament expression. Intracellular bacterial numbers of $\Delta fliC$ and $\Delta fliC\&\Delta fljB$ were significantly reduced after 180 min infection compared to the wild-type.

Flagella primarily function as motility organelles. Therefore, strains unable to express either flagella filament proteins FliC or FljB ($\Delta fliC\&\Delta fljB$) are non-motile. Motility plays an important role in infection and it was important to recognise that the differences between wild-type and double flagellin mutant, $\Delta fliC\&\Delta fljB$, in adherence and invasion assays may be attributable to lack of motility by $\Delta fliC\&\Delta fljB$. To try to address this issue, adherence and invasion assays were repeated using centrifugation to negate any difference attributable to motility (Figure 3.7). Centrifugation of the bacteria onto the cells reduced the differences between wild-type and flagella mutant strains. Indeed, differences between strains were not found to be statistically different ($P>0.08$). However, differences in the number of intracellular bacteria at 180min between the wild-type and $\Delta fliC$ mutant strains did near statistical significance ($P=0.08$). This suggests a subtle difference may exist between strains which might have been detected had there been less variation between experiments.

Motility tests shown in Figure 3.1 showed that $\Delta fliC$ and wild-type strains exhibited a very similar motility phenotype. However, adherence and invasion assays shown in Figure 3.5 identified significant differences between wild-type and $\Delta fliC$ strains ($P < 0.05$), suggesting motility was not the only factor affecting adherence in this assay.

Centrifugation of bacteria onto the cells could have negated the requirement for surface factors important for initial adherence, as the bacteria were forced onto the cells. An alternative experiment to show that lack of motility was not the sole explanation for the lower bacterial numbers of the mutant strains (Figure 3.5 and 3.6) would be to use a motility mutant which still expresses flagella, (mot^-). Unfortunately there was insufficient time to make mot^- strains for this study.

While $\Delta fliC$ and wild-type strains were very similar in motility tests (Figure 3.1), the $\Delta fljB$ strain had a reduced motility phenotype in comparison. However, binding and invasion tests showed no statistical difference between $\Delta fliC$ and $\Delta fljB$ on either porcine or human intestinal epithelial cells ($P > 0.4203$). If the reduction in adherence and invasion of flagella mutant strains was as a result of loss of motility, the $fljB$ mutant would have exhibited significantly less adherence and invasion of intestinal epithelial cells in comparison to the $\Delta fliC$ mutant which appears to be considerably more motile (Figure 3.1). $\Delta fliC$ and $\Delta fljB$ were not statistically different in either adherence or invasion of either porcine or human intestinal epithelial cells. This suggests that differences in binding and invasion by wild-type and flagella mutant strains shown in Figure 3.5 and 3.6 were not solely attributable to motility. Adherence and invasion assays indicate that flagella may have an early role in infection of porcine intestinal epithelium.

The apparent loss of motility of the $\Delta fljB$ strain shown in Figure 3.1 could be due to the expression of $fljA$, which represses expression of $fliC$ (Macnab, 1996). Flagellar phase variation is achieved by stochastic inversion of a promoter, the *hin* switch that produces both FljB flagellin and an inhibitor (FljA) of FliC flagellin formation. When the $fljB$ - $fljA$ operon is expressed, only FljB flagellar filaments are produced; when the operon is not transcribed, the gene for FliC flagellin ($fliC$) is released from

inhibition and FliC flagellar filaments are formed (Macnab, 1996). In the $\Delta fljB$ strain the *fljB* gene has been removed, resulting in loss of expression of FljB. However, *fljA* remains and may be expressed when *hin* is in the *fljB/fljA* orientation. If the $\Delta fljB$ strain is expressing FljA, *fliC* will not be expressed, resulting in lack of motility due to reduced expression of flagella filament proteins.

S. Typhimurium flagella expression is regulated by a number of external factors including the environment. *S. Typhimurium* is capable of switching flagella phase type, but little is known about the specific function of this switching. *S. Typhimurium* motility assays with and without the addition of flagella specific IgG, shown in Figure 3.2 suggested a flagella phase switch could be induced. The motility assays and subsequent Western blots to determine flagella phase identified that flagella proteins isolated from antibody treated bacteria, switched from one flagella phase type to the other (Figure 3.2). These preliminary findings suggest that *Salmonella* may be able to “sense” the restriction of one flagella phase type and be able to switch to the other. More research is required to confirm this hypothesis, but if confirmed, the ability of *S. Typhimurium* to switch flagella phase type could be linked to evasion of the immune system. Although both FliC and FljB are recognised by TLR-5 (Simon and Samuel, 2007a), switching from one phase type to another may help avoid recognition by circulating antibody.

Following the study of *S. Typhimurium* flagella function as an adhesin by bacterial adherence and invasion assays, it was also determined that purified FliC and FljB filaments both adhered to porcine and human intestinal epithelial cells. Figure 3.8 shows an in-cell ELISA with FliC and FljB. The relative binding determined by pixel densitometry shows that FliC and FljB proteins bound both porcine (IPEC-J2) and human (Caco-2) intestinal epithelial cells. These findings also support the hypothesis that flagella play a role in *S. Typhimurium* adherence to intestinal epithelial cells during infection.

To determine if the binding of flagella to porcine intestinal epithelial cells could be inhibited by the addition of specific antibodies, adherence assays were carried out using flagella specific IgG and *S. Typhimurium* wild-type SL1344. The adherence

assays shown in Figure 3.9 were carried out on both IPEC-J2 cells and on intestinal explants from porcine colons obtained from a local abattoir. The adherence assays showed that the addition of FliC and FljB flagella specific IgG together reduced bacterial binding.

Additional studies with porcine tonsil tissues were also conducted to determine if the presence of flagella antibodies reduced bacterial binding to tonsils as well as intestinal explants. The results from these preliminary studies (data not shown) appeared to show no reduction in bacterial binding. Further studies are required to confirm that flagella are not involved in adherence to porcine tonsils. However, if confirmed this could suggest that *S. Typhimurium* flagella are involved specifically in adherence to intestinal epithelial cells.

The expression of *S. Typhimurium* flagella during infection of porcine intestinal epithelial cells was confirmed by employing microscopy techniques. SEM micrographs in Figures 3.4 and 3.10 show expression of flagella-like structures at different infection times. Furthermore, Figure 3.10 appears to show *S. Typhimurium* flagella penetrating the cell surface and weaving in and out of the cell surface as though anchoring the bacterium. Confocal microscopy was used to investigate the presence of intracellular flagella. Wheat-germ agglutinin, which stains the cell surface, and phalloidin, which stains actin filaments, were both used to confirm flagella penetrates the cell surface. Figure 3.11 shows the presence of flagella in the plane (or z-stack) of the image as actin filaments indicating flagella are intracellular. Furthermore, these flagella filaments appear to be interacting with actin filaments appearing to co-localise with one another. Indeed, other experiments performed within our group have shown that FliC and FljB bind purified $\beta\gamma$ human actin (Eliza Wolfson, personal communication). Further work is currently underway within our group to determine if FliC and FljB interactions with actin can affect actin polymerisation.

The levels of *Salmonella* infection in pigs in the UK are amongst the highest in Europe (EFSA, 2008b). National Control Plans for *Salmonella* are working towards

the reduction of *Salmonella* prevalence in pigs. The use of effective vaccination is viewed as a reasonable tool, amongst others, in reducing *Salmonella* prevalence in the UK and elsewhere in Europe. The primary aim of vaccination would be to limit initial colonisation, so no Salmonellae enter the porcine system. The findings of this study qualify these bacterial adhesion factors as potential immuno-protective antigens, and thus *S. Typhimurium* vaccine candidates in pigs.

The *in vivo* testing of *S. Typhimurium* FliC and FljB proteins to assess their protective immunogenicity and potential for a *Salmonella* vaccine in pigs is detailed in Chapter 4.

**4. A vaccine efficacy study in pigs
demonstrated an immuno- protective role
induced by *Salmonella* Typhimurium Flagella
Filament Proteins FliC and FljB**

4.1. Introduction

S. Typhimurium is a zoonotic enteric pathogen of worldwide importance and pigs are a significant reservoir of human infection. Following infection, pigs can be asymptomatic carriers of the pathogen and can shed *S. Typhimurium* in their faeces for several months post-exposure (Berends et al., 1996, Swanenburg et al., 2001). Such infected animals can contaminate the food chain, via meat, and the environment, via faeces, and represent a significant human health hazard (Hurd et al., 2002).

During a nationwide survey of *Salmonella*, *S. Typhimurium* was detected as the predominant serovar in pigs presented for slaughter in UK abattoirs (Davies et al., 2004). *S. Typhimurium* has subsequently been reported as the most prominent serovar by the Veterinary Laboratories Agency (VLA) since 1998 (AHVLA, 2002, AHVLA, 2006, AHVLA, 2011). The most recent report from the VLA shows *S. Typhimurium* and *S. Typhimurium*-like strains accounting for ~75% of *Salmonella* serovars, from 172 samples that were serotyped (Figure 4.1) (AHVLA, 2011).

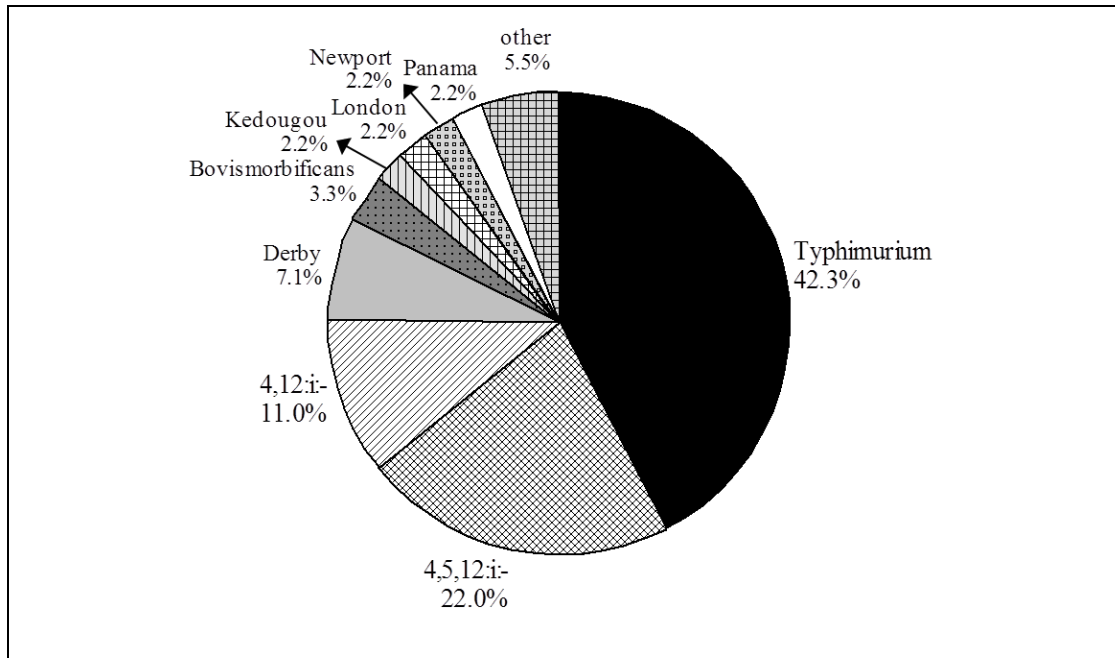


Figure 4.1. Most common *Salmonella* serovars isolated from pigs in 2011 (AHVLA, 2011)

The most common *Salmonella* serovars isolated in the UK by the Veterinary Laboratories Agency in 2011. The most common serovar was Typhimurium followed by Typhimurium-like serovars 4,5,12:i- and 4,12:i-, which are monophasic variants of Typhimurium. The number of samples serotyped was 182 and the raw data originated from the VLA (AHVLA, 2011).

The levels of infection in pigs in the UK are amongst the highest in Europe (EFSA, 2008). A slaughterhouse survey in 2004 identified that the frequency of infection was 23% (Davies et al., 2004). A European Food Standards Agency (EFSA) study reported the prevalence of *Salmonella* species on pig carcasses from different countries across Europe. The European Union average was 10.3% of pig carcasses testing positive for *Salmonella*. The same study reported the UK as testing *Salmonella* positive on 13.5% of carcasses tested, with 7.2% carcasses identified as positive for *S. Typhimurium* (EFSA, 2008b).

A National Control Plan (NCP) for *Salmonella* in pigs was scheduled to be implemented in all EU Member States by 2012-2013 (as required by EC Reg. 2160/2003). According to EU law, the NCPs will work towards reduction of prevalence of *Salmonella* in pigs against national targets set by the EFSA. Since the introduction of *Salmonella* vaccines in the poultry industry, the number of human *S.*

Enteritidis cases in the UK has fallen (HPA, 2011). It is likely that the reduction of *Salmonella* cases related to this serovar in humans is largely due to the introduction of the *Salmonella* vaccine in the poultry industry. The use of effective vaccination is therefore viewed as a pragmatic tool, amongst others, in reducing *Salmonella* prevalence in pigs in the UK and elsewhere in Europe.

No porcine *S. Typhimurium* vaccine is currently available in the UK. The only licensed vaccine is a live vaccine “Salmoporc STM” (IDT, Germany), which covers only 5% of the EU market. However, because animals vaccinated with a live vaccine cannot be discriminated serologically from animals infected by wild-type strains, the use of this vaccine in herds is problematic.

Many of the animal health pharmaceutical companies, for example Boehringer Ingelheim (Enterisol[®] SC-54) and Novartis Animal Health (Salmo Shield[®] Live), offer live-attenuated porcine vaccines using another serovar, *S. Choleraesuis*. Some studies (Schwarz et al., 2011, Husa et al., 2009) have shown that there is benefit in the administration of these vaccines to protect against *Salmonella*. Pigs vaccinated with *S. Choleraesuis* displayed fewer symptoms than those that were not vaccinated, but only limited cross protection against *S. Typhimurium* was demonstrated (Schwarz et al., 2011). Therefore, there is still a need to develop a recombinant vaccine with cross-protective antigens to give protection against different *Salmonella* serovars. Such a vaccine could also incorporate specific markers for differential diagnostics, as a part of a Differentiating Infected from Vaccinated Animals (DIVA) strategy.

4.2. Flagellar antigenic properties and the immune response

Flagella have been shown to be immunogenic and interact with the immune system via TLR-5 (Hayashi et al., 2001) and Ipaf (Franchi et al., 2006). The flagellin monomer consists of α -helical domains D₀ and D₁ positioned in the filament core, and folded β -sheet domains D₂ and D₃ that are on the outer surface of the filament. The filament core domains D₀ and D₁ are important for flagellar filament assembly and are largely conserved across bacterial species. It is this conserved region of the flagellin monomer that TLR-5 recognises. TLR signalling results in transcription of

pro-inflammatory genes via activation of nuclear factor (NF)- κ B and mitogen activated protein kinase (MAPK) pathways (Akira and Takeda, 2004).

Flagellin stimulation of epithelial cells upregulates production of inducible nitric oxides (iNOS), nitric oxide, matrilysin, human β -defensin 2, and chemokines such as macrophage inflammatory protein (MIP)-2 α and IL-8, a chemokine essential for recruitment of neutrophils and macrophages (Zeng et al., 2003, Eaves-Pyles et al., 2001, Sierro et al., 2001). Furthermore, these pro-inflammatory signals also induce dendritic cell maturation (Gewirtz et al., 2001). As flagella are pro-inflammatory, they may be efficacious as part of a vaccine. Indeed, several studies have utilised flagella as a vaccine adjuvant with promising results, as reviewed by Mizel and Bates 2010.

4.3. Preliminary results

Through both *in vitro* and *ex vivo* techniques, *S. Typhimurium* FliC and FljB flagella filament proteins were identified as adherence factors to porcine intestinal epithelial cells. Flagellin proteins have also been shown to be immunogenic in other studies (Mizel and Bates, 2010). Furthermore, addition of specific flagellin-IgG significantly reduced *S. Typhimurium* adherence to porcine epithelia. Serological studies on samples from known *S. Typhimurium* infected pigs confirmed generation of antibody to flagellin in this reservoir host (personal communication, Edith Paxton, data not shown). The techniques employed for these studies included porcine primary intestinal epithelial cell culture (detailed in chapter 2), porcine intestinal epithelial cell line IPEC-J2 and porcine intestinal explants.

These findings (detailed in chapter 3) identified *S. Typhimurium* flagella proteins as potential immuno-protective antigens, and thus *S. Typhimurium* vaccine candidates in pigs. *In vivo* testing of these *S. Typhimurium* factors was therefore performed, to assess their protective immunogenicity, and validate their potential for use in a vaccine preparation to limit *S. Typhimurium* colonisation and shedding from pigs.

