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**Rational design of vaccines for the control  
of *Campylobacter* in chickens**

**Cosmin Chintoan-Uta**



**Doctor of Philosophy**

**The University of Edinburgh**

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## **Declaration**

I hereby declare that the research described within this thesis is my own work, unless acknowledged in the text. I certify that the work has not been submitted for any other degree or professional qualification.

Date: 30.09.2015

Cosmin Chintoan-Uta BVSc, MSc(R), MRCVS  
Roslin Institute  
University of Edinburgh  
Easter Bush  
Roslin  
Midlothian  
EH25 9RG  
United Kingdom

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## Abstract

*Campylobacter* is the leading cause of bacterial food-borne diarrhoeal disease in the developed world and a significant cause of infant morbidity and mortality in developing countries. Epidemiological studies implicate poultry as a key source of infection, with up to 80% of human cases being attributable to the avian reservoir. An effective vaccine for broilers is predicted to limit the incidence of human campylobacteriosis. Vaccination of chickens with CjaA, either in recombinant form or vectored in live-attenuated *Salmonella*, has been reported to significantly reduce caecal colonisation by *C. jejuni*, with more invasive carriers eliciting greater protection. However, protection remains modest and is slow to develop. I therefore sought to improve such vaccines, first by vectoring codon-optimised CjaA in a licensed avian pathogenic *E. coli*  $\Delta$ aroA vaccine. In two independent trials, White Leghorn birds were vaccinated subcutaneously on the day of hatch and 14 days later then challenged with *C. jejuni* M1 at 28 days post-hatch. No protection was observed despite significant induction of CjaA-specific serum IgY, however, a previously described *S. Typhimurium*  $\Delta$ aroA vaccine vectoring CjaA also failed to protect.

Owing to the variability observed with live CjaA-based vaccines in these and previous studies, other candidate antigens were sought and evaluated as subunits. Twenty-one candidate *C. jejuni* antigens were cloned and expressed as glutathione-S-transferase (GST) fusions. Nine of these could be purified in adequate soluble quantities to be tested *in vivo*. The intervals of vaccination and challenge were as above, with GST alone or GST-CjaA acting as negative and positive controls, respectively. Each antigen was administered subcutaneously in TiterMax Gold<sup>®</sup> adjuvant at the molar equivalent of the doses of GST-CjaA. Repeated testing of initially promising candidates revealed that, when averaged across three independent trials, GST-SodB and GST-FliD induced statistically significant reductions in caecal colonisation of 1-2 log<sub>10</sub> colony-forming units of *C. jejuni* at 48 and 56 days post-hatch compared to negative controls. Induction of antigen-specific serum IgY was measured by enzyme linked-immunosorbent assays using maltose-binding protein fusions to each antigen. This revealed significant induction of antigen specific serum IgY for the majority of the antigens tested, even when no protection was observed. In the SodB- and FliD-vaccinated groups, the peak of antigen-specific serum IgY was not coincident with the onset of protection and the fold-change in specific IgY levels in individual birds did not correlate with caecal *Campylobacter* numbers. Furthermore, sera from SodB-vaccinated birds failed to detect SodB in the outer membrane or surface of *Campylobacter* cells, indicating that SodB-specific antibodies are unlikely to be neutralising.

Taken together, these studies identified two novel protective antigens that, with further optimisation, could form part of an anti-*Campylobacter* vaccine for broilers. However further studies are required to define the nature and consequences of immune responses required for protection.

## Lay summary

*Campylobacter* is the most common cause of bacterial food-borne diarrhoeal disease in the developed world. Consumption or handling of contaminated chicken meat is the main source of human infections. *Campylobacter* reaches very high numbers in the intestines of chickens and carcasses often become contaminated with gut contents during the slaughter process. Strategies to control *Campylobacter* at source in chickens are expected to lower the incidence of human disease. Some laboratories have achieved this by vaccinating chickens, but protection is often modest or slow to develop. Towards the goal of an improved *Campylobacter* vaccine for chickens I initially modified existing live vaccines for birds based on *E. coli* or *Salmonella* by engineering them to express a *Campylobacter* protein previously reported to confer immunity. These so-called ‘vectored’ vaccines were evaluated in two independent trials in chickens but they did not elicit protection, despite inducing antibodies specific to the *Campylobacter* protein.

In order to identify other constituents of *Campylobacter* that may be protective in birds I surveyed the literature and identified 21 proteins that may be suitable for use as vaccines. It was feasible to purify nine of these on a large scale and these were tested individually in chickens for their ability to induce immune responses and protect birds against experimental *Campylobacter* infection. From three independent trials, two of these vaccines reduced colonisation of the chicken intestines by *Campylobacter* by up to 100-fold. These two vaccines were based on *Campylobacter* proteins called superoxide dismutase (SodB), which helps the bacteria respond to oxidative stress, and a flagellum protein (FliD) which is part of a structure the bacteria use to swim. It was shown previously that SodB and the flagellum are required for chicken colonisation. We were able to detect antibodies against the SodB and FliD proteins after vaccination, however the levels of these antibodies did not correlate with the protection against colonisation. Furthermore, SodB was not detectable on the bacterial surface, suggesting that antibodies against SodB would be unable to locate their target. These findings suggest that antibodies may not play a major role in vaccine-induced protection against *Campylobacter* in birds and further studies are needed to understand which immune responses are important. My research nevertheless identified two new protective vaccines that can be added to the limited repertoire available. This knowledge will aid the further development of anti-*Campylobacter* vaccines for meat-producing birds.