4.4. Objectives

- To test the prophylactic efficacy of identified adherence factors FliC and FljB as potential vaccine candidates against *S. Typhimurium* colonisation and persistence in pigs.
- Characterise systemic and mucosal humoral immune responses following immunisation and subsequent *S. Typhimurium* challenge of pigs.

4.5. Experimental design

S. Typhimurium infection studies were carried out in collaboration with Moredun Scientific Limited, at the Moredun Research Institute. The experimental design is summarised in Table 4.1. Pigs approximately 6 weeks in age were sourced from a commercial pig breeder. The piglets were litter matched and all male. The pigs were transported and placed in semi-containment facilities in 2 groups of 10.

Table 4.1. Porcine *Salmonella Typhimurium* (STm) vaccine trial

Group	n	Treatment	Treatment Regime	Challenge	Challenge Regime	End of Study	Purpose
1	10	<i>S. Tm</i> vaccine (FliC and FljB) & adjuvant QuilA	2 x Intra-muscular (IM) immunisations (days 0, 21)	<i>S. Tm</i> SL1334	10 ⁸ cfu day 28, 10 ⁹ cfu day 32 (Oral)	Day 49	To test efficacy of <i>S. Tm</i> vaccine protection against <i>S. Tm</i> challenge
2	10	PBS & adjuvant QuilA	2 x IM immunisations (days 0, 21)	<i>S. Tm</i> SL1334	10 ⁸ cfu day 28, 10 ⁹ cfu day 32 (Oral)	Day 49	Control group to monitor colonisation in non-immunised animals.

Pigs were bedded on straw and provided with concentrated feed twice daily and *ad lib* water. After a week's acclimatisation, the pigs had blood samples taken from the vena cava and faeces collected from the anus. Sera were screened for *Salmonella* O4 antibodies by agglutination test. Faeces were screened for live *Salmonella* by culture. Each faecal sample was diluted in 10ml Rappaport-Vassiliadis (RV) broth. Broths were cultured at 37°C, 18h and streaked onto brilliant green agar (BGA). BGA was

incubated at 37°C, 18h. *Salmonella* growth was determined by colony morphology on BGA; pink, round colonies were considered to be *Salmonella*.

A previous study in cattle found the use of QuilA adjuvant effective when delivering a flagella based vaccine. In addition, cattle that received intra-muscular (IM) immunisations generated higher titres of IgA in rectal secretions compared to cattle receiving mucosal immunisation using coated beads (McNeilly et al., 2008). Due to the comparative ease of administering IM immunisation compared with mucosal immunisation, and the success of a previous study in generating IgA at the mucosal surface with this approach, IM immunisation was chosen. Therefore, the pigs were vaccinated into muscle on the left side of the neck with 60µg of each antigen FliC and FljB, with 1mg/dose of QuilA adjuvant, in PBS. Control pigs were given 1mg/dose of QuilA adjuvant in PBS. A second, booster dose was given 3 weeks later.

A week after the second vaccine, the pigs were challenged orally with 10ml, containing 10⁸cfu naladixic acid resistant (nal^r) *S. Typhimurium* SL1344. The pigs were dosed with *Salmonella* in a sterile syringe. Pigs were given 20ml of 10% sodium bicarbonate to help neutralise stomach acid 1h prior to challenge, to prevent stomach acid killing some of the *Salmonella* in the challenge dose. The pigs were also challenged a second time, 4d later with 10ml containing 10⁹ cfu *S. Typhimurium* SL1344 nal^r. On this occasion the pigs were challenged using a 10ml dosing gun.

Post-bacterial challenge, animals were clinically observed once daily with standard clinical parameters (demeanour, faecal consistency, appetite, body condition) as well as rectal temperature. Faecal samples were collected from all animals to quantify bacterial shedding. Faecal samples alternated between rectal faeces collected with a spatula and swab samples. Faecal samples collected with a spatula are referred to as weighed samples. Blood samples were collected on days 0, 21 and 49 for serology.

The pigs were euthanised on day 49 by stunning and exsanguination. Relevant tissue samples were collected and processed microbiologically to quantify bacterial persistence and colonisation. In addition, spleen and mesenteric lymph nodes (MLN) were collected for cell-based immunological assays.

4.6. Materials and methods

4.6.1. Bacterial strains

Table 4.2 Bacterial strains

Strain	Characteristics	Source & Reference
SL1344 (CMG 109)	WT naladixic acid resistant	Roberto La Ragione, Veterinary Laboratories Agency
SL1344 $\Delta fliC$ (CMG 83)	λ red removal of <i>fliC</i> gene	Angus Best (Arques et al., 2009)
SL1344 $\Delta fljB$ (CMG 84)	λ red removal of <i>fljB</i> gene	Angus Best (Arques et al., 2009)
<i>S. Dublin</i> (CMG 33)		Tim Wallace Institute for Animal Health
<i>S. Cholerasuis</i> (CMG 32)		Tim Wallace Institute for Animal Health
<i>S. Derby</i> (CMG 111)		Roberto La Ragione, Veterinary Laboratories Agency
<i>S. Enteritidis</i> (CMG 41)		Lohmann Animal Health

4.6.2. Media and growth conditions

Strains (Table 4.2) were grown in LB broth (Invitrogen) with and without shaking (200rpm), and on LB agar and BGA at 37°C for 18h. BGA_{nal} was purchased from E&O and contained naladixic acid (20µg/ml) and novobiocin (1µg/ml). RV broth was incubated at 37°C, 18h.

4.6.3. Purification of bacterial flagella

Bacterial flagella were acquired by mechanical shearing of the bacteria with a homogeniser (IKA, T10). *S. Typhimurium* SL1344 flagella mutant strains were grown statically for 18h at 37°C in 500ml LB. Bacteria were collected by centrifugation at 5000×g (4°C, 15min) then suspended in an equal volume of PBS (Sigma) and collected again before being suspended in 20ml PBS. The suspension was homogenised on ice, speed 6 for 60s. Whole bacteria were removed by centrifugation (5,000×g 4°C, 15 min). The remaining suspension was further centrifuged (16,300×g 4°C, 10 min) to ensure removal of bacteria. To pellet the flagella, the supernatant was centrifuged (145,000×g 4°C, 90min) and the pellet was

suspended in 500µl PBS (Ikeda et al., 2001). Flagella suspensions were stored at -20°C.

4.6.4. Sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE)

The flagella suspensions were diluted in Laemmli sample buffer (4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.004% (v/v) bromphenol blue and 125mM Tris HCl, pH 6.8) then heated to 95-100°C for 5min. Suspensions were then loaded onto a 4% (v/v) polyacrylamide stacking gel and subsequently run through a 12% (v/v) polyacrylamide, 1% (w/v) SDS, 1mm thick resolving gel. The gel was run in 25mM Tris, 192mM glycine, 0.1% (w/v) SDS at 200V for 60min. To stain proteins in the resolving gel, gelcode blue safe (Thermo Scientific) stain was used. Gels were incubated for a minimum of 1h in protein stain with gentle rocking, at RT, before de-staining in distilled water, with gentle rocking at 4°C for 16h (Laemmli, 1970). Gel pictures were obtained using a scanner (Epson).

4.6.5. Western blotting

Flagella proteins in 12% (v/v) polyacrylamide gels were Western-blotted onto nitrocellulose (Amersham, GE Healthcare) using a semi-dry method. Using a Trans-blot semi-dry transfer cell (Bio-Rad), the semi-dry method was carried out at RT in Schaeffer-Nielsen buffer (48mM Tris, 39mM glycine, 20% (v/v) methanol, 0.04% (v/v) SDS, pH 9.2), and proteins were transferred at 15V for 30min.

Blots were blocked in 5% (w/v) skimmed milk powder (Marvel), 0.1% (v/v) Tween20 in PBS (PBST) at 4°C for 16h. Primary antibodies used were either anti-FliC rabbit IgG or anti-FliB rabbit IgG antibodies (1:500, *Salmonella* Hi, Mast, and 1:500 *Salmonella* H2, Difco respectively). Primary antibodies were diluted in 5% (w/v) skimmed milk powder, 0.1% (v/v) PBST, and incubated with blots for at least 1h at RT, with gentle rocking. Blots then were washed three times in 0.1% (v/v) PBST at 15min intervals with gentle rocking at RT. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibodies were diluted in 5% (w/v) skimmed milk powder, 0.1% (v/v) PBST at a 1:1000 dilution and were incubated with blots for

at least 1h at RT with gentle rocking. Following incubation, blots were washed as above.

Blots were developed using enhanced chemi-luminescence reagent; Pico-West SuperSignal ECL (Thermo Scientific). The subsequent signal from the blots was captured using GBOX iChemiXT from Syngene and GeneSnap (Syngene) software. Blots captured on the GBOX, were placed on an acetate sheet and covered with a thin layer of ECL reagent. The settings used were a focus of 180 at a distance of 285mm, with the highest quality capture time (Towbin et al., 1979).

4.6.6. Immunisation, serum collection and processing

Moredun Scientific Ltd staff immunised the pigs and collected whole blood samples from the vena cava. The blood was allowed to clot and serum was transferred to centrifuge tubes. Blood cells were removed by centrifugation at 1000×g for 15min. Serum was transferred to CryoTubes and stored at -80°C.

4.6.7. Faecal sample collection and processing

Trained animal handlers from Moredun Scientific Ltd collected faecal samples using a swab or a sterile spatula. Faecal samples were weighed if collected by spatula into a sample tube then sub-cultured 1:10 with PBS. Samples were both plated onto BGA_{nal} (100µl) for enumeration and sub-cultured 1:10 into RV broth for detection. BGA_{nal} and RV were incubated at 37°C, 18 h. Post incubation, 10µl RV was streaked onto BGA_{nal} for *Salmonella* detection. BGA_{nal} was incubated at 37°C, 18h.

4.6.8. Tissue sample collection and processing

Pigs were euthanised by Moredun Scientific Ltd trained staff and tissue samples excised by vet, Dr Arvind Mahajan. Tissue samples (1g) were diluted 1:10 in PBS. Samples were plated onto BGA_{nal} agar plates for enumeration. For *Salmonella* detection 1 ml from each diluted sample was sub-cultured 1:10 into RV broth and incubated (static, 37°C, 16-18h). RV samples (10µl) were then streaked onto BGA_{nal} and incubated (statically, 37°C, 16-18h). *Salmonella* presence was indicated by large pink colonies on BGA plates.

4.6.9. Detection of porcine *S. Typhimurium* FliC and FljB specific antibodies by enzyme linked immuno-sorbent assay (ELISA)

Flagella proteins were diluted in sodium bicarbonate buffer (Sigma), and 1µg of flagella was added to wells of a 96 well plate and incubated (4°C, 18h). Wells were washed with 0.05% (v/v) PBST three times. Wells were blocked with 5% skimmed milk powder in 0.05% (v/v) PBST, 4°C, 18h. After blocking, flagella specific antibodies; anti-FliC rabbit IgG (1:10000 dilution, Hi Mast) and anti-FljB rabbit IgG (1:10000, H2 Difco), were added to positive control wells diluted in 5% skimmed milk powder in 0.05% (v/v) PBST. Porcine serum was serially diluted 1:2 and added to the plate in triplicate. Plates were incubated at 4°C, 18h. Wells were then washed as before and the positive control wells had anti-rabbit IgG (goat) conjugated to HRP (BD) added diluted in 5% skimmed milk powder in 0.05% (v/v) PBST (1:10000 dilution) added. To the test wells, anti-porcine rabbit IgG conjugated to HRP (Sigma) or anti-porcine goat IgA conjugated to HRP (AbD serotec) diluted in 5% skimmed milk powder in 0.05% (v/v) PBST (1:10000 dilution) was added. Secondary antibodies were incubated for 2h at RT. Wells were then washed as before. Binding was detected using 3,3',5,5'-Tetramethylbenzidine (TMB, Sigma) which was added to the wells for 20min, followed by 1M sulphuric acid (Fisher Scientific). Florescence was measured at 450nm using Biotek Synergy HT plate reader.

4.6.10. Detection of porcine *S. Typhimurium* FliC and FljB specific mucosal antibodies by Western blot

Flagella proteins FliC and FljB were separated by SDS-PAGE then trans-blotted to nitrocellulose membrane, as described earlier in section 4.6.5. Blots were blocked in 5% (w/v) skimmed milk powder (Marvel), 0.1% (v/v) PBST at 4°C for 16h. At post-mortem, mucosal scrapings from the ileum were collected from control and vaccinated pigs. Mucus from vaccinated pig (591) and control pig (550) was diluted (1:10) and centrifuged (3000×g, 5min). The supernatant was further diluted (final concentration 1:500) in PBS, and added to the blots (blots were incubated at 4°C for 18h). Blots then were washed three times in 0.1% (v/v) PBST at 15min intervals with gentle rocking at RT. Secondary antibodies, anti-porcine goat IgA conjugated to

HRP were diluted in 5% (w/v) skimmed milk powder, 0.1% (v/v) PBST at a 1:1000 dilution and were incubated with blots for at least 1h at RT with gentle rocking. Following incubation, blots were washed as above. Blots were developed using enhanced chemi-luminescence reagent; Pico-West SuperSignal ECL (Thermo Scientific). The subsequent signal from the blots was captured using GBOX iChemiXT from Syngene and GeneSnap (Syngene) software. Blots captured on the GBOX, were placed on an acetate sheet and covered with a thin layer of ECL reagent. The settings used were a focus of 180 at a distance of 285mm, with the highest quality capture time.

4.6.11. *In vitro* lymphocyte proliferation of porcine spleens

Single cell suspensions from the spleens of control and vaccinated pigs were plated at 5×10^5 cells/well of a 96-well, flat-bottom plate in 200 μ l final volume. Graded doses of FliC, FljB or ConA were added. Proliferation was measured by the addition of 0.5 μ Ci of 35 Ci/mmol-thymidine (Perkin Elmer) to each well, 16h before the end of a 4d culture period. Plates were harvested onto filter mats (Wallac, Turku, Finland) using a 96-well MachIIM Tomtec harvester (Wallac). The mats were dried on a hot plate and a solid scintillation wax melted into them (Meltilex™ A; Wallac). When the wax had re-solidified, the mats were read using a Trilux 1450 Microbeta liquid scintillation and luminescence counter and software (Trilux, Arnsberg, Germany). Tissue isolation, cell preparations and FACS measurements were carried out by Dr Simmi Mahajan.

4.6.12. Intracellular cytokine staining for IFN γ + cells: flow cytometry

Splenocytes from 9 control and 10 vaccinated pigs were stimulated in the presence of 100ng/ml phorbol 12-myristate 13-acetate (PMA) and 1 μ g/ml ionomycin (both from Sigma) for 5h at 37°C. GolgiStop™ (Pharmingen), inhibitor of protein transport was included at a dilution of 1:1500, as recommended by the manufacturer. Cells were stained with CD4-FITC (1:50), CD8-PE (1:20) and CD21-Biotin (1:50) followed by Streptavidin-per CP eFluor 710 (1:2000) to detect surface CD4, CD8 and CD21

expression, washed and then fixed and permeabilised by re-suspending in Cytofix/Cytoperm™ reagent (PharMingen) for 20min on ice. Cells were washed in permeabilisation buffer (provided in the Cytofix/Cytoperm™ kit) and stained with AlexaFluor 647-labelled antibodies against IFN γ for 30 min on ice. Subsequent washes were again performed in permeabilisation buffer and the cells finally resuspended in 2% (v/v) FBS in PBS. Samples were acquired with a FACScalibur flow cytometer and CellQuest Pro software (Becton Dickinson, Mountain View, CA, USA), using a live cell gate set by forward and side scatter characteristics. The analysis was done using FlowJo software (TreeStar, California, USA). Tissue isolation, cell preparations and flowcytometry measurements were carried out by Dr Simmi Mahajan.

4.6.12. *Salmonella* agglutination test

To confirm that the pigs did not possess antibodies to *S. Typhimurium* at the beginning of the trial, agglutination tests with SL1344 were carried out. *Salmonella* were grown at 37°C on LB agar plates. One colony from an LB agar plate was added to a drop of pre-immunisation serum from each pig on glass slides. Agglutination was determined visually, and there were no positive agglutination tests prior to vaccination. At the end of the trial, both pre-and post-immunisation sera from each pig were tested for agglutination against the strains listed in Table 4.2. Agglutination was determined visually and recorded.

4.6.13. Maintenance of cell lines

Porcine intestinal epithelial cell line, IPEC-J2 cells were maintained in DMEM/F-12; 100U/ml penicillin, 30 μ g/ml streptomycin (Invitrogen); 5% (v/v) FBS (Schierack et al., 2006) at 37°C, 5% CO₂. The medium was changed every 48h.

4.6.14. Adherence Inhibition Assay of IPEC-J2 cells by in-cell ELISA

IPEC-J2 cells were grown to confluence in 24 well tissue culture plates (Corning). *Salmonella* Derby were sub-cultured from a streaked LB agar plate into LB and

cultured at 37°C, 200rpm for 18h. Cultures were centrifuged at 4000×g for 5min and pellets re-suspended in MEM-HEPES (Lonza), to an OD₆₀₀ of 0.3. Pre-immunisation and post-immunisation serum were added to separate bacterial suspensions at a 1:10 dilution prior to addition to the cells, and incubated at RT with gentle rocking for 30min. Cells were washed with MEM HEPES and bacteria were added at an MOI of 10-20, and incubated for 3h at 37°C, 5% CO₂. Cells were washed three times with PBS, fixed with 2% (w/v) PFA for 15min at RT then permeabilised with 0.1% (v/v) Triton X-100 for 1min. Cells were then washed 3 times with PBS, before being treated with 1% (v/v) H₂O₂ in methanol to quench endogenous peroxidase activity at RT for 20min. Cells were then washed as above before being blocked with 3% (w/v) BSA (fraction V, Sigma) in PBS at 4°C for at least 16h.

After blocking, anti-O4 rabbit IgG (Mast) was added at 1:500 to 3% (w/v) BSA and incubated with cells for 1h at RT. Cells were washed as before then anti-rabbit goat IgG-HRP (BD) was added 1:1000 in 3% (w/v) BSA, incubated for 1h at RT and washed as before. Binding was detected using SuperSignal West Pico ECL reagents (Thermo Fisher) using GBOX iChemiXT running GeneSnap (Syngene) the focus setting was 50 and the distance was set at 625mm. Densitometry was performed on inverted images using GeneTools software (Syngene) with a 65 spot radius. To indicate how confluent the cells were; cells were washed with PBS and 0.1% (v/v) crystal violet stain (Sigma) was added, incubated at 4°C for 30min then washed three times with PBS. 20% (v/v) acetone in ethanol (both Fisher Scientific) was added, incubated for 30min at RT and the absorbance was measured at 595nm using Synergy HT plate reader (BioTek). Results from in-cell binding assays are expressed as relative binding, using the densitometry results and the following formula:

$$\text{Relative protein binding} = \frac{\text{Densitometry result} - \text{No primary antibody control}}{\text{Crystal Violet result} - \text{No protein added control}}$$

4.6.15. Statistical analyses

Statistical analyses were carried out using R i386 version 3.0.1

4.6.15.1. *Salmonella* shedding

To determine if the number of *Salmonella* positive days from pigs in the vaccinated group was significantly different from pigs in the control group, a GLM with Poisson errors was employed, in order to take into account the integer nature of the number of *Salmonella* positive days' data. Examination of the residuals revealed over dispersion of the deviant and therefore a quasi-Poisson structure was employed. P-values of <0.05 were taken as significant (Crawley, 2007).

4.6.15.2. Intestinal colonisation of *Salmonella* in vaccinated and control pigs

Differences in the colonisation status of intestinal tissues between vaccinated and control group was assessed using Wilcoxon rank sum (WRS) (equivalent to Mann-Whitney). P-values of <0.05 were taken as significant.

4.6.15.3. *Salmonella* tissue distribution and *Salmonella* shedding

In order to try and assess whether there were differences between vaccinated and control pigs in tissue distribution of *Salmonella*, principal component techniques were used. For each tissue pigs were ascribed as *Salmonella* positive (1) or negative (0). In addition, whether pigs were positive for more than 10 days was also included as a binary variable. Graphical representations of the first, second and third principal components (PC1, PC2 and PC3) were examined visually to determine if separation between groups was evident.

4.6.15.4. Porcine *S. Typhimurium* FliC and FljB specific antibody ELISAs

To analyse differences between antibody titres in the control and vaccinated groups a set of WRS tests and Wilcoxon signed rank (WSR) tests were carried out. First, control and vaccinated pre- immune titre results were analysed using a WRS. Second, changes in titre levels in controls and changes in titre levels in vaccinated pre- to post- immunisation were analysed using WSR, this was to take into account of the fact that pre- and post- paired values from the same pig were been compared (non-parametric equivalent to the paired t-test). Next, differences between control and

vaccinated pigs post immunisation were compared using a WRS. Finally, the changes in titres, pre- to post- immunisation, were assessed between control and vaccinated pigs using a WRS.

4.6.15.5. Relationship between number of *Salmonella* shedding days with increased recognition of FliC and FljB by humoral antibodies

To determine if the number of *Salmonella* positive days during shedding was dependent on the antibody titre ratio, a GLM with Poisson errors was employed. P-values of <0.05 were taken as significant.

4.6.15.6. Relationship between *Salmonella* positive tissues with increased recognition of FliC and FljB by humoral antibodies

To determine if *Salmonella* detection in intestinal tissues was dependent on the antibody titre ratio, analysis using a GLM with binomial errors was carried out. P-values of <0.05 were taken as significant.

4.6.15.7. Splenocyte proliferation

Data were assessed for normality using a Kolmogorov-Smirnov test and were found not to be normally distributed ($p < 0.05$). Therefore, analyses of differences between groups were assessed using WSR. P-values of <0.05 were taken as significant.

For the range of antigen concentrations, shown in Figure 4.7, the standard error was calculated using the formula:

$$\text{Standard error of the mean} = \text{SD} \div \sqrt{n}$$

Where SD is the standard deviation calculated using the following formula:

$$\text{SD} = \sqrt{[\sum(x - \tilde{x})^2/n]}$$

Where \tilde{x} is the average value of x for the entire population and n is the population size.

4.6.15.8. Flowcytometry analysis of CD4, CD8 and IFN γ stained cells

Analyses of differences in the proportion of distinct populations of cells between groups were assessed using WSR. P-values of <0.05 were taken as significant.

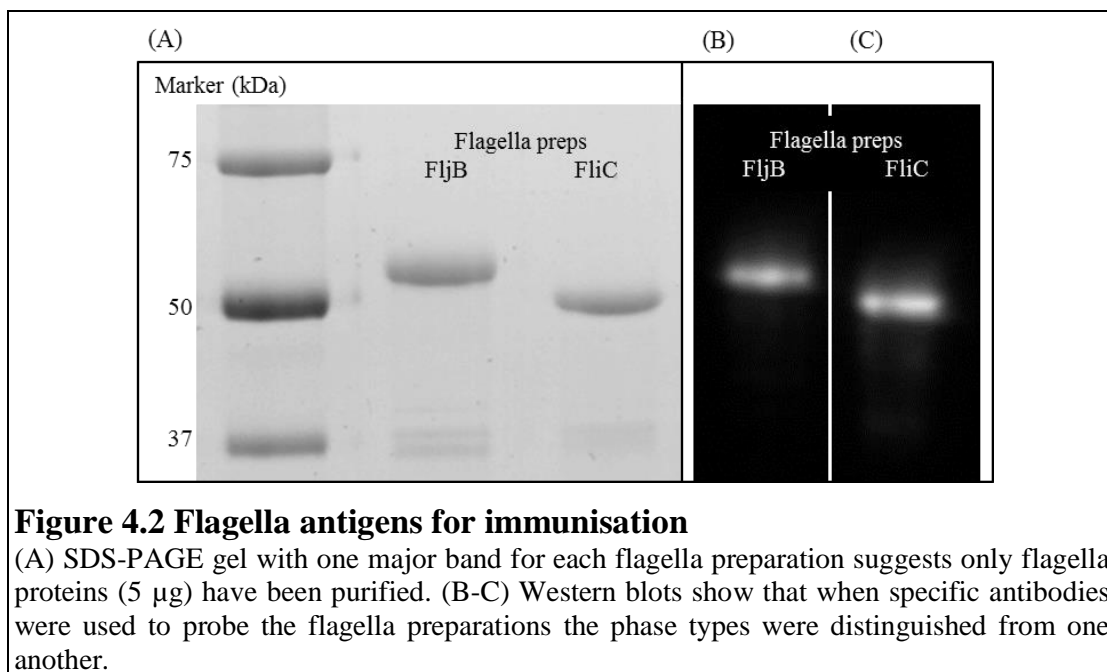
4.6.15.9. Adherence Inhibition Assay of IPEC-J2 cells

Data were assessed for normality using a Kolmogorov-Smirnov test (normal, $p > 0.05$). Analysis of differences between groups were therefore assessed by using GLMs to look at overall differences, where p-values of < 0.05 were taken as significant.

4.7. Results

4.7.1. Preparation of flagella antigens for vaccination

S. Typhimurium flagellin proteins, FliC and FljB were purified from SL1344 flagella knock-out strains; $\Delta fliC$ and $\Delta fljB$. The flagella preparations were separated by SDS-PAGE and imaged to determine purity. Western blots were performed to confirm flagella phase and recognition by specific antibodies (Figure 4.2).



4.7.2. Clinical observations

The clinical parameters of the pigs were assessed continuously throughout the trial. The parameters were recorded according to Table 4.3. Throughout the trial all the pigs scored 0 for all the parameters shown in Table 4.3, with the exception of pigs 606 and 608 on days 1, 2, 3 post challenge. Rectal temperatures for pig 606 did not fall within normal parameters; 40.3, 37.8, 39.6°C respectively. Rectal temperatures for pig 608 also did not fall within normal parameters; 39.9, 39.8, 39.6°C for days 1, 2, 3 post challenge respectively. However, pigs 606 and 608 did score 0 for all the other parameters throughout the trial. An increase in rectal temperature was expected following challenge with *Salmonella*, along with higher scores in faecal consistency (diarrhoea). However, the majority of pigs had normal temperature and all pigs had

normal faecal consistency throughout the testing period. The *Salmonella* challenge did not result in clinical symptoms that we were able to measure.

Table 4.3 Clinical observation scoring

Rectal temperature		Demeanour	
$\geq 38.0 \leq 39.5^{\circ}\text{C}$	0	Normal	0
$> 39.5 \leq 40.0^{\circ}\text{C}$	1	Depressed	1
$> 40.0 \leq 41.0^{\circ}\text{C}$	2	Very Dull	2
< 38.0 or $> 41.0^{\circ}\text{C}$	3	Recumbent	3
Faecal consistency		Body condition	
Normal	0	Normal	0
Soft	1	Thin	1
Fluid	2	Anorexis	2
Fluid and copious	3		

4.7.3. *Salmonella* Typhimurium flagella vaccination effect on *Salmonella* shedding and intestinal colonisation

Pigs were immunised with *S. Typhimurium* purified flagella, consisting primarily of FliC and FljB flagellin proteins. Pigs were subsequently challenged to determine if immunisation with purified flagella could provide pigs with a protective immune response against *S. Typhimurium* infection. A group of 10 pigs were vaccinated with purified FliC and FljB plus adjuvant and a group of 10 control pigs were given adjuvant only. Vaccinated and control pigs were both challenged with naladixic acid resistant *S. Typhimurium* SL1344. Porcine faecal samples were tested for *Salmonella* to determine the colonisation status of each pig. Analysis using WRS revealed no significant difference in the number of *Salmonella* positive days during the shedding test period between the control and vaccinated groups (Figure 4.3).

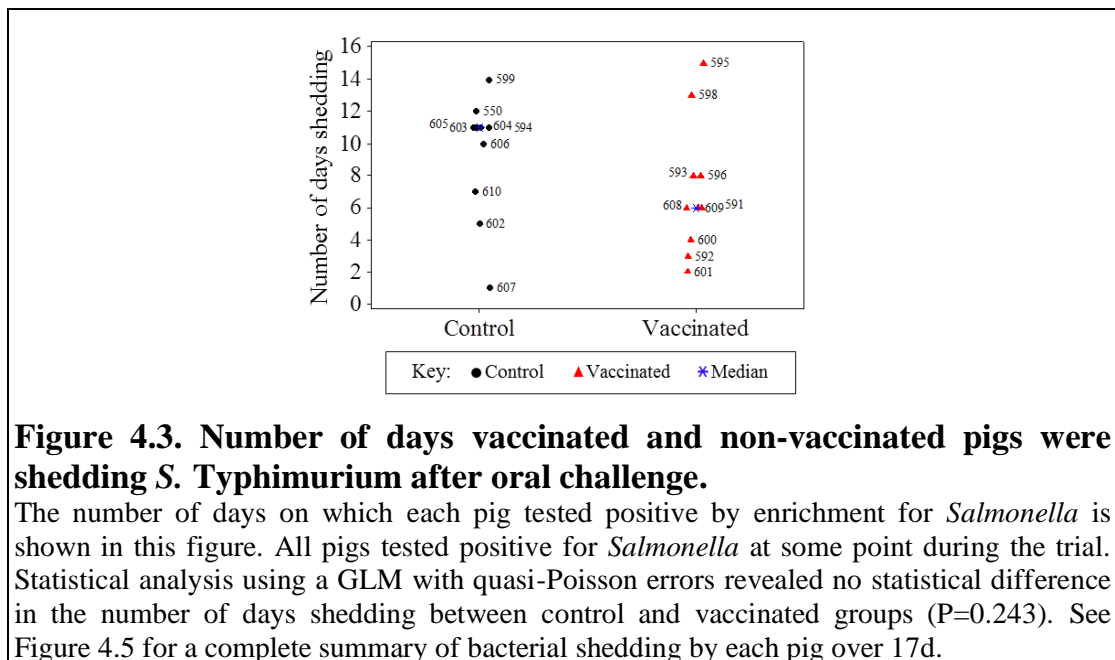


Figure 4.3. Number of days vaccinated and non-vaccinated pigs were shedding *S. Typhimurium* after oral challenge.

The number of days on which each pig tested positive by enrichment for *Salmonella* is shown in this figure. All pigs tested positive for *Salmonella* at some point during the trial. Statistical analysis using a GLM with quasi-Poisson errors revealed no statistical difference in the number of days shedding between control and vaccinated groups ($P=0.243$). See Figure 4.5 for a complete summary of bacterial shedding by each pig over 17d.

At post mortem, samples were taken to determine the presence and distribution of *Salmonella* within the gut tissues and contents. Intestinal tissues tested were as follows; colon, colon contents, caecum, caecum contents, ileum, ileum contents, MLN and ileocecal lymph nodes (IcLN, Figure 4.4). Analysis of intestinal tissue *Salmonella* status using WRS revealed that significantly more pigs in the control group tested positive for *Salmonella* in ileum tissue samples compared to the vaccinated group ($P=0.036$). However, there was no significant difference between the control and vaccinated groups for any of the other tissues or the intestinal contents tested.

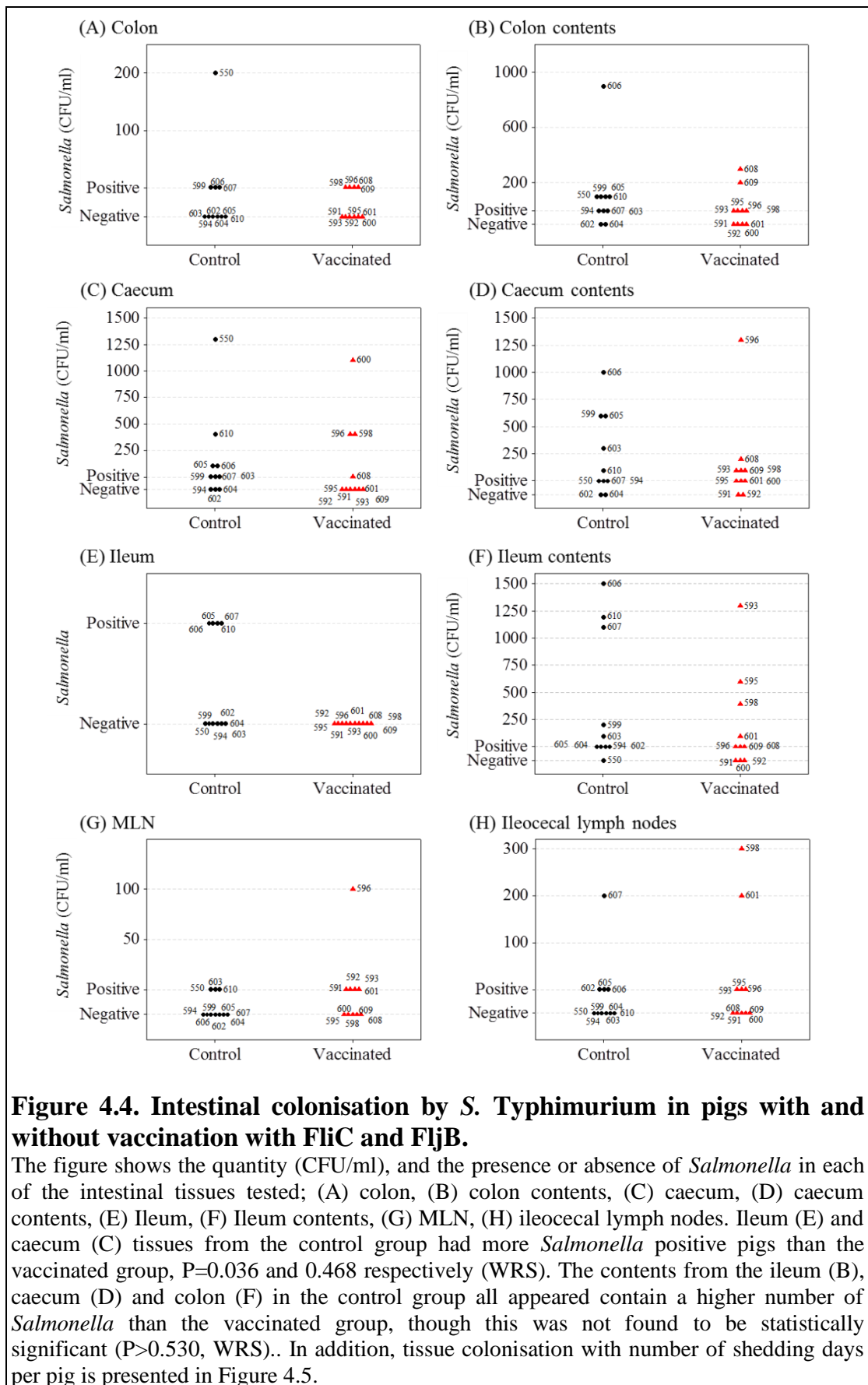


Figure 4.4. Intestinal colonisation by *S. Typhimurium* in pigs with and without vaccination with FliC and FljB.