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# 1. Introduction

## 1.1 *Campylobacter* biology

### 1.1.1 General characteristics

*Campylobacter* is a Gram-negative spiral-shaped bacterium and part of the class *Epsilonproteobacteria* (On, 2001). It is part of the *Campylobacterales* order, which includes other important pathogens such as *Helicobacter pylori*, associated with gastric ulceration and cancer in humans (Bauer and Meyer, 2011). Gupta (2006) described unique molecular features of this order than can be used in its identification and also unique molecular markers for individual genera and species. Currently, the genus *Campylobacter* comprises 17 species and 6 subspecies, of which *C. jejuni* subsp. *jejuni* and *C. coli* are the most important species causing food-borne diarrhoeal disease in humans (WHO, 2011). Other species such as *C. lari* and *C. upsaliensis* have also been isolated from human clinical cases but are less frequent (Miller *et al*, 2008; Parsons *et al*, 2012). In addition to humans, *Campylobacter* can infect a large number of different hosts including shellfish (Soonthornchaikul and Garelick, 2009) and all of the vertebrate classes. They have been isolated from fish (as reviewed in Novotny *et al*, 2004), possibly from amphibians (Gossling *et al*, 1982), from reptiles (Gilbert *et al*, 2014) and it is widely known that they infect birds and mammals. Clinical signs within each of the hosts vary from asymptomatic carriage in lower vertebrates and birds to severe in mammals, e.g. abortion induced by *Campylobacter fetus* in sheep (The Merck Veterinary Manual (a), 2014) and dogs (Sahin *et al*, 2014), reproductive losses in cattle (The Merck Veterinary Manual (b), 2014) and gastrointestinal disease in dogs and cats (Marks *et al*, 2011), non-human primates (Kalashnikova *et al*, 2002) and humans (reviewed below).

### 1.1.2. Genetic characteristics

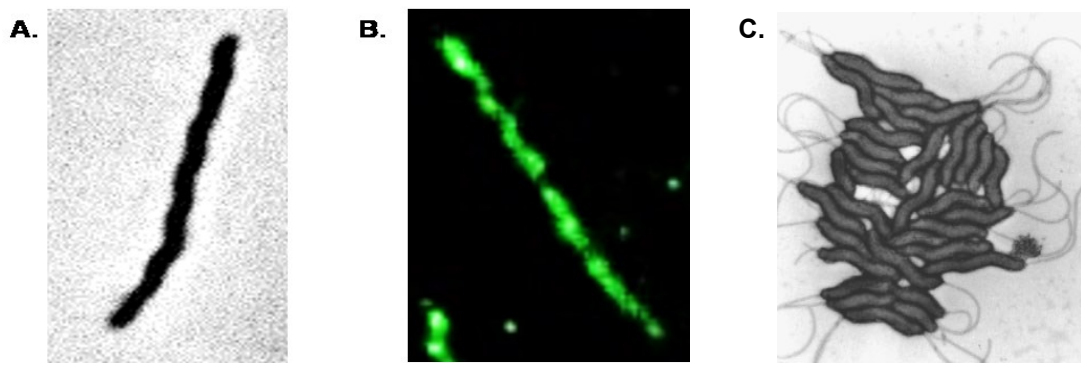
*Campylobacter* have a relatively small but variable AT-rich genome, comprised of approximately 2000 genes in the core-genome with approximately as much again in the pan-genome of *C. jejuni* (Friis *et al*, 2010), all encoded on a single chromosome. Parkhill *et al* (2000) sequenced the first complete genome of *C. jejuni* and revealed a structure lacking features commonly found in other *Proteobacteria*; for example there are no prophage-associated sequences, obvious pathogenicity islands and few typical regulators. Its genome was shown to contain homopolymeric tracts (Parkhill *et al*, 2000), particularly in genes



predicted to encode biosynthesis-associated molecules and modifications of surface-exposed structures. Slipped-strand mis-pairing of these homopolymeric tracts during DNA synthesis can lead to variation in number of these homopolymeric repeats and phase variation with on/off switching of the encoded genes (Bayliss *et al*, 2012). This high rate of genomic variation is believed to be involved in survival strategies of *Campylobacter* and evasion of the immune system. Gilbert *et al* (2002) showed that phase variation induces variability in the structure of surface lipooligosaccharides (LOS) and Thompson (2002) showed that genetic variation induces changes in surface-expressed S-layer proteins and allows evasion of the immune system by *C. fetus*.

### 1.1.3 Phenotypic characteristics

*In vitro*, campylobacters have variable motility and morphology, ranging from highly motile long spiral-shaped rods (Fig. 1) to non-motile coccoid forms, depending on the phase of the growth cycle and the culturing conditions (Ng *et al*, 1985; Thomas *et al*, 1999). They are microaerophilic organisms, requiring culture in reduced oxygen conditions, most commonly in 5% oxygen, although they can grow in levels of oxygen of up to 10% (Davies and DiRita, 2008). Most *Campylobacter* species are thermophilic, with ideal growth temperatures of around 40-42 °C, which corresponds to the body temperature of chickens and may partly explain their ability to efficiently colonise the avian intestines. Furthermore, *Campylobacter* is fastidious in terms of the requirements of its culture media – they grow best in Mueller Hinton broth or agar (Davies and DiRita, 2008), often used with the addition of 5% (v/v) lysed horse blood and beta-nicotinamide adenine dinucleotide ( $\beta$ -NAD) when used for standardised culture and antibiotic sensitivity testing (EUCAST, 2015). A selective medium commonly used for isolation of *Campylobacter* from animal or environmental samples is modified charcoal cefoperazone deoxycholate agar (mCCDA), which selects for *Campylobacter* through the presence of cefoperazone and sodium desoxycholate, to which *Campylobacter* is intrinsically resistant and aids growth through the presence of sodium pyruvate and ferrous sulphate as nutrients and charcoal as detoxifying agent that reduced oxygen tension.