The figure shows the quantity (CFU/ml), and the presence or absence of *Salmonella* in each of the intestinal tissues tested; (A) colon, (B) colon contents, (C) caecum, (D) caecum contents, (E) Ileum, (F) Ileum contents, (G) MLN, (H) ileocecal lymph nodes. Ileum (E) and caecum (C) tissues from the control group had more *Salmonella* positive pigs than the vaccinated group, $P=0.036$ and 0.468 respectively (WRS). The contents from the ileum (B), caecum (D) and colon (F) in the control group all appeared contain a higher number of *Salmonella* than the vaccinated group, though this was not found to be statistically significant ($P>0.530$, WRS).. In addition, tissue colonisation with number of shedding days per pig is presented in Figure 4.5.

Figure 4.4 shows that 4 of the 10 ileum tissue samples from the control group tested positive for *Salmonella*, yet all 10 ileum tissues collected from vaccinated pigs were negative ($P=0.036$). In addition, caecum tissue from 4 pigs of the vaccinated group tested positive for *Salmonella*, compared to 7 in the control group, though this was not statistically significant ($P=0.468$). Furthermore, the number of bacteria (CFU/ml) recovered from the contents of the caecum and the ileum from the control group appeared to be higher than those from the vaccinated group, however, these were not statistically significant $P=0.614$ and $P=0.530$ respectively.

Figure 4.5 shows the *Salmonella* shedding results per pig per day and the *Salmonella* status of each of the tissues tested. Graphical representations of first, second and third principal components of Figure 4.5 were examined visually to determine if separation between groups was evident (Figure 4.6). This analysis suggests that immunisation with flagella had no effect on *Salmonella* shedding or on colonisation overall.

These results suggest that immunisation with flagella may have reduced colonisation of the ileum in vaccinated pigs. However, it is difficult to conclude this definitively given the small number of *Salmonellae* in the tissues of the intestine which in most pigs was less than 100CFU/ml, much less than previously reported by similar studies (Carnell et al., 2007). Furthermore, the number of *Salmonella* isolated during the shedding test period was again much lower than previously reported (also <100CFU/ml) (Carnell et al., 2007, Pullinger et al., 2010). A possible explanation for this difference between studies was the use of SL1344 nal_r. This strain is a histidine auxotroph and in previous studies was used as a challenge strain on younger pigs (personal communication, Roberto La Ragione). This could account for the lack of colonisation by this strain in older pigs which have more developed immune systems making them more difficult to colonise with an attenuated *Salmonella* strain.

Despite however, the apparent failure of the challenge strain to colonise the pigs to the levels which were expected the vaccinated pigs did appear to be afforded some protection for colonisation in the ileum, perhaps indicating vaccination did provide

some protection against colonisation. However, further studies would be required to confirm this, given the overall poor *Salmonella* colonisation status that was observed.

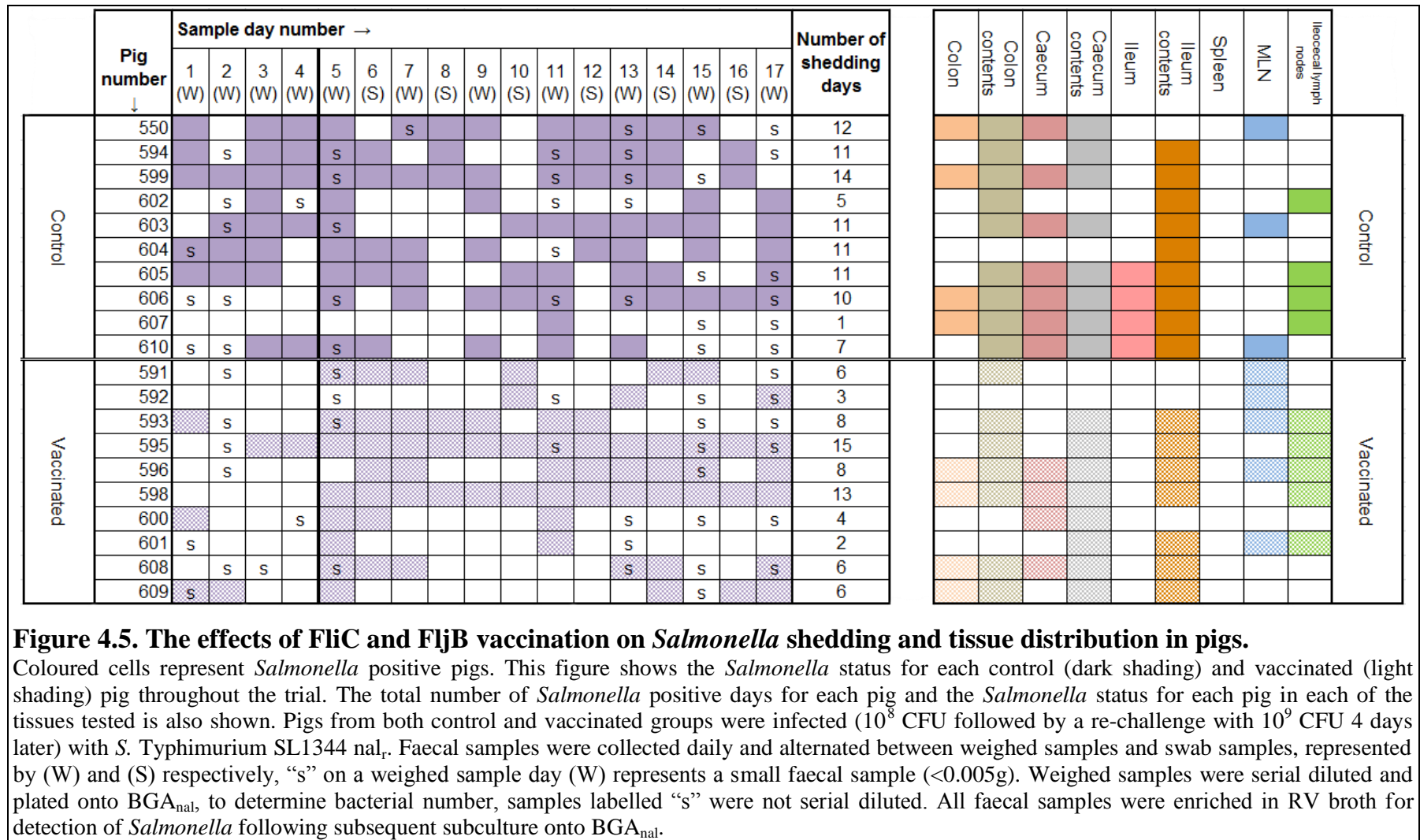


Figure 4.5. The effects of FliC and FljB vaccination on *Salmonella* shedding and tissue distribution in pigs.

Coloured cells represent *Salmonella* positive pigs. This figure shows the *Salmonella* status for each control (dark shading) and vaccinated (light shading) pig throughout the trial. The total number of *Salmonella* positive days for each pig and the *Salmonella* status for each pig in each of the tissues tested is also shown. Pigs from both control and vaccinated groups were infected (10^8 CFU followed by a re-challenge with 10^9 CFU 4 days later) with *S. Typhimurium* SL1344 nal_r. Faecal samples were collected daily and alternated between weighed samples and swab samples, represented by (W) and (S) respectively, “s” on a weighed sample day (W) represents a small faecal sample (<0.005g). Weighed samples were serial diluted and plated onto BGA_{nal}, to determine bacterial number, samples labelled “s” were not serial diluted. All faecal samples were enriched in RV broth for detection of *Salmonella* following subsequent subculture onto BGA_{nal}.

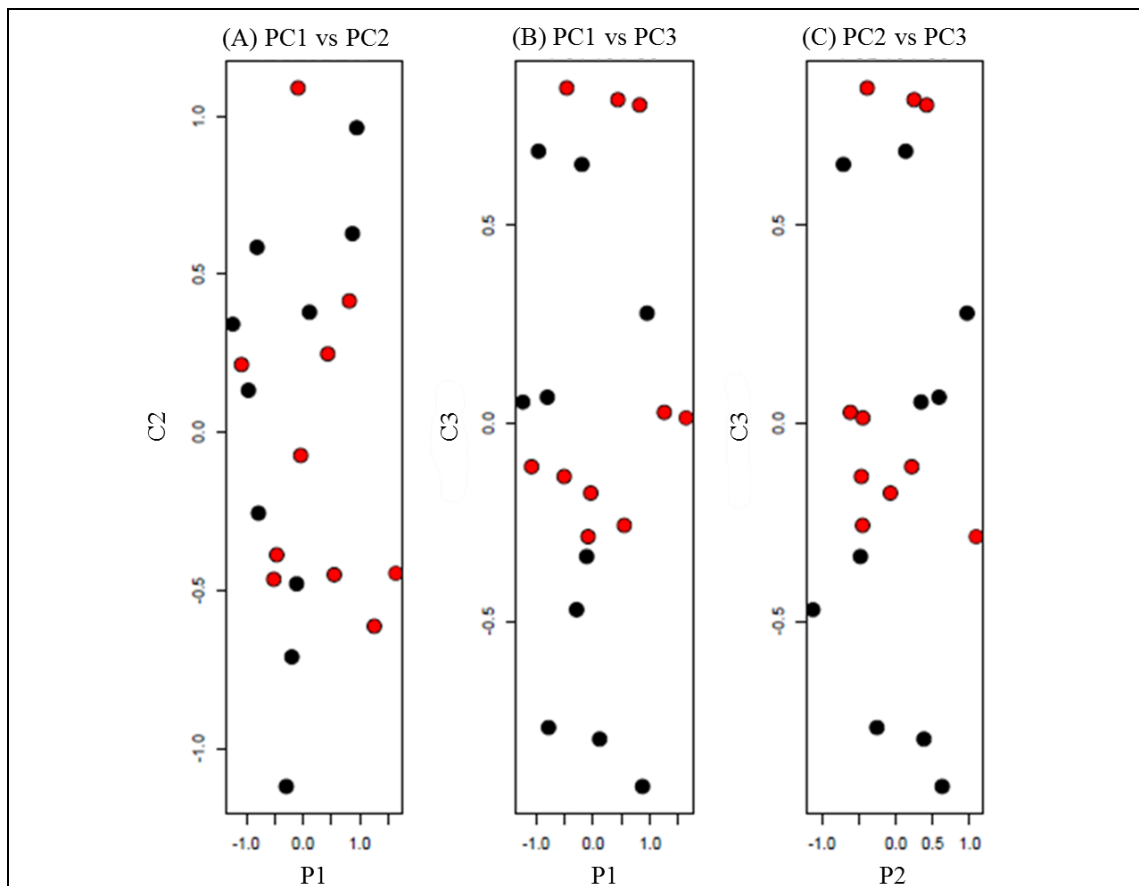


Figure 4.6. Principal components analysis of the effect of FliC and FljB vaccination on *S. Typhimurium* colonisation of host tissues and shedding into the lumen and the external environment.

Graphical representations of the principal components analysis for the *Salmonella* status of tissues, contents and shedding is shown above. FliC and FljB vaccinated pigs are shown in red, control pigs are shown in black. No distinct grouping was determined. Principal components techniques were used in order to evaluate differences between control and vaccinated groups in *Salmonella* status for tissues, contents and shedding for more than 10 days. Pigs were ascribed a value of 1 if tissues were *Salmonella* positive or 0 if negative. Pigs positive for more than 10 days during shedding were also ascribed a value of 1, the remaining pigs were ascribed a value of 0.

4.7.4. Vaccinated pigs exhibit increased recognition of flagellin proteins FliC and FljB by IgG and IgA humoral antibody

To determine if immunisation with flagella antigens FliC and FljB increased recognition of these antigens by IgG and IgA humoral antibodies, both pre- and post-immunisation sera were tested by ELISA. Titres were determined by identifying the highest dilution which was positive. A positive ELISA result was defined as absorbance values measured at 450nm exceeding 0.1. The titre results are shown in Figure 4.7.

Analysis of pre-immune sera titres between groups using WRS revealed that pre-immune titres were not significantly different between vaccinated and control groups. In addition, analysis by WSR showed no significant difference between post-immune titres and pre-immune titres in the control group. In contrast, the vaccinated group had a significantly higher titre in post-immune sera compared with pre-immune sera. This was found to be the case for each isotype and flagella type tested, for both control and vaccinated groups. This indicates an increase in recognition of FliC and FljB by both IgG and IgA in the vaccinated group, suggesting that both IgG and IgA antibodies were generated to FliC and FljB, following immunisation with these proteins. However, no significant change in titre for either FliC or FljB by IgG or IgA in the control group suggests the level of FliC and FljB recognition by IgG and IgA remained the same for the control group.

Further analysis by WRS was carried out on the ratio of titre change (post-immune titre divided by pre-immune titre) between vaccinated and control groups. This test demonstrated a significant difference between the vaccinated and control group in the post- to pre-immune titre ratio for both FliC and FljB for both IgG ($P < 0.001$) and IgA (FliC= $P = 0.004$, FljB $P = 0.001$). This data analysis appears to support the hypothesis that there is an increase of FliC and FljB recognition by IgG and IgA in the vaccinated group and suggests there was no increase or decrease in the level of FliC and FljB recognition by the control group.

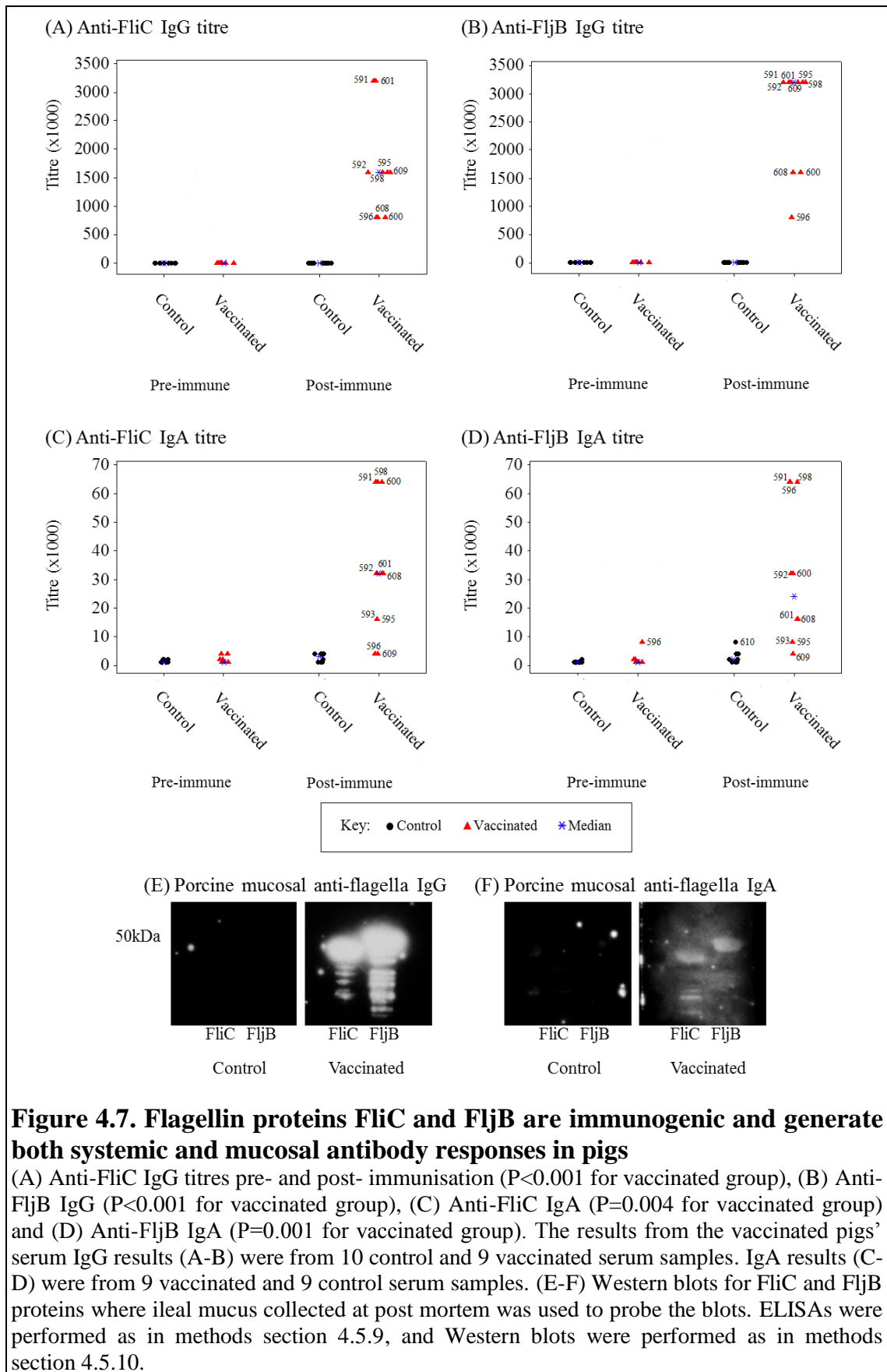


Figure 4.7. Flagellin proteins FliC and FljB are immunogenic and generate both systemic and mucosal antibody responses in pigs

(A) Anti-FliC IgG titres pre- and post- immunisation ($P < 0.001$ for vaccinated group), (B) Anti-FljB IgG ($P < 0.001$ for vaccinated group), (C) Anti-FliC IgA ($P = 0.004$ for vaccinated group) and (D) Anti-FljB IgA ($P = 0.001$ for vaccinated group). The results from the vaccinated pigs' serum IgG results (A-B) were from 10 control and 9 vaccinated serum samples. IgA results (C-D) were from 9 vaccinated and 9 control serum samples. (E-F) Western blots for FliC and FljB proteins where ileal mucus collected at post mortem was used to probe the blots. ELISAs were performed as in methods section 4.5.9, and Western blots were performed as in methods section 4.5.10.