**Figure 1.1 Morphology of *Campylobacter jejuni*.** **A.** The spiral-shaped rod form of *C. jejuni* illustrated through light microscopy (100x). **B.** The same morphology illustrated through immunofluorescent staining with an anti-*C. jejuni* whole cell antibody. Both images are my own production. **C.** Transmission electron microscopy of *C. jejuni* showing polar flagella (Stevens laboratory, The Roslin Institute),

## 1.2 *Campylobacter* as a cause of human diarrhoeal disease

*Campylobacter* infections are the main cause of acute food-borne diarrhoeal illness in humans worldwide (WHO, 2011). It invades intestinal epithelial cells and causes a severe inflammatory reaction and diarrhoeal disease (as reviewed by Young *et al*, 2007).

### 1.2.1 Clinical manifestations of *Campylobacter* infection in humans

The dose required to establish infection in humans can be as low as 500 colony-forming units (CFU) in milk as demonstrated by Robinson (1981) who infected himself with *C. jejuni* contaminated milk. This was confirmed in other experimental studies on larger cohorts, e.g. Black *et al* (1988) demonstrated that a dose as low as 800 CFU of two different strains results in clinical infections in each case. The same study also demonstrated that the severity of the diarrhoeal disease induced by infection varies with the strain of *Campylobacter* and with the individual infected. Further modelling work on the data obtained by Black *et al* (1988) showed a slowly rising dose-response relation when considering infection only as the outcome (Medema *et al*, 1996), however the relationship between challenge dose and the occurrence of clinical signs did not follow a dose-related trend as the highest illness-to-infection ratio was found at a dose of  $9 \times 10^4$  CFU. Additional modelling work on field-acquired infection showed a nearly sigmoidal relationship between dose and clinical manifestation (Teunis *et al*, 2005), highlighting potential differences between laboratory-grown inocula and naturally occurring ones.

Following ingestion of infectious *Campylobacter* cells, the incubation period varies from 2 days to over a week, depending on the dose ingested. This data was determined by Skirrow and Blaser (1995) through calculations following outbreak investigations. Blaser (1997) describes the clinical signs of *Campylobacter* infections observed in developed countries as involving non-specific prodromal symptoms such as headache, myalgia and pyrexia lasting over 24h. These are followed by acute diarrhoeal illness, which is the main clinical manifestation and can often involve acute abdominal pain. The diarrhoea is watery and frequently bloody, with over eight bowel movements per day at its peak. Abdominal cramps may appear as severe as the pain of appendicitis. Following a peak which usually lasts 24-48h, the illness then gradually resolves over approximately a week, however it can be longer lasting in a proportion of cases. If no treatment is provided, relapses occur in as many as 20% of infections. To exemplify the severity of *Campylobacter* infections, an outbreak at a military base in the United States (U.S.) due to contamination of water supply resulted in 249 people exhibiting clinical signs of whom as many as 32% required hospitalisation. Infection rates varied amongst companies but were as high as 70% of 141 recruits in the company situated in immediate vicinity to the infected water tank (DeFraités *et al*, 2014).

In contrast, in developing countries, *Campylobacter* infection mainly affects children under the age of 5, being a major cause of morbidity and mortality (Blaser, 1997). A study undertaken in children under 5 years old admitted to hospital with confirmed *Campylobacter* infection in Kenya highlighted a mortality rate as high as 8.8% (O'Reilly *et al*, 2007). In the same age group, Taylor *et al* (1993) demonstrated potent seroconversion in 80% of confirmed infections and also showed that the duration of excretion following infection declines with age. This is suggestive of an age-related development of protective immunity within the population in developing countries. This is further supported by the short duration and less intense clinical signs in adults in developing countries (as reviewed in Coker *et al*, 2002).

### **1.2.2 Sequelae of *Campylobacter* infection in humans**

Although most *Campylobacter* infections resolve without complications, links have been shown to various long-lasting sequelae. Evidence is mounting to suggest that in a minority of cases, infection with *Campylobacter* can lead to chronic gastrointestinal clinical signs, namely inflammatory bowel syndrome (IBS) and inflammatory bowel disease (IBD), including Crohn's disease. Borgaonkar *et al* (2006) describes that 4.2% of patients with *Campylobacter* infection developed IBS in a three month follow-up study compared to 2.6%

of *Salmonella*-infected patients. Furthermore, Spiller *et al* (2000) proposed an immunological mechanism that may contribute to the induction of IBS by *Campylobacter* and demonstrates that increased enteroendocrine cells, T lymphocytes and gut permeability can persist for over a year following campylobacteriosis. These findings have been confirmed to be a feature of patients that develop IBS following infections with enteric pathogens (reviewed by DuPont, 2008). Regarding IBD, it has been shown that *Campylobacter* and *Salmonella* infections result in an increased risk of this clinical manifestation, both in the short term as well as long term (15 years) and the IBDs developed include Crohn's disease (Gradel *et al*, 2009). A novel mechanism for this disease following *Campylobacter* infection has been proposed, involving translocation of the commensal microflora through the intestinal wall via lipid rafts (Khalischuk *et al*, 2009). The increase in intestinal permeability and the breakdown of the normal barrier function is in line with the aforementioned studies that demonstrated long-term increased gut permeability following campylobacteriosis. In contrast, other studies found no links between the two and *Campylobacter* infection. Jess *et al* (2010) suggested that the perceived increased risk of post-infectious IBD is only due to detection bias around the time of first-hospitalisation due to clinical signs of IBD.

More severe sequelae can occur, namely inflammatory neuropathies and reactive arthritis. A link has been shown between *C. jejuni* infections and Guillain-Barré syndrome (GBS; Tam *et al*. 2007), an inflammatory neuropathy that can involve transient paralysis. Due to the similarity of the lipooligosaccharide (LOS) present on some strains of *C. jejuni* with human neural gangliosides, antibodies raised against *C. jejuni* LOS during infection may cross-react later with neural gangliosides (Ang *et al*. 2002). Certain *C. jejuni* strains, e.g. ICDCC07001, have even been associated with outbreaks of campylobacteriosis and GBS. This strain was isolated from a 36-case outbreak of GBS in China in 2007 (Zhang *et al*, 2010). The mechanisms behind GBS induction by *Campylobacter* still warrant further elucidation but it is evident that different alleles of certain bacterial virulence factors are associated with the presentation or not. Koga *et al* (2005) showed that *Campylobacter* strains that have the *cst-II(Thr5)* gene, involved in the biosynthesis of ganglioside-like lipooligosaccharide, are more likely to cause neurological clinical signs than strains than those with *cst-II(Asn55)*. A link between *Campylobacter* infections and Miller Fisher syndrome, a variant of GBS, has also been identified (Neisser *et al*. 1997).

In addition, *Campylobacter* infections also cause reactive arthritis or Reiter's syndrome. A review by Pope *et al* (2007) estimates that reactive arthritis following *Campylobacter* infection can occur in between 1 and 5% of those infected and that its annual

incidence may be 4.3/100,000 cases. Work on reactive arthritis due to gastrointestinal infections is limited and further research is warranted to shed light into this manifestation and its causes.

### 1.2.3 Pathological mechanisms and immune responses in acute diarrhoeal disease

#### 1.2.3.1 Descriptive studies in human infections

Histopathologically, campylobacteriosis is classified as inflammatory diarrhoea as it has been demonstrated through colonic biopsies of infected humans that diffuse inflammatory colitis is induced by these bacteria and that polymorphonuclear cells and red blood cells (RBCs) are present in the stool of over two thirds of patients (Blaser *et al*, 1982). Pyrexia is often observed during campylobacteriosis and is another sign typical of inflammatory conditions. These observations are suggestive of a mechanism of pathogenesis involving bacterial adherence, invasion and replication within the intestinal cells, with an associated inflammatory response leading to blood-containing inflammatory diarrhoea (as reviewed in Janssen *et al*, 2008).

In human patients it has been difficult to establish *in vivo* the roles of different arms of immunity but studies suggest that innate, humoral and cell-mediated arms of immunity are all involved in control and/or pathogenesis of this disease to varying degrees. Young *et al* (2007) reviewed the immune responses to *Campylobacter* infection in humans and suggest that a predominantly Th1 polarised (cell-mediated) immunity is likely to be responsible for control and clearance of infection (Fig. 1.2), with dendritic cells (DCs) and macrophages playing a key role. DCs were suggested to encounter and internalise *C. jejuni*, which induces secretion of NF- $\kappa$ B, TNF $\alpha$  and several other cytokines associated with maturing DCs. The role of macrophages in this response is less clear as different studies highlighted in this review gave conflicting evidence using different cell lines and strains of *Campylobacter*.

This model of infection control is supported by studies undertaken in varied systems. Local cellular and cytokine responses are difficult to describe in humans as they would require invasive colonic biopsies. However, this technique has been used in a limited number of studies. Enocksson *et al* (2004) described that patients with gastroenteritis symptoms infected with *Campylobacter* have higher levels of nitric oxide release from rectal tissue. Following natural infection with *C. jejuni* for 8-12 hours, it was shown that IFN $\gamma$ , IL-22 and IL-17A were induced suggesting induction of both innate and adaptive immunity in the initial phase of the infection. Furthermore, flow cytometry indicated that cells with markers indicative of Th1 and Th17 responses and cells with double positive Th1/Th17 markers were

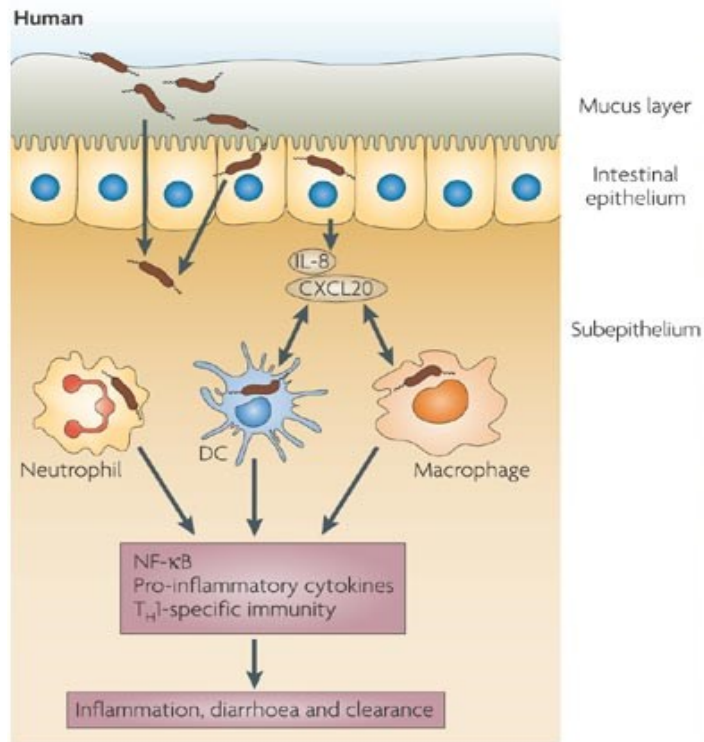
significantly increased suggesting an involvement of cell-mediated immunity in the control of this disease.