Further to the humoral antibody ELISAs, mucosal scrapings taken at post mortem from the ileum were tested for the presence of mucosal antibody. Western blots were used as an alternative method to ELISAs, as the high sensitivity of the ELISA test resulted in saturation of the signal. This is most likely due to the presence of the many peptides within the mucus preparation, resulting in non-specific binding. To test the mucus by ELISA, antibodies could be purified from the mucus preparation before testing. However, this method was beyond the scope of this study. Though, future studies would include this additional stage.

Mucus from the ileum of one pig from the control group and one from the vaccinated group were tested by Western blot for the presence of IgG and IgA that recognise FliC and FljB (Figure 4.7(E-F)). These Western blots indicate the presence of both IgG and IgA that can bind to FliC and FljB in the vaccinated pig mucus, but no recognition of either FliC or FljB was detected in the control pig mucus. The results from Western blots using mucus suggest that antibodies were generated at the mucosal surface following immunisation with flagella. Data from all the animals is required to conclude that more FliC/FljB antibodies were present at the mucosal surface in either group. However, results by Western blot show that antibodies were detected in one of the vaccinated animals at the mucosal surface, potentially indicating the generation of mucosal antibody following intramuscular immunisation. This has previously been shown in another study for an *E. coli* O157:H7 vaccine in cattle (McNeilly et al., 2008, McNeilly et al., 2010).

4.7.5. Relationship between number of *Salmonella* shedding days with increased in recognition of FliC and FljB by humoral antibodies

To determine if the number of *Salmonella* positive days during shedding was dependent on the antibody titre ratio, antibody titre ratios and the number of *Salmonella* positive days during shedding for each pig were analysed. Figure 4.8 shows titre ratios for each antibody isotype and flagella tested, plotted against the number of *Salmonella* positive days for each pig. Analysis revealed no relationship between titre ratio and number of *Salmonella* positive days. The lack of an apparent relationship between antibody titre and shedding may be due to the low numbers of *Salmonella* isolated from both groups.

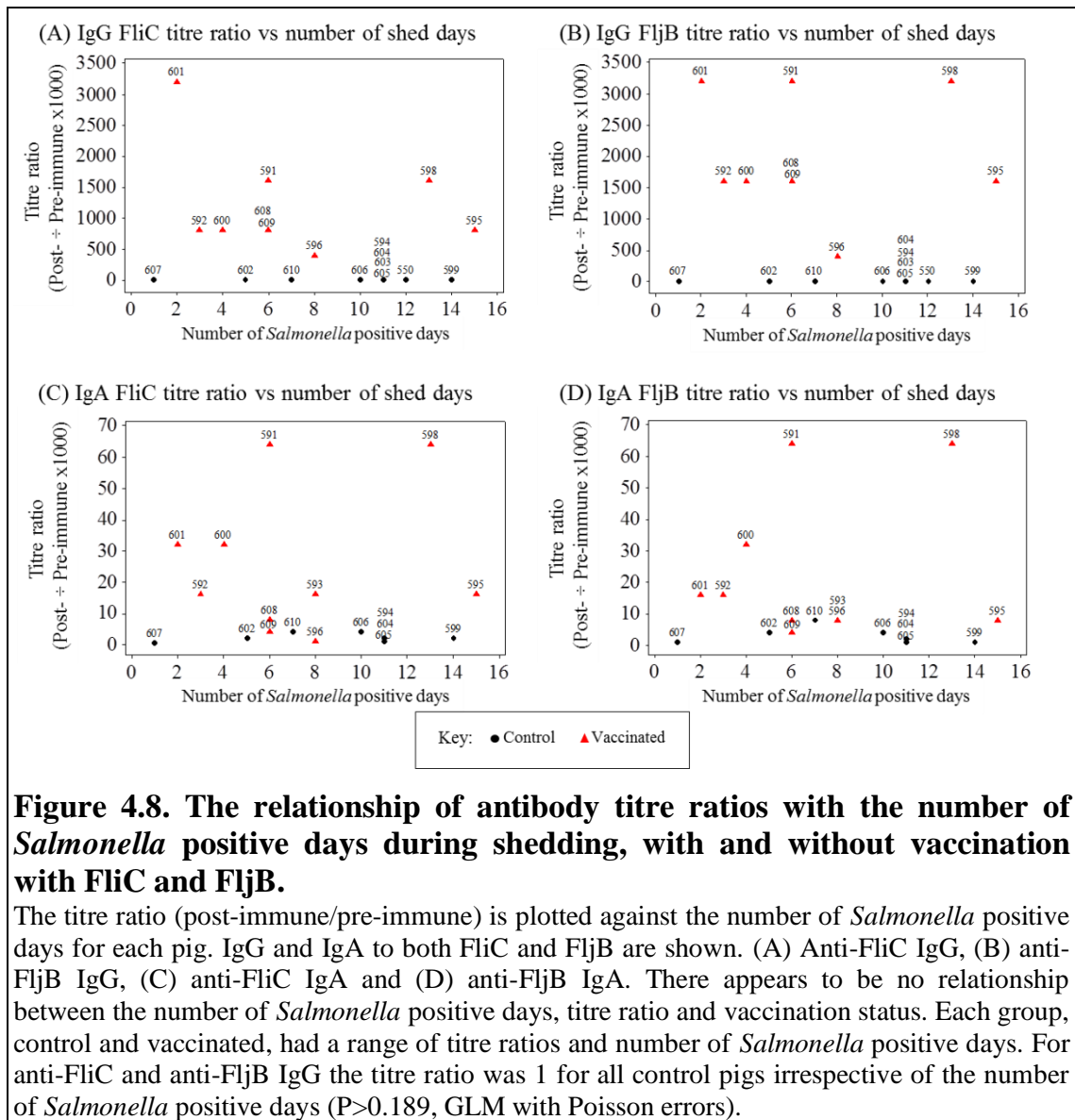


Figure 4.8. The relationship of antibody titre ratios with the number of *Salmonella* positive days during shedding, with and without vaccination with FliC and FljB.

The titre ratio (post-immune/pre-immune) is plotted against the number of *Salmonella* positive days for each pig. IgG and IgA to both FliC and FljB are shown. (A) Anti-FliC IgG, (B) anti-FljB IgG, (C) anti-FliC IgA and (D) anti-FljB IgA. There appears to be no relationship between the number of *Salmonella* positive days, titre ratio and vaccination status. Each group, control and vaccinated, had a range of titre ratios and number of *Salmonella* positive days. For anti-FliC and anti-FljB IgG the titre ratio was 1 for all control pigs irrespective of the number of *Salmonella* positive days ($P > 0.189$, GLM with Poisson errors).

4.7.6. Relationship between *Salmonella* positive tissues with increased recognition of FliC and FljB by humoral antibodies

To determine if *Salmonella* detection in intestinal tissues was dependent on the antibody titre ratio, antibody titre ratios and the number of *Salmonella* positive days during shedding for each pig were analysed. Figure 4.9 shows titre ratios for anti-FljB IgA plotted against each intestinal tissue result (positive or negative) for each pig. Analysis revealed no relationship between titre ratio and *Salmonella* status of intestinal tissues, for anti-FljB IgA, anti-FliC IgA, anti-FljB IgG or anti-FliC IgG. This may be due to the low *Salmonella* colonisation experienced in both groups. The P-values for IgA and IgG analysis are displayed in Tables 4.4 and 4.5 respectively. Where two or less pigs were positive or negative in a particular tissue no analysis could be performed. For IgG results from the control group, no analysis could be performed as there was no change in antibody titre for either FliC or FljB. This is detailed in Tables 4.4 and 4.5.

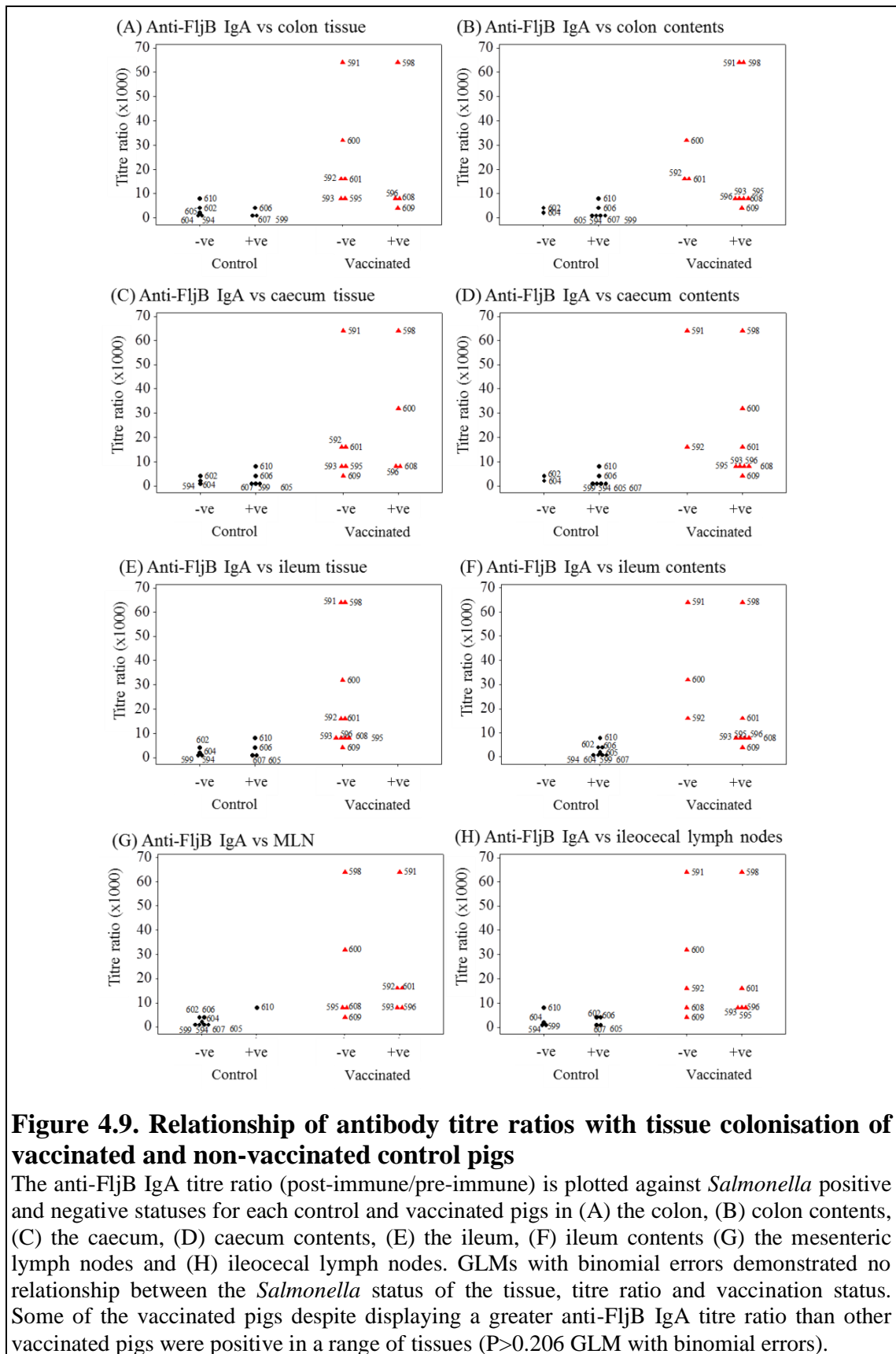


Table 4.4. Statistical analysis of tissue *Salmonella* status and IgA antibody titre ratio

Tissue	Isotype and flagella	Vaccinated or Control pig	Probability value (p=)
Colon	Anti-FljB IgA	Vaccinated	0.832
Colon	Anti-FljB IgA	Control	0.502
Colon contents	Anti-FljB IgA	Vaccinated	0.890
Colon contents	Anti-FljB IgA	Control	2 -ve pigs
Caecum	Anti-FljB IgA	Vaccinated	0.542
Caecum	Anti-FljB IgA	Control	0.619
Caecum contents	Anti-FljB IgA	Vaccinated	2 -ve pigs
Caecum contents	Anti-FljB IgA	Control	2 -ve pigs
Ileum	Anti-FljB IgA	Vaccinated	0 +ve pigs
Ileum	Anti-FljB IgA	Control	0.396
Ileum contents	Anti-FljB IgA	Vaccinated	0.206
Ileum contents	Anti-FljB IgA	Control	0 -ve pigs
MLN	Anti-FljB IgA	Vaccinated	0.954
MLN	Anti-FljB IgA	Control	1 +ve pig
IcLN	Anti-FljB IgA	Vaccinated	0.774
IcLN	Anti-FljB IgA	Control	0.763
Colon	Anti-FliC IgA	Vaccinated	0.483
Colon	Anti-FliC IgA	Control	0.854
Colon contents	Anti-FliC IgA	Vaccinated	0.896
Colon contents	Anti-FliC IgA	Control	2 -ve pigs
Caecum	Anti-FliC IgA	Vaccinated	0.910
Caecum	Anti-FliC IgA	Control	0.495
Caecum contents	Anti-FliC IgA	Vaccinated	2 -ve pigs
Caecum contents	Anti-FliC IgA	Control	2 -ve pigs
Ileum	Anti-FliC IgA	Vaccinated	0 +ve pigs
Ileum	Anti-FliC IgA	Control	0.485
Ileum contents	Anti-FliC IgA	Vaccinated	0.276
Ileum contents	Anti-FliC IgA	Control	0 -ve pigs
MLN	Anti-FliC IgA	Vaccinated	0.942
MLN	Anti-FliC IgA	Control	1 +ve pig
IcLN	Anti-FliC IgA	Vaccinated	0.942
IcLN	Anti-FliC IgA	Control	0.670

Table 4.5. Statistical analysis of tissue *Salmonella* status and IgG antibody titre ratio