In contrast, antibody responses are easier to characterise in human patients as only blood samples are required. As described above, epidemiological studies suggest involvement of antibodies in control of the infection as observations of increasing antibody levels and decreasing severity of clinical symptoms has been described. Furthermore, the increased severity of the disease in agammaglobulinaemic or hypogammaglobulinaemic patients confirms the importance of antibodies in control of *Campylobacter* infection (Melamed *et al*, 1983). Both local mucosal IgA and serum IgG are likely to be involved in playing a role in different outcomes of the disease as a study of a patient that was deficient in IgA and IgM described long-term intestinal carriage with transient bacteraemia that cleared upon the induction of serum IgG (Johnson *et al*, 1984). Furthermore, a role of maternal antibodies has been suggested in humans. Children that had *Campylobacter* infection within the first 6 months of life had significantly lower maternal-derived anti-flagellum antibody titres at birth compared to those that did not exhibit infection (Martin *et al*, 1989), implying a possible protective role.

#### 1.2.3.2 Animal models of diarrhoeal disease

The nature of the immune response and the molecular pathological mechanisms in humans associated with *Campylobacter* infection have been difficult to characterise due to the lack of adequate models of this disease. Good animal models of human diarrhoeal disease caused by infection with *C. jejuni* or *C. coli* are lacking, as laboratory animals are either not easily colonised by *Campylobacter* infection is either asymptomatic or elicits milder clinical signs. Mice are not easily colonised by *Campylobacter* unless they are infant or their intestinal flora is depleted before infection. Lee *et al*, 1986 described the use of a model in which gnotobiotic BALB/c mice had to be treated with magnesium sulphate and three antibiotics to acquire *C. jejuni* infection.

More recently, transgenic mice have been used to develop models of human campylobacteriosis and have provided novel insights into the nature of the immune responses to this infection, however their findings are seldom validated in human patients. For example, Watson *et al* (2006) report efficient colonisation of MyD88-deficient mice by *C. jejuni* which suggests a role for toll-like receptors (TLRs) in the immune response in this



**Figure 1.2 Model of the immune response in human *Campylobacter* infection leading to pathogenesis and clearance.** (Proposed by and adapted from Young *et al*, 2007). *C. jejuni* interacts with human intestinal epithelial cells and invades them. Secretion of IL-8 and other pro-inflammatory cytokines by these cells attracts neutrophils, macrophages and dendritic cells at the site of infection and sets up the milieu for a highly pro-inflammatory environment.

host as MyD88 is involved in signal transduction through most TLRs. It was also demonstrated that such mice can resolve phenotypes of bacterial mutants lacking known virulence factors, however, their usefulness for immunological studies may be precluded by impaired immune response in an arm of native immunity known to be essential in defences against bacterial infections. In contrast, Stahl *et al* (2014) demonstrate that Sigirr<sup>-/-</sup> C57BL/6 mice that have upregulated MyD88 signalling exhibit severe gastroenteritis, similar to the human condition. It was shown that in this infection model, the mice have an increased Th1/Th17 cytokine profile, suggesting a role of cellular immune responses in the pathogenesis and possibly resolution of this disease. Furthermore, the same study described a protective role of TLR2 in preserving mucosal integrity and reducing clinical symptoms and a pro-inflammatory role of TLR4 in the enteritis symptoms observed. The increased Th1 cytokine profile in this study is in line with the observations of Edwards *et al* (2010) described above.

Other animal models include a model of haemorrhagic diarrhoea in gnotobiotic colostrum-derived neonatal piglets (Babakhani *et al*, 1993), however, using animals at such a young age without a fully-developed immune system and intestinal microbiota may not adequately replicate the nature of the immune response in human adult diarrhoea. Ferrets have provided a more adequate model of human infection as they can become colonised by *C. jejuni* and develop diarrhoea. However, they are not as widely available and the disease is mild and transient in adult ferrets (Bell and Manning, 1990) and only mimics human adult diarrhoea in weaning-age animals (Fox *et al*, 1987). Some of the closest models of human infection have been developed in primates (Russell *et al*, 1989; Baqar *et al*, 1995), however, these are only rarely available.

A contribution of the human microbiota to the outcome of the infection and the nature of the immune responses has been described. Bereswill *et al* (2011) reported that gnotobiotic mice given human microbiota can be easily and stably colonised by *C. jejuni*. In contrast to mice with typical murine microbiota, those with human microbiota exhibited a more severe infiltration of T- and B-lymphocytes, regulatory T cells and neutrophils and increased concentration of TNF $\alpha$ , IL-6 and monocyte chemoattractant protein 1 (MCP-1) in their colonic mucosa. This study confirms the findings of Stahl *et al* (2014) that TLR4 plays a central role in pathogenesis but also demonstrated a role for TLR6 in this processes.

In addition to the above studies pointing towards a Th1-biased response in *Campylobacter*-induced colitis, Malik *et al* (2014) further describe the nature of the immune responses in a mouse colitis model. Their study shows a role for both innate and adaptive immunity in the induction of colitis as Thy1<sup>+</sup> lymphocytes were shown to be required for the appearance of this presentation but they were insufficient as Rag1<sup>-/-</sup> mice did not exhibit colitis. Furthermore, this study also showed that colitogenic (*C. jejuni* 11168) or GBS-inducing strains (*C. jejuni* 260.94) induce different responses, with colitogenic strains biasing the response towards Th1 immunity and antibodies of the IgGb, IgG2c and IgG3 isotypes and GBS-inducing strains biasing responses towards Th2 immunity and antibodies of the IgG1 isotype. While Th2-type immunity was required for the induction of IgG1 antibodies cross-reacting to human neural gangliosides GM1 and GD1a, it appeared that their cross-reaction was a property of the strain used as IgG1 antibodies against 11168 raised in a Th2-biased response in IL-17 and IFN $\gamma$  depleted mice did cross-react with human neural gangliosides (Malik *et al*, 2014).