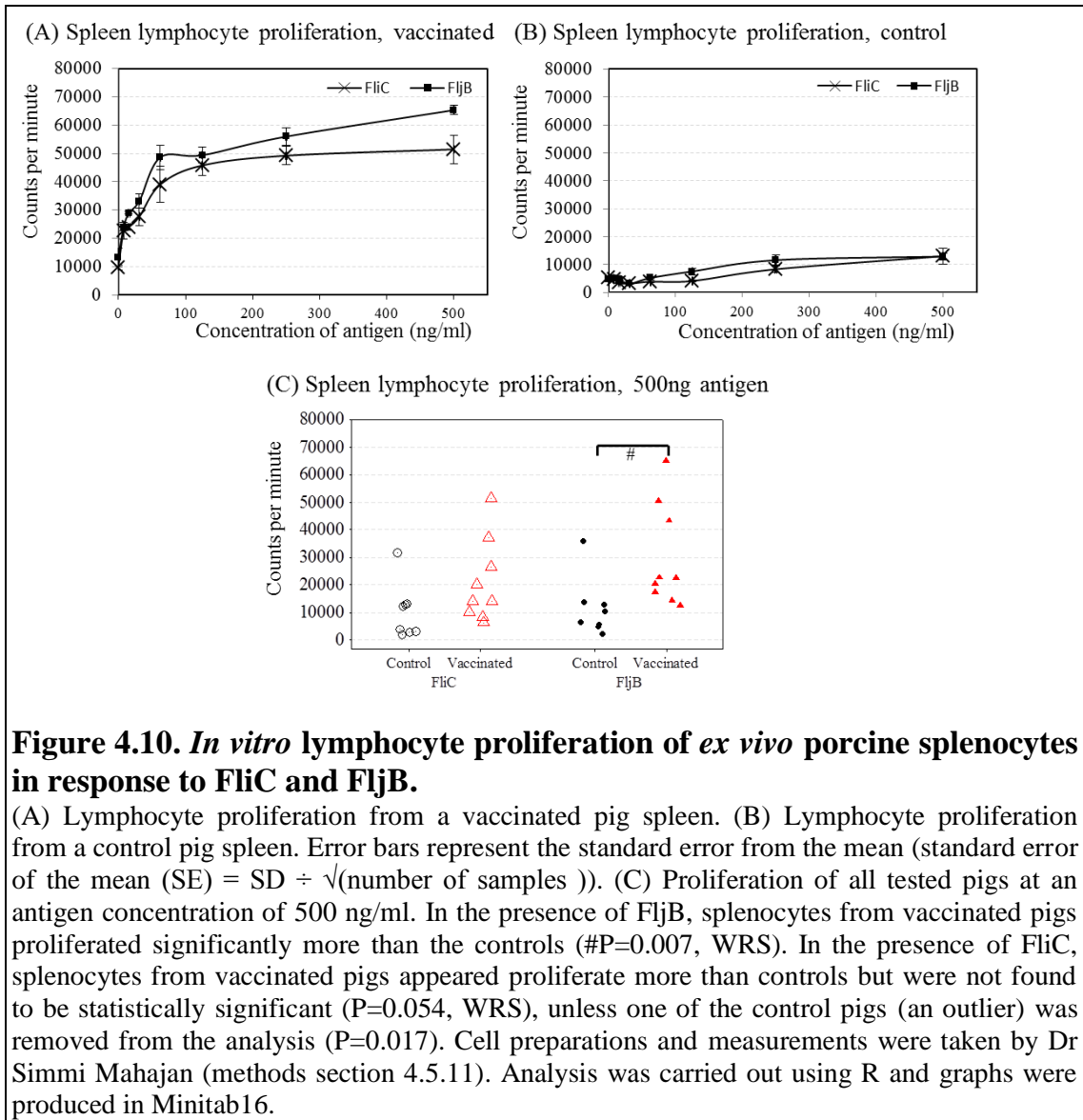
Tissue	Isotype and flagella	Vaccinated or Control pig	Probability value (p=)
Colon	Anti-FljB IgG	Vaccinated	0.399
Colon	Anti-FljB IgG	Control	0.748
Colon contents	Anti-FljB IgG	Vaccinated	0.760
Colon contents	Anti-FljB IgG	Control	2 -ve pigs
Caecum	Anti-FljB IgG	Vaccinated	0.399
Caecum	Anti-FljB IgG	Control	0.501
Caecum contents	Anti-FljB IgG	Vaccinated	2 -ve pigs
Caecum contents	Anti-FljB IgG	Control	2 -ve pigs
Ileum	Anti-FljB IgG	Vaccinated	0 +ve pigs
Ileum	Anti-FljB IgG	Control	0.997
Ileum contents	Anti-FljB IgG	Vaccinated	0.760
Ileum contents	Anti-FljB IgG	Control	0 -ve pigs
MLN	Anti-FljB IgG	Vaccinated	0.772
MLN	Anti-FljB IgG	Control	1 +ve pig
IcLN	Anti-FljB IgG	Vaccinated	0.772
IcLN	Anti-FljB IgG	Control	0.997
Colon	Anti-FliC IgG	Vaccinated	0.376
Colon	Anti-FliC IgG	Control	No change in titre
Colon contents	Anti-FliC IgG	Vaccinated	0.336
Colon contents	Anti-FliC IgG	Control	No change in titre
Caecum	Anti-FliC IgG	Vaccinated	0.376
Caecum	Anti-FliC IgG	Control	No change in titre
Caecum contents	Anti-FliC IgG	Vaccinated	2 -ve pigs
Caecum contents	Anti-FliC IgG	Control	No change in titre
Ileum	Anti-FliC IgG	Vaccinated	0 +ve pigs
Ileum	Anti-FliC IgG	Control	No change in titre
Ileum contents	Anti-FliC IgG	Vaccinated	0.727
Ileum contents	Anti-FliC IgG	Control	No change in titre
MLN	Anti-FliC IgG	Vaccinated	0.363
MLN	Anti-FliC IgG	Control	No change in titre
IcLN	Anti-FliC IgG	Vaccinated	0.363
IcLN	Anti-FliC IgG	Control	No change in titre

4.7.7. Vaccinated pigs exhibited increased lymphocyte proliferation to flagella antigens

To determine if vaccination with FliC and FljB induced a T-cell immune response, a lymphocyte proliferation assay was carried out by culturing splenocytes *in vitro* in the presence of FliC or FljB with ConA by Dr Simmi Mahajan (methods section 4.5.11).

Figure 4.10(A-B) shows a dose-dependent lymphocyte proliferation in both the sample vaccinated and control pig. Splenocytes from vaccinated pigs proliferated significantly more than those from the controls in response to FljB ($P=0.007$) suggesting a memory response from T-cells. In addition, splenocytes from the majority of vaccinated pigs also seemed to proliferate more than control pigs in response to FliC. This is excepting the splenocytes from one outlier pig in the control group (pig 606), which appeared to proliferate much more than those from other pigs in the control group. When this outlier pig's splenocyte data was removed from the analysis, the vaccinated group proliferated significantly more than the control group $P=0.017$ as determined by WSR test.

Figure 4.10(C) shows porcine lymphocyte proliferation after addition of 500 ng/ml of antigen. When the vaccinated group was compared with the control group, FljB specific proliferation was significantly increased in the vaccinated group compared to the control ($P=0.007$). FliC specific proliferation was significantly increased in the vaccinated group compared to the control group when the control outlier (pig 606) was removed from analysis ($P=0.054$ with 606, $P=0.017$ without 606). These results suggest that the vaccinated group developed immunological memory to flagella antigens while the control group did not.



4.7.8. Spleen and mesenteric lymph nodes from vaccinated and control pigs contain IFN γ ⁺ cells

To determine if vaccinated pigs demonstrated a “primed” immune system phenotype, cells from the spleen and MLN of pigs post-challenge were collected and stimulated before FACS analysis. Cells were stained with antibodies recognizing CD4, CD8 and IFN γ (methods section 4.5.12). FACS plots of the cells were gated for “live” cells then gated for the following cell populations; CD4⁺CD8⁻ (T helper), CD4⁻CD8⁺ (cytotoxic lymphocytes), CD4⁺CD8^{dim} (memory T-cells) and CD4⁻CD8^{dim} ($\gamma\delta$ T-cells and NK cells). Figure 4.8 shows representative FACS plots of the gated cell populations. There was no discernible difference in the proportion of any of the gated cell populations between vaccinated and control pigs, in either the mesenteric lymph nodes (Figure 4.12) or the spleen (Figure 4.13). In addition, the proportion of IFN γ positive cells within each of the gated populations was not significantly different between vaccinated and control pigs, in either the mesenteric lymph nodes (Figure 4.14) or the spleen (Figure 4.15).

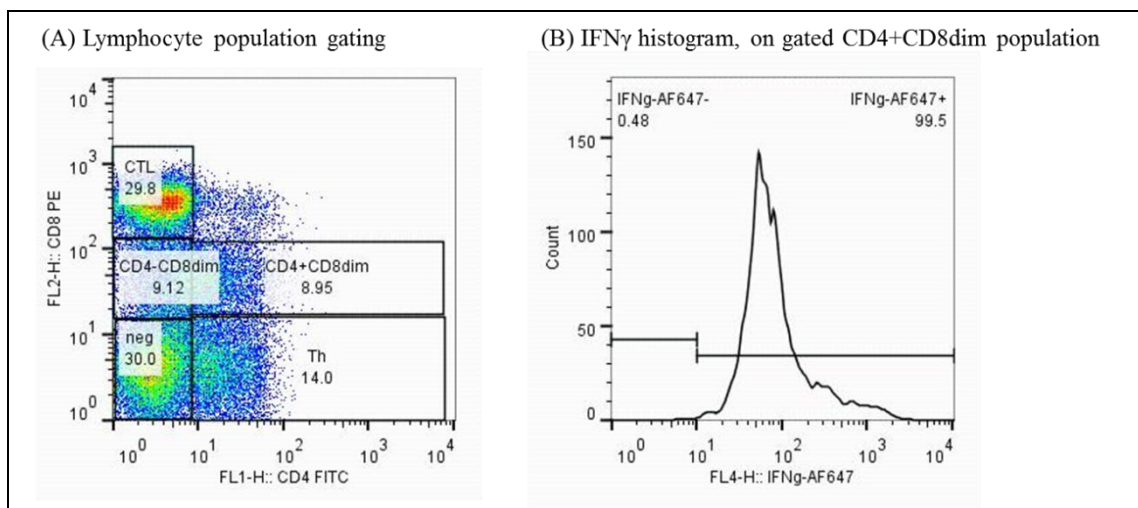


Figure 4.11. Representative flowcytometry plots of PMA-stimulated lymphocytes isolated from the porcine spleen.

(A) An example of gated populations on which further analysis was carried out, shown in Figures 4.9-4.10. (B) Histogram of IFN γ staining for the CD4⁺CD8^{dim} gated population. The IFN γ ⁺ population of cells was calculated within each of the gated populations as a proportion of the total number of live cells, these results are plotted in Figures 4.11 and 4.12. Cells were treated with PMA (100ng/ml) and Ionomycin (1 μ g/ml), in the presence of an inhibitor of protein transport-Golgistop. Cells were then surface stained for either CD8 or CD4, before permeabilisation and then IFN γ staining. Tissue isolation, cell preparations and FACS measurements were undertaken by Dr Simmi Mahajan. FACS gating and analysis carried out by J. Elvidge.

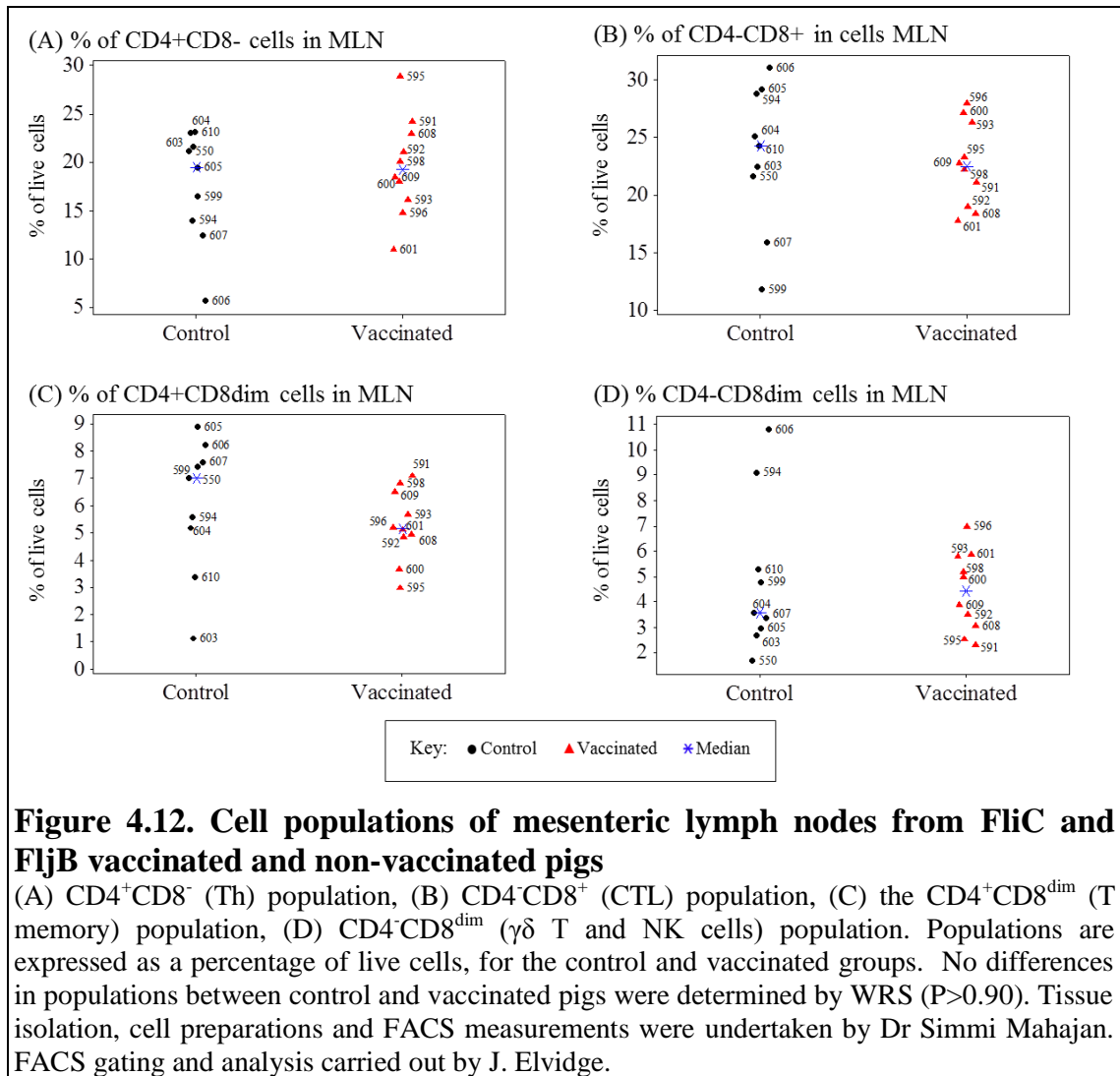


Figure 4.12. Cell populations of mesenteric lymph nodes from FliC and FljB vaccinated and non-vaccinated pigs

(A) CD4⁺CD8⁻ (Th) population, (B) CD4⁻CD8⁺ (CTL) population, (C) the CD4⁺CD8^{dim} (T memory) population, (D) CD4⁻CD8^{dim} (γδ T and NK cells) population. Populations are expressed as a percentage of live cells, for the control and vaccinated groups. No differences in populations between control and vaccinated pigs were determined by WRS (P>0.90). Tissue isolation, cell preparations and FACS measurements were undertaken by Dr Simmi Mahajan. FACS gating and analysis carried out by J. Elvidge.