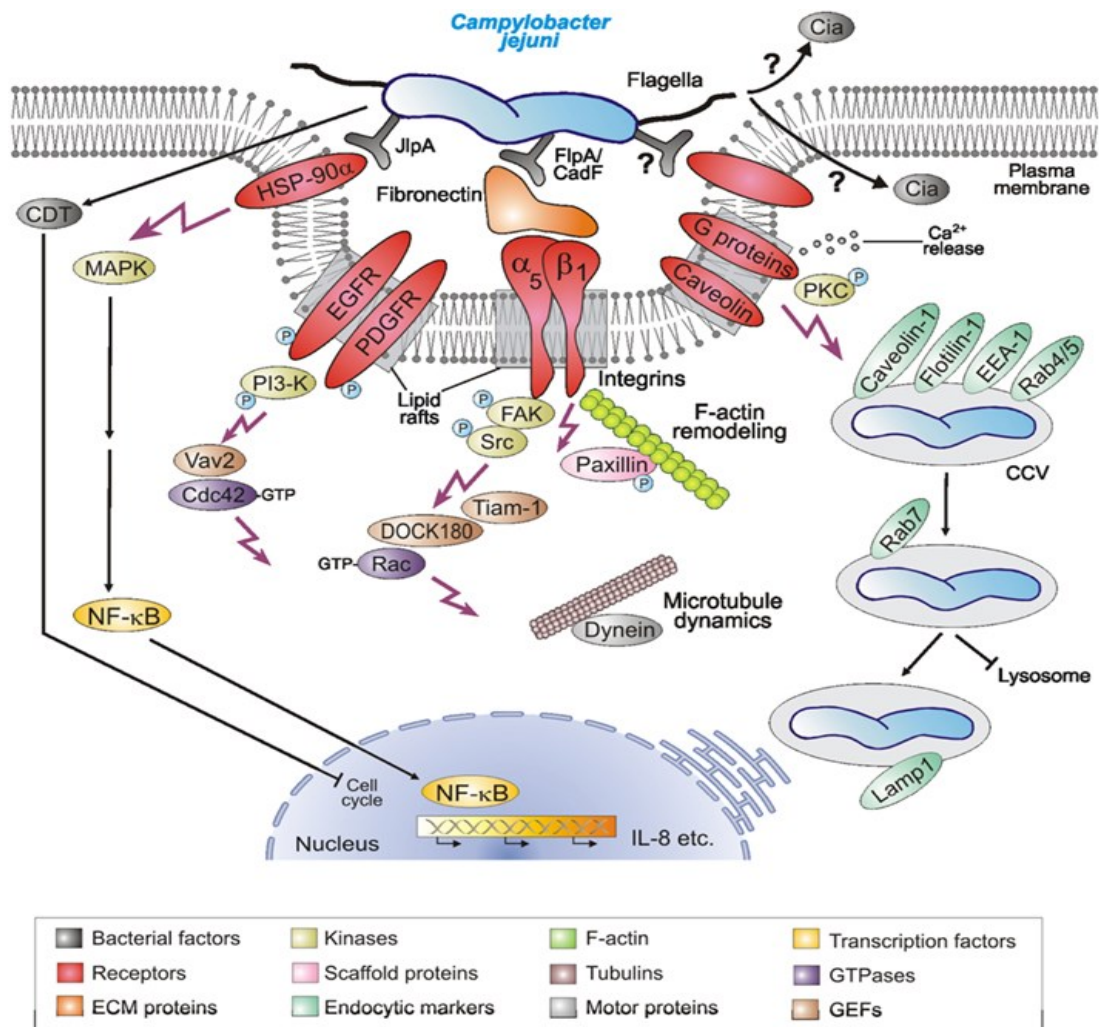
### 1.2.3.3. *In vitro* studies using human tissue cultures

Due to the above limitations, the immune response in humans has also been characterised through studies undertaken *in vitro* in human cell cultures. Hickey *et al* (1999) showed that *C. jejuni* induces secretion of interleukin 8 (IL-8) from INT407 cells, that it required live bacterial cells and invasion, and that induction and release of IL-8 occurs early in the interaction of bacterial and eukaryotic cells. Al-Saloom *et al* (2003) showed that *C. jejuni* induces release of IFN- $\gamma$ , IL-10, TNF- $\alpha$  and IL-4 from INT407 cells and Bakhiet *et al* (2004) showed that the same cells presented with *C. jejuni* produce IL-8, CCL4 and CCL2 but not CCL3 or CCL5. Caco-2 cells, a human colonic epithelial line, have been proposed as a model of intestinal epithelia as a polarised layer of these cells was successfully used as a model for invasion and transcytosis (Everest *et al*, 1992). Furthermore, they were shown to have increased mitogen-activated protein kinase (MAPK), which plays a role in many host signalling pathways, following *C. jejuni* infection, similarly to primary human colonic tissue (MacCallum *et al*, 2005). Based on *in vitro* studies, a model of invasion of intestinal cells has been described by O’Croinin and Backert (2012), highlighting host factors involved in this process (summarised in Fig 1.3).

A limitation of such studies is that monocellular tissue cultures lack the complexity of immune responses induced in whole organisms. While they can identify individual molecules involved in immune responses, the relative contribution to these molecules to the immune response generated in an entire organism is difficult to assess without further confirmatory studies. Furthermore, different cells lines vary in their response to infection. MacCallum *et al* (2006) confirmed IL-8 secretion from INT407 cells but demonstrated minimal IL-8 secretion from Caco-2 cells and levels of IL-8 secretion from HeLa and T84 cells matching that of primary human intestinal tissue.

### 1.2.4 Bacterial virulence factors involved in human diarrhoeal disease

The majority of *Campylobacter* virulence factors that have been well-characterised, such as the flagellum and systems for nutrient acquisition, chemotaxis and oxidative stress resistance play a conserved role in the colonisation of most hosts that *Campylobacter* is able to infect (reviewed by Wassenaar and Blaser, 1999). However, there are some virulence factors that have been described to be implicated in human disease, including the interplay with the particular types of immune responses elicited by the human hosts.



**Figure 1.3 A hypothetical model of *C. jejuni* invasion into host intestinal cells.** This diagram, reproduced from O’Croinin and Backert (2012) summarises some of the bacterial and host factors leading to attachment and successful establishment of *Campylobacter* infection in the human host. Authors propose that, following interaction between numerous described and unknown virulence factors (marked with “?”) of *C. jejuni* and the described host cellular factors, local actin and tubulin re-arrangement at the site of attachment of *C. jejuni* result in engulfment and bacterial uptake.

*Campylobacter* invasion into intestinal cells is considered more common in human infections than in infections of other hosts. With regards to the model of invasion described above, while both microfilaments and microtubules are likely to be involved in membrane re-shaping around the invading *C. jejuni* cells, it is unclear as to which are the major contributors. Hu and Kopecko (1999) showed that the interaction between microtubules and *Campylobacter* is crucial for invasion as inhibition of actin polymerisation did not reduce *C. jejuni* invasion but inhibition of tubulin polymerisation nearly abolished invasion. In

contrast, Biswas *et al* (2003) described that inhibition of microfilament formation had a greater inhibitory effect on *C. jejuni* invasion than inhibition of microtubule formation.

In addition to direct invasion, production of toxins has been implicated in the pathogenesis of diarrhoeal disease. Although typical enterotoxins (cytotoxic enterotoxins similar to those of *V. cholerae* and *E. coli*) have not been identified in *Campylobacter* (as reviewed by Wassenaar and Blaser, 1999), cytotoxins have been described. Wassenaar (1997) reviewed the *Campylobacter* proteins that were shown to have cytotoxic activity and may be involved in pathogenesis of the human disease and classified them into six groups: a 70kD cytotoxin, a Vero/HeLa cell cytotoxin, a cytolethal distending toxin (CDT), a Shiga-like toxin, haemolytic cytotoxins and hepatotoxins. The sequencing of the *C. jejuni* genome (Parkhill *et al*, 2000) revealed that *C. jejuni* 11168H did not contain a cholera-like toxin gene but confirmed the presence of CDT and highlighted the presence of a contact-dependent haemolysin (CJ0588), a membrane protein with a haemolysin domain (Cj0183) and a phospholipase (PldA).

The activity of most of the *Campylobacter* proteins that have been described as cytotoxins remains poorly characterised. The *Campylobacter* CDT (one of the bacterial virulence factors well characterised in other pathogens) has been shown to have a similar role to that from *E. coli*, in that it arrests the cell cycle and leads to cell death (Johnson *et al*, 1988). It has also been shown to be recognised by the human immune system but not by the chicken (AbuOun *et al*, 2005). Even so, it is insufficient to cause the observed clinical signs on its own as Mortensen *et al* (2011) showed that no differences in the clinical outcome of patients infected with *Campylobacter* strains with different titres of CDT. Although well characterised in other bacterial species, the *Campylobacter* Shiga-like toxin has only been reported in one study and as such its presence and role remains anecdotal (Moore *et al*, 1988) and was not confirmed by the sequencing of *C. jejuni*'s genome (Parkhill *et al*, 2000).

Furthermore, even though *Campylobacter* do not possess a classical type III secretion system (T3SS), they have been proposed to be able to secrete effectors into host cells through flagella, which are considered T3SS analogues (Neal-McKinney and Konkel, 2012). Approximately 10% of *C. jejuni* strains have also been shown to possess a type VI secretion system (T6SS) which induces haemolysis in capsule-deficient strains and has been isolated from patients that developed bacteraemia (Bleumink-Pluym *et al*, 2013). Konkel *et al* (1999) described the *Campylobacter* invasion antigens (Cia) excreted through the flagellum and showed that they are involved in invasion of host cells as mutants lacking CiaB bound equally well to host cells as the wild-type (WT) but significantly lower numbers of *C. jejuni* were found intracellularly. Furthermore, it was shown that for these proteins to

exert their function an intact flagellar hook (composed of FlgE, FlgK and FlgL) was required for their delivery into host cells (Neal-McKinney and Konkel, 2012).

With regards to other possible factors involved in human disease, Jin *et al* (2003) has shown that JlpA, a surface lipoprotein mediates attachment to host epithelial cells (Jin *et al*, 2001), interacts with Hep-2 cell surface heat shock protein and induces activation of NF-kappaB and p38 MAP kinase, that stimulate inflammatory responses in host cells. CadF (Konkel *et al*, 2003) and FlpA (Flanagan *et al*, 2009) have also been described as adhesins. More recently, transposon mutagenesis of a hyper-invasive clinical isolate identified additional virulence factors and highlighted Cj01/51\_0497 as a potential adhesin (Javed *et al*, 2010). Another transposon mutagenesis screen identified aspA, aspB and sodB as required for survival in within epithelial cells (Novik *et al*, 2010).