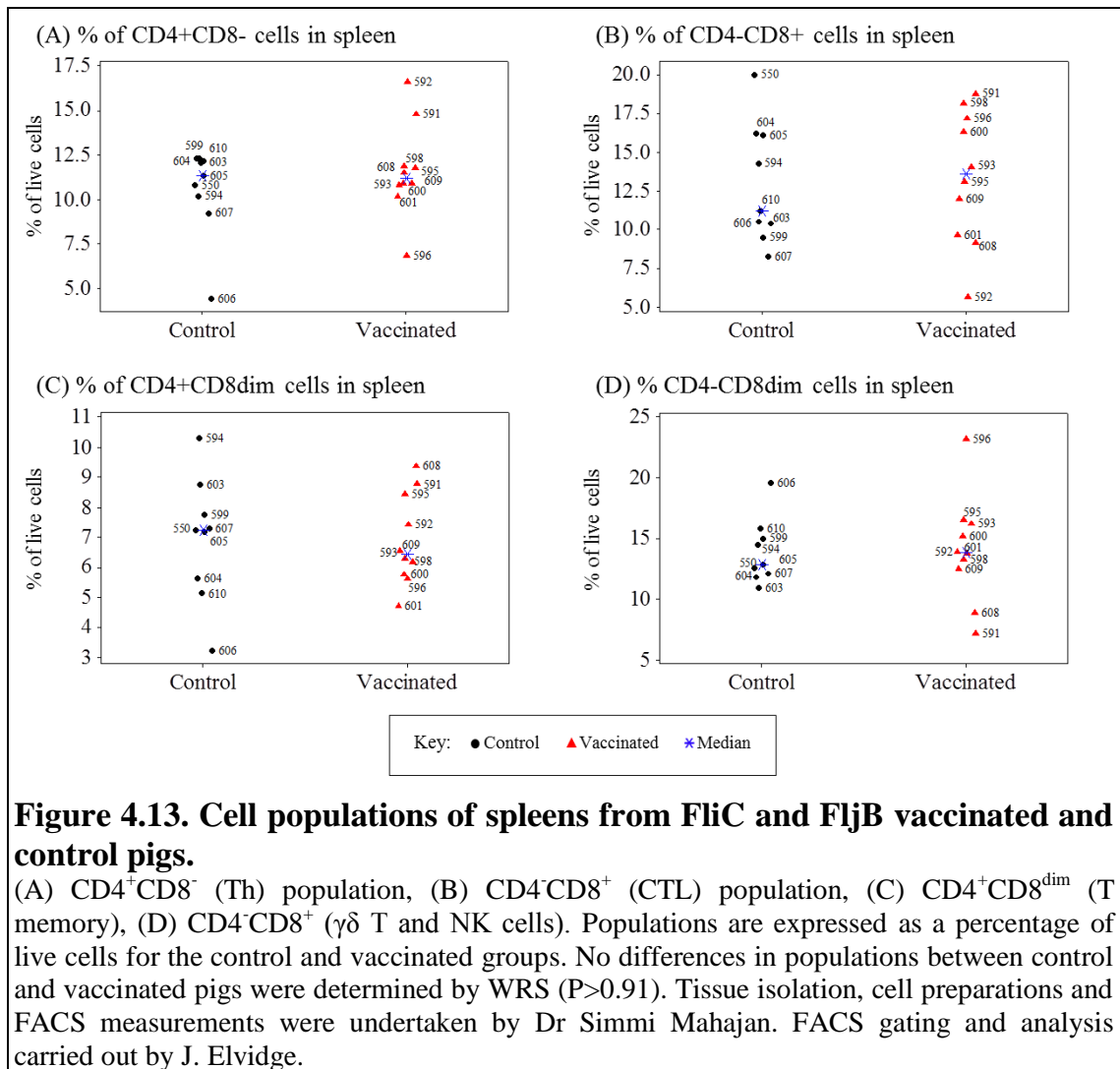


Figure 4.13. Cell populations of spleens from FliC and FljB vaccinated and control pigs.

(A) CD4⁺CD8⁻ (Th) population, (B) CD4⁻CD8⁺ (CTL) population, (C) CD4⁺CD8^{dim} (T memory), (D) CD4⁻CD8⁺ (γδ T and NK cells). Populations are expressed as a percentage of live cells for the control and vaccinated groups. No differences in populations between control and vaccinated pigs were determined by WRS (P>0.91). Tissue isolation, cell preparations and FACS measurements were undertaken by Dr Simmi Mahajan. FACS gating and analysis carried out by J. Elvidge.

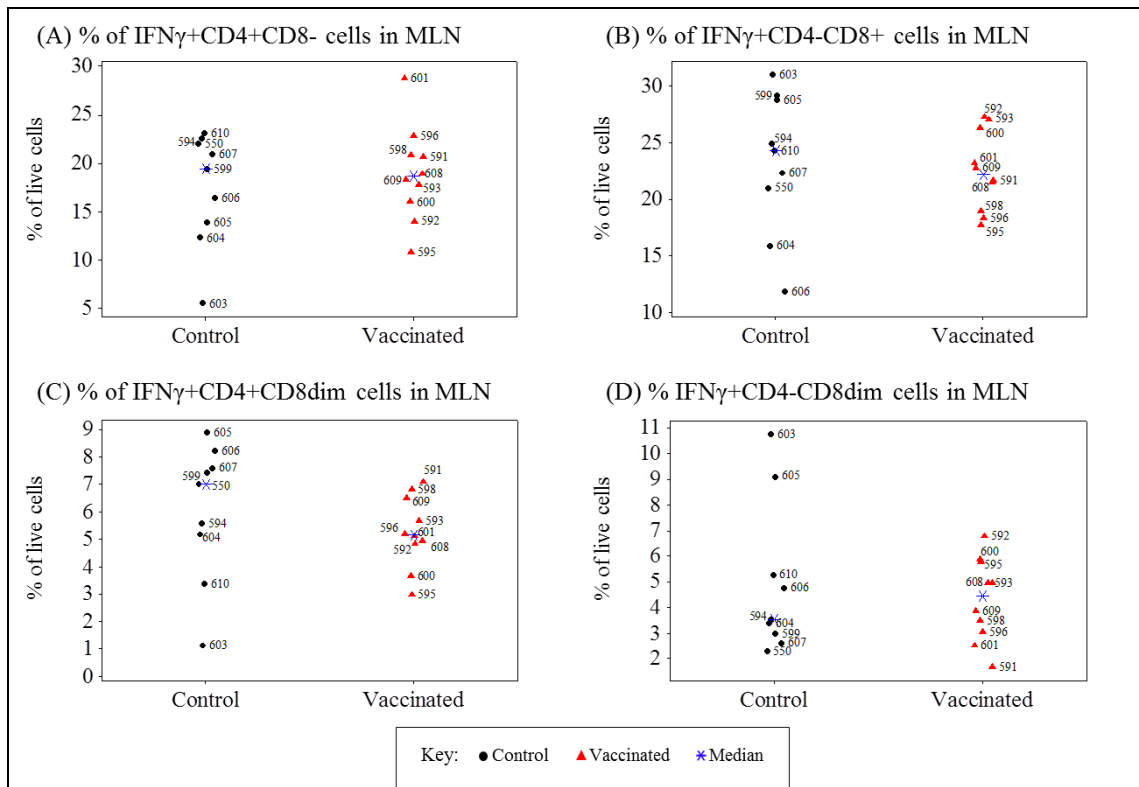


Figure 4.14. IFN γ^+ cell populations of mesenteric lymph nodes from FliC and FljB vaccinated and non-vaccinated control pigs

(A) IFN γ^+ CD4 $^+$ CD8 $^-$ (Th) population, (B) IFN γ^+ CD4 $^-$ CD8 $^+$ (CTL) population, (C) IFN γ^+ CD4 $^+$ CD8dim (T memory) population, (D) IFN γ^+ CD4 $^-$ CD8dim ($\gamma\delta$ T and NK cells) population. IFN γ^+ populations expressed as a percentage of live cells for the control and vaccinated groups. No differences in populations between control and vaccinated pigs were determined by WRS ($P > 0.97$). Tissue isolation, cell preparations and FACS measurements were undertaken by Dr Simmi Mahajan. FACS gating and analysis carried out by J. Elvidge.

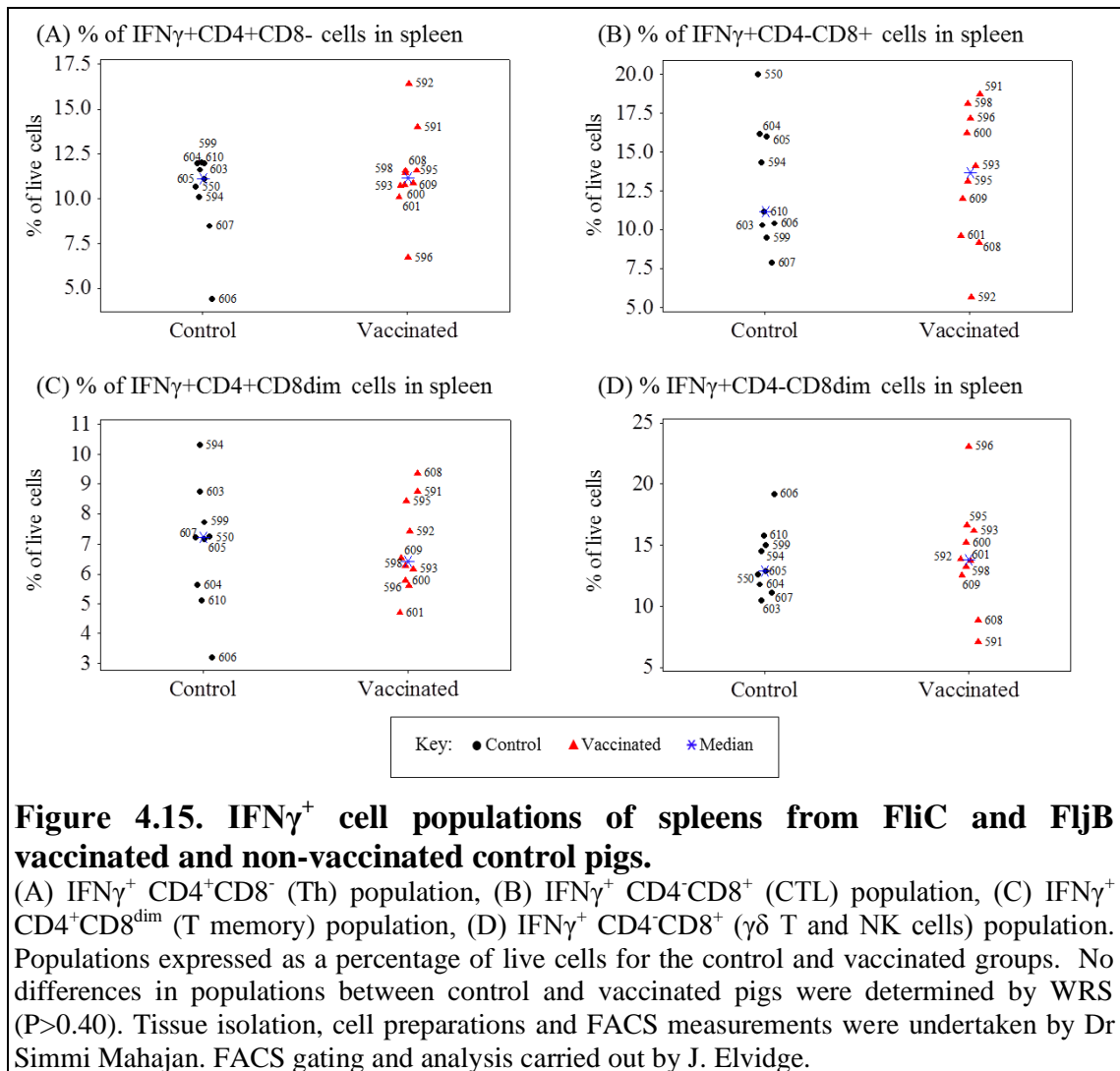


Figure 4.15. IFN γ ⁺ cell populations of spleens from FliC and FljB vaccinated and non-vaccinated control pigs.

(A) IFN γ ⁺ CD4⁺CD8⁻ (Th) population, (B) IFN γ ⁺ CD4⁻CD8⁺ (CTL) population, (C) IFN γ ⁺ CD4⁺CD8^{dim} (T memory) population, (D) IFN γ ⁺ CD4⁻CD8⁺ ($\gamma\delta$ T and NK cells) population. Populations expressed as a percentage of live cells for the control and vaccinated groups. No differences in populations between control and vaccinated pigs were determined by WRS ($P > 0.40$). Tissue isolation, cell preparations and FACS measurements were undertaken by Dr Simmi Mahajan. FACS gating and analysis carried out by J. Elvidge.

4.7.9. *Salmonella* antibody cross-reactivity

To determine the antibody cross-reactivity of serum from vaccinated and control pigs, these sera were tested with different *Salmonella* serovars for agglutination (Table 4.6). Sera from control pigs were unreactive to any of the strains tested. The vaccinated serum samples all reacted to *S. Typhimurium* and *S. Derby*. Some of the vaccinated samples also reacted to *S. Dublin* and *S. Enteritidis*. All sera from vaccinated pigs were unreactive to *S. Choleraesuis*.

Serum agglutination tests from vaccinated pigs suggest that immunisation with FliC and FljB proteins from *S. Typhimurium* generate antibodies that cross-react with *S. Derby*. *S. Derby* was the fourth most common serovar isolated from pigs in the UK in 2011, behind only *S. Typhimurium* and *S. Typhimurium* monophasic variants 4,[5],12:i:- and 4,12:i:- (AHVLA, 2011). Given this antibody cross-reactivity, it is possible that vaccination with FliC and FljB from *S. Typhimurium* might provide some level of protection to *S. Derby*, though additional tests would have to be carried out to ensure this is the case.

The antigenic formulas of the strains tested are shown in Table 4.7 (Grimont and Weill, 2007). As shown in Table 4.7, the phase 2 flagella antigens expressed by *S. Derby* are the same as for *S. Typhimurium*. In addition, the O antigens expressed are also similar. These flagella antigenic similarities between *S. Derby* and *S. Typhimurium* may explain the reasons for the antibody cross-reactivity seen from the vaccinated pig serum. The other strains tested have very little antigenic similarity to *S. Typhimurium* and perhaps would not be expected to cross-react.

Some of the pig serum from vaccinated pigs also reacted to serovars *Dublin* and *Enteritidis*. It is possible that vaccinated pigs were exposed to a very small amount of additional *Salmonella* antigens present in flagella preparations that was not detected by SDS-PAGE and subsequent gel staining. Flagella preparations were made from live mutant *S. Typhimurium* strains (detailed in 4.5.3) that were subjected to high speed homogenisation to mechanically remove flagella. Whilst whole bacteria were removed by high speed centrifugation, any additional bacterial antigens present with the flagella in the supernatant may remain. Flagella were subsequently centrifuged out of

suspension and re-suspended; this process should have removed any small bacterial antigens which do not form part of the flagella pellet, post-centrifugation. However, contamination with other bacterial antigens is possible. Therefore, vaccinated pigs may have been exposed by vaccination to other *Salmonella* antigens. This may help explain why some pigs had cross reactivity to other *Salmonellae* species and some did not. If the antigen was only present in a very small amount, B cell clonal expansion following recognition of this unknown antigen may have occurred only in some the pigs, leaving other pigs without these antibodies.

Table 4.6. Post-immunisation serum agglutination with *Salmonella* strains

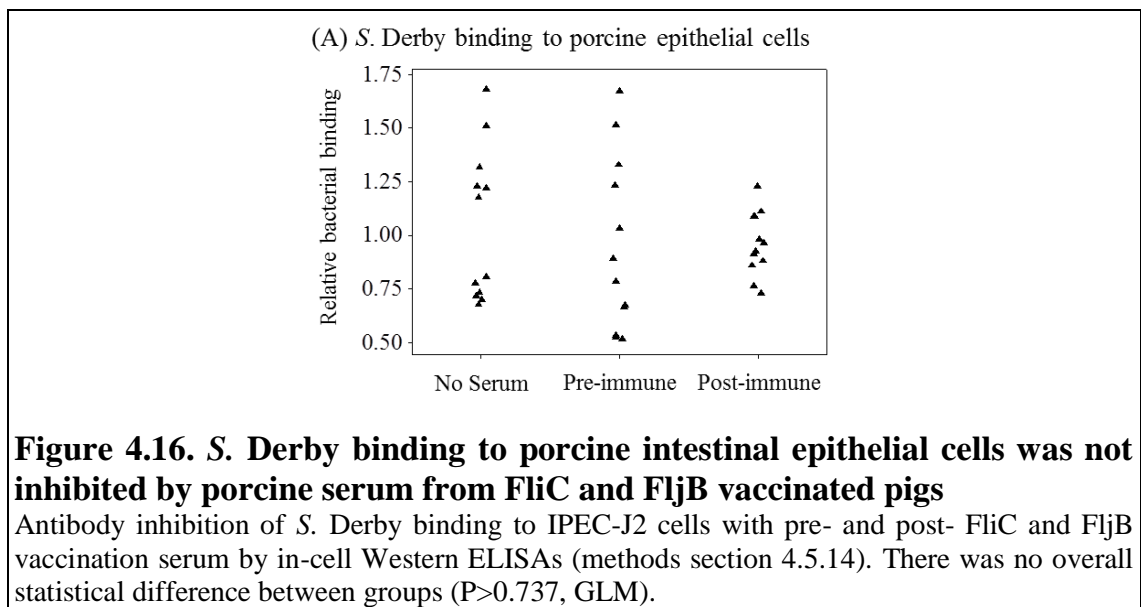
Pig Number	<i>S.Typhimurium</i>	<i>S.Dublin</i>	<i>S.Cholerasuis</i>	<i>S.Derby</i>	<i>S.Enteritidis</i>
591	+ve	-ve	-ve	+ve	+ve
592	+ve	-ve	-ve	+ve	+ve
593	+ve	-ve	-ve	+ve	+ve
595	+ve	+ve	-ve	+ve	+ve
596	+ve	-ve	-ve	+ve	-ve
598	+ve	+ve	-ve	+ve	+ve
600	+ve	-ve	-ve	+ve	-ve
601	+ve	+ve	-ve	+ve	-ve
608	+ve	-ve	-ve	+ve	-ve
609	+ve	-ve	-ve	+ve	-ve

Table 4.7. Antigenic formulae of *Salmonella* strains

Strain	Antigenic formulae		
	O antigens	Phase 1 flagella antigen	Phase 2 flagella antigen
SL1344 (CMG 109)	4, [5], 12	i	1, 2
<i>S. Dublin</i> (CMG 33)	1, 9, 12 [Vi]	g, p	-
<i>S. Cholerasuis</i> (CMG 32)	6, 7	c	1, 5
<i>S. Derby</i> (CMG 111)	1,4,[5],12	f, g	[1, 2]
<i>S. Enteritidis</i> (CMG 41)	1, 9, 12	g, m	-

4.7.10. *Salmonella* Derby binding inhibition assay

Serum from FliC and FljB vaccinated pigs agglutinated with *S. Derby*. It has been shown previously (Chapter 3) that antibodies to FliC and FljB inhibited *S. Typhimurium* binding to porcine intestinal epithelial cells, suggesting that flagella may be important for interacting with the host cells. To determine if antibodies present in the vaccinated pig serum inhibited *S. Derby* binding to porcine intestinal epithelial cells, binding inhibition assays were carried out. The addition of vaccinated pig serum to *S. Derby* did not reduce bacterial binding in the conditions used (Figure 4.16).



4.8. Discussion

Previous experiments detailed in Chapter 3 have indicated that *S. Typhimurium* SL1344 FliC and FljB may be important for initial adherence to porcine intestinal epithelial cells. Research targeted at “Identification and characterisation of *Salmonella* enterica serovar Typhimurium factors playing a role in colonisation of the porcine gut”, identified two bacterial surface proteins, FliC and FljB that act as adhesins to porcine intestinal epithelial cells. In addition, specific anti-sera to FliC and FljB were shown to significantly reduce *S. Typhimurium* adherence to the porcine epithelium.

On the basis of the above findings (detailed in Chapter 3), both these bacterial adhesins were considered as potential immuno-protective antigens. They were therefore tested for their prophylactic efficacy as putative vaccine candidates against *S. Typhimurium* infection in pigs. The vaccine efficacy study was funded by the BBSRC (follow-on funding pathfinder scheme), a small-scale project grant and BPEX.

The number of days for which pigs from the vaccinated group tested positive for *Salmonella* during the shedding test period appeared to be lower than the control group. However, this was not found to be statistically significant. Unfortunately the pigs were insufficiently colonised by the *Salmonella* test strain to recover countable numbers of bacteria from the faecal samples tested, and results were reported as either positive or negative following overnight enrichment (RV). Bacterial colonisation of the tissues representative of *Salmonella* colonisation and persistence in swine was determined by serial dilution of samples and by enrichment. Again, the pigs were insufficiently colonised by the *Salmonella* test strain to recover countable numbers of bacteria from the majority of tissues tested, though some did yield low numbers of *Salmonella*. Bacteraemia of both the ileum and caecum tissues appeared to be reduced in the vaccinated group compared to the control group, and the number of vaccinated pigs testing positive for *Salmonella* in the ileum was found to be significantly less than the control group (0 and 4 respectively, $P=0.036$, WRS). The number of the vaccinated pigs testing *Salmonella* positive in the caecum was not significantly different from the control group (4 and 7 respectively, $P=0.468$, WRS). This suggests that the vaccine may have provided some protection against *Salmonella* colonisation of the ileum. However, further studies using a more virulent *Salmonella* test strain would

need to be carried out in order to validate these results following the low colonisation status of all the pigs tested in this study.

To examine the relationship between *Salmonella* colonisation and immunisation status of the pigs a principal components analysis was employed, to take into account as much of the information collected as possible. The analysis included the positive and negative statuses of each pig from each of the tissues and contents tested, as well as whether the pig had shed *Salmonella* for more than 10 of the shedding test days. This analysis revealed no obvious relationship between colonisation and immunisation status. However, *Salmonella* shedding counts were indiscernible from either vaccinated or control pigs and very few *Salmonella* were found in the intestinal tissues post-mortem. These results suggest that the pigs may have been insufficiently colonised to observe any difference between groups.

Importantly, it was shown that parental administration with both these antigens elicited humoral immune responses, as recognition of FliC and FljB by IgA and IgG was increased following immunisation, as demonstrated by ELISA and Western blot (Figure 4.7). Additional experiments performed within our research group (Dr Simmi Mahajan) have confirmed the generation of an immune response to flagella by investigation of splenocyte proliferation. These studies showed that splenocytes from vaccinated pigs proliferated significantly ($P < 0.05$) more than those from control pigs in the presence FljB and FliC (500ng/ml). This suggests vaccination with flagella antigens primed the immune response and produced immunological memory. The production of immunological memory in response to FliC and FljB and the production of antibodies, demonstrated by ELISA, shows that these flagella are immunogenic in pigs. However, flowcytometry analysis of stimulated cells from the spleen and MLN of pigs post exposure to *Salmonella* showed no difference in the populations cells between groups or the number of IFN γ positive cells. The vaccinated pigs did not appear to exhibit a primed immune system phenotype as might have been expected if a protective immune response was generated. Studies using recombinant FliC from *S. Typhimurium* in mice showed a Th2 response rather than Th1 (Bobat et al., 2011, Cunningham et al., 2004). The apparent lack to T cell memory generated in response to vaccination with FliC and FljB appears to support this finding in pigs as well. These

results suggest that for the flagella to be used as part of a multivalent subunit vaccine it would need to be delivered along with an antigen resulting in a Th1 response and the generation of T cell memory, which is likely to be important for clearance of *Salmonellae*.

To determine if the antibodies generated in response to *S. Typhimurium* FliC and FljB had the potential to be cross-protective with other *Salmonella* serovars, agglutination tests were carried out. The second most common *Salmonella* serovar isolated from pigs in Europe and fourth most common in the UK is *S. Derby* (AHVLA, 2011, EFSA, 2008b). Interestingly, agglutination tests revealed that serum from vaccinated pigs reacted with *S. Derby*, suggesting perhaps some cross protection may be afforded by vaccination with *S. Typhimurium* FliC and FljB antigens. However, binding inhibition assays using serum from vaccinated pigs did not reduce *S. Derby* binding to porcine intestinal epithelial cell line IPEC-J2 in the conditions tested, though additional testing may be required to confirm this. While *S. Derby* bacterial binding appeared not to be reduced in the presence of serum from FliC and FljB vaccinated pigs, it is possible that some priming of the immune response using these flagella antigens may reduce the time it takes for pigs to clear a *S. Derby* infection.

The *S. Typhimurium* SL1344 strain is a histidine auxotroph of ST4/74, a wild-type strain isolated from a calf bowel (Hoiseh and Stocker, 1981, Jones et al., 1991). Challenging the pigs in this study with the attenuated SL1344 strain did not yield discernible bacterial counts from faecal samples or from tissues at post-mortem. Future studies will aim to repeat the vaccine efficacy study using a different *S. Typhimurium* strain and also immunologically characterise these antigens (FliC and FljB). One possible route for further study could involve the identification of other immunogenic *Salmonella* antigens. A combination of immunogenic antigens could be applied alone or in combination with other inactivated porcine vaccines as a potential combinatorial multi-subunit *S. Typhimurium* porcine vaccine. Current porcine vaccines on the market include FosterTM PCV (Pfizer Animal Health) against porcine circovirus. In the future it may be possible to combine an effective, multi-subunit *Salmonella* vaccine with an existing vaccine.

4.8.1. Commercial route

The commercialisation end-point for this project would most likely be a licence to an animal health pharmaceutical company with expertise in porcine vaccines. Due to the upcoming EU legislation, there is a growing interest in developing an effective vaccine solution for *Salmonella* in pigs. Initial discussions with animal pharmaceutical companies have indicated that the preliminary data presented here were not sufficient for commercial development without further *in vivo* proof of concept. For a successful licence it is anticipated that a patent will be required, and an initial discussion with a patent agent has indicated that this research would be patentable.

These preliminary data indicated that vaccination with FliC and FljB antigens generated antibodies to FliC and FljB, demonstrated by increased recognition of both antigens following immunisation. Furthermore, immunological memory to these antigens appears to have been generated, demonstrated by increased proliferation of splenocytes from the vaccinated group compared to the control group following flagella antigen challenge. Unfortunately, this study did not result in sufficient bacterial colonisation to draw conclusions about a *Salmonella* protective immune response, as the pigs did not shed countable numbers of bacteria. Further experiments are therefore required to determine the effectiveness of these flagella proteins in a porcine *S. Typhimurium* vaccine.

5. Conclusions

5.1. Concluding statements

The intestinal epithelium is comprised of multiple cell types containing M-cells, enterocytes, goblet cells, and paneth cells (Appleton et al., 1980). In mice it has been shown that *Salmonella* exhibits tissue tropism for the distal end of the small intestine (Wray and Wray, 2000) and it has been shown in multiple species that *Salmonella* enters the cells the distal end of the small intestine. Although, in contrast to the mouse model of infection where *S. Typhimurium* appears to preferentially invade M-cells (Jones et al., 1994), evidence from cattle (Santos et al., 2002) and pigs (Reed et al., 1986, Bolton et al., 1999) shows no evidence of an M-cell to enterocyte preference. These studies indicate a difference in the interaction of *S. Typhimurium* with the intestinal epithelium from different species. It is possible the apparent preference for M-cells by *S. Typhimurium* mice is related to *S. Typhimurium* becoming systemic in this host. More studies will be required to elucidate the reasons for M-cell preference in mice. However, these studies highlight the necessity for further study of enteritis caused by *Salmonella* in relevant species, rather than using mice as a substitute model.

Salmonella enter both M-cells and enterocytes and a relevant *in vitro* infection model is required to study *Salmonella* in the porcine host. During the course of this study a protocol for establishment of a porcine primary intestinal cell culture was developed. This cell culture contained a mixed population of cells and resembled native intestinal epithelium. The culture consisted mainly of enterocytes though, a subset of cells were identified as M-cells. Since *Salmonella* target both enterocytes and M-cells this primary culture model would provide a very relevant *in vitro* infection model.

Previous studies with other bacterial species have shown a role for flagella in adherence to host cells. It was hypothesised that *S. Typhimurium* flagella play a role in adherence to porcine epithelia. This was investigated using WT and flagella mutant *S. Typhimurium* SL1344 strains. Adherence and invasion assays indicated that *S. Typhimurium* flagella do play a role in adherence and invasion of porcine intestinal epithelium. In addition, purified flagella proteins bound to both porcine and human intestinal epithelial cells. It was also found that the presence of flagella specific antibodies inhibited bacterial adherence to porcine intestinal epithelia. This result potentially indicates that *S. Typhimurium* flagella play a role specifically in adherence

to intestinal epithelium. In addition, preliminary studies carried out suggested that flagella were not important for interaction with porcine tonsils. Although the tonsil explants were small, there appeared to be no reduction in bacterial binding to porcine tonsil tissue upon addition of flagella specific antibodies.

S. Typhimurium is able to switch its flagella filament protein. It is widely thought that this switching is an immune evasion mechanism, with no other function currently attributed to flagella phase switching. The preliminary findings presented here indicate that *Salmonella* may be able to “sense” the restriction of one flagella phase type and be able to switch to the other.

S. Typhimurium flagella are expressed during infection of porcine intestinal epithelial cells as confirmed by microscopy. Flagella were shown to penetrate the cell surface. Furthermore, confocal microscope images indicated that flagella interact with actin filaments. Other experiments performed within the group appeared to confirm this flagella-actin interaction (Eliza Wolfson, personal communication).

The findings presented here qualify FliC and FljB bacterial adhesion factors as potential immuno-protective antigens. These purified proteins were tested *in vivo* to assess their protective immunogenicity and potential for forming part of a *Salmonella* multivalent subunit vaccine in pigs.

Bacterial shedding data from FliC/FljB vaccinated, *S. Typhimurium* challenged pigs did not reveal significant differences between the vaccinated group and control group ($P > 0.05$). However, there were issues with the colonisation of the *Salmonella* challenge strain (SL1344 na_r). None of the pigs yielded countable numbers of bacteria during the shedding period, < 100 CFU/ml, significantly less *Salmonellae* than was expected following similar studies which found between 10^6 and 10^7 *Salmonellae* per gram of faeces in the first few days following exposure, which then reduced to between 10^4 and 10^5 for days 5 to 16 post exposure to ST4/74 (Pullinger et al., 2010). Admittedly one of the difficulties with this pig study was the small amount of faecal matter, provided on sampling days where a weighed sample was requested. However, despite the small faecal samples, depicted by ‘s’ in Figure 4.5 countable numbers of

bacteria were expected. Post mortem analysis of the intestinal tissues representative of tissue persistence showed no colonisation of the ileum tissue in the vaccinated group following overnight enrichment, where 4 of the control pigs were colonised. This was found to be statistically significant by WRS $P=0.036$. The colonisation of the other intestinal tissues tested however, was found not to be significantly different between groups. Although the number of positive caecum tissue samples was less in the vaccinated group, it was not found to be statistically significant ($P=0.468$, WRS).

Parental administration with FliC and FljB induced humoral IgG and IgA antibody responses in vaccinated pigs demonstrated by ELISA. In addition, Western blots showed that a sample pig from the vaccinated group following experimental challenge showed recognition of both FliC and FljB by IgA antibodies. This indicates that in this sample vaccinated pig at least, mucosal antibody was generated. The sample control pig which was tested in the same way showed no recognition of either FliC or FljB. Indeed generation of mucosal antibody using systemic vaccination has previously been demonstrated by other studies (McNeilly et al., 2010). Additional experiments performed within our research group (Dr Simmi Mahajan) confirmed generation of an immune response to FljB. Splenocytes from vaccinated pigs proliferated significantly ($P<0.05$) more than those from control pigs in the presence FljB and FliC (500ng/ml). The production of immunological memory in response to flagella and the production of antibodies, demonstrated by ELISA shows that flagella are immunogenic in pigs. Flowcytometry analysis of stimulated cells from the spleen and MLN of pigs post exposure to *Salmonella* was carried out to determine if vaccinated pigs exhibited a primed immune system phenotype in comparison to the control pigs. It may have been expected that lymphocytes from the spleen and MLN of the vaccinated pigs were more likely to produce increased amounts of IFN γ upon stimulation. However, intracellular staining for IFN γ was not significantly different between groups ($P>0.97$, WRS). It may also have been expected that successful protective vaccination would result in T cell memory. T cell memory in pigs are CD4⁺CD8^{dim} cells (Zuckermann, 1999). The gated populations of these cells were not significantly different between vaccinated and control pigs ($P>0.90$, WRS). Previous studies using recombinant FliC from *S. Typhimurium* in mice have shown a Th2 response rather than Th1 (Bobat et al., 2011, Cunningham et al., 2004). The lack to T cell memory generated here in response to

vaccination with these flagella proteins appears to support this finding in pigs as well. These results suggest that for the flagella to be used as part of a multivalent subunit vaccine it must be delivered with an antigen that results in a strong enough Th1 response to result in T cell memory. This will be important for clearance of *Salmonellae*.

A *Salmonella* vaccine for use in pigs would ideally be cross protective against serovars. To determine if antibodies generated in response to *S. Typhimurium* FliC and FljB were cross reactive with other *Salmonella* serovars, agglutination tests were carried out. The second most common *Salmonella* serovar isolated from pigs in Europe and fourth in the UK is *S. Derby* (AHVLA, 2011, EFSA, 2008b). Interestingly agglutination tests revealed that serum from vaccinated pigs reacted with *S. Derby* suggesting perhaps some cross protection may be afforded by vaccination with *S. Typhimurium* FliC and FljB antigens, assuming *S. Derby* flagella are also important for intestinal epithelial cell interactions. Binding inhibition assays using serum from vaccinated pigs did not reduce *S. Derby* binding to porcine intestinal epithelial cell line IPEC-J2, suggesting that *S. Derby* flagella may not play a role in adherence to porcine intestinal epithelium. While, *S. Derby* bacterial binding may not be reduced in the presence of FliC and FljB antibodies, the priming of the immune response using FliC and FljB antigens from *S. Typhimurium* may reduce the time it takes for the animals to clear *S. Derby* infection.

The work described here, could form the basis of further study into the generation of a *Salmonella* vaccine for use in pigs. Although, further *in vivo* proof of concept is required before commercial companies will invest. Vaccine development is extremely costly and commercial companies want to be assured of positive outcomes and patent availability before further investment.

5.2. Future work

Future studies will aim to repeat the vaccine efficacy study using a wild type *S. Typhimurium* strain. Another porcine study would also further study the immune response to flagella antigens with antigen specific lymphocyte proliferation assays prior to bacterial challenge. In addition, a cytokine profile would be generated and compared to similar studies where flagella were used as an adjuvant. Vaccination of source animals is viewed as a key strategy to reduce *Salmonella* in pigs. An effective vaccine would limit initial *Salmonella* colonisation and subsequently reduce *Salmonella* prevalence in pigs. A combination of immunogenic antigens could be applied alone or in combination with other inactivated porcine vaccines as a potential combinatorial porcine vaccine. Future studies will attempt to identify additional immunogenic antigens from *Salmonella* which could form part of a multi-subunit vaccine.

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