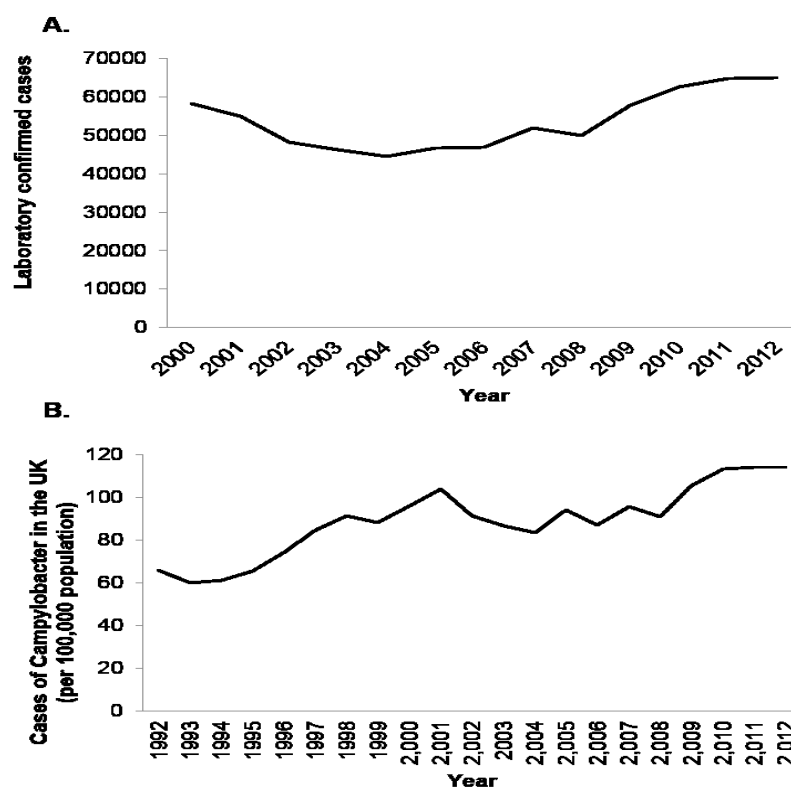
### 1.2.5 Epidemiology of *Campylobacter* infection in humans

*Campylobacter* is the leading cause of acute food-borne diarrhoeal illness in humans in the developed world. DEFRA (2011) reported that following the introduction of an effective anti-*Salmonella* vaccine in the poultry industry in late 1990's, *Campylobacter* replaced non-typhoidal *Salmonella* as the most common foodborne gastrointestinal pathogen in the UK.

#### 1.2.5.1 Incidence and economic burden

In 2013, 66,575 laboratory-confirmed cases of human campylobacteriosis were recorded in the UK, most due to *C. jejuni* subspecies *jejuni* and *C. coli* (DEFRA, 2015). Furthermore, the Infectious Intestinal Diseases (IID) II study estimated that for every case captured by national surveillance, a further 9.3 cases occur in the community but are not recorded owing to the mostly self-limiting nature of the disease and the lack of laboratory investigations (Tam *et al*. 2011). As such the true burden of human campylobacteriosis in the UK may be c. 700,000 infections per annum. The majority of human campylobacteriosis represents sporadic cases, however outbreaks are occasionally recorded. The most common cause for these is the consumption of undercooked chicken liver parfait (HPA, 2011). The latest Zoonoses Report (DEFRA, 2015) highlights 19 outbreaks during 2013, a significant increase on the 8 reported in 2012, with 14 being food-borne and 9 of these due to chicken liver parfait. Figure 1.4 below highlights the increasing number of laboratory-confirmed *Campylobacter* infections in humans between 1992 and 2012 and it is apparent that apart from a period of reduction between 2000 and 2004, the incidence of human infections has generally risen. These infections have a high recurring economic and societal cost. The cost

of foodborne illness in the UK has been estimated at approximately £1.8 billion per year (FSA, 2013). As *Campylobacter* is responsible for ~60% of foodborne illness and 92% of hospital admissions due to these (FSA, 2013), it may be inferred that the cost of human campylobacteriosis is in the region of £1 billion/year in the UK. The problem is not limited to the UK as the European Food Standards Agency estimates that there are nine million cases of human campylobacteriosis per year across EU27 countries, with the disease and its sequelae causing 0.35 million disability-adjusted life years per annum at an annual cost of €2.4 billion (EFSA, 2011).

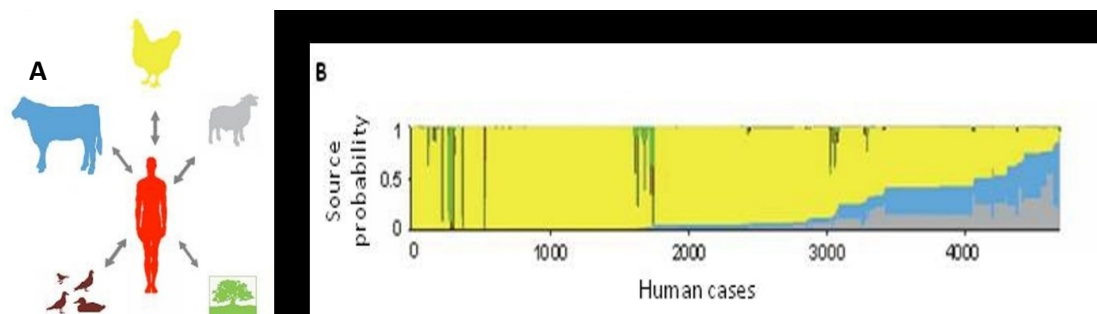


**Figure 1.4 The trend of laboratory-confirmed cases of *Campylobacter* infections in the UK between 1992 and 2012. A.** The number of laboratory-confirmed cases between 2000 and 2012, as given in the DEFRA Zoonoses report for each year, demonstrating the reduction in *Campylobacter* cases between 2000 and 2004. **B.** Incidence of *Campylobacter* in the UK between 1992 and 2012. Numbers are given as cases per 100,000 of population. (Data adapted from DEFRA Zoonoses Report 2000 and DEFRA Zoonoses Report 2012 for the number of *Campylobacter* infections and the Office for National Statistics for population estimates between 1992 and 2012).

### 1.2.5.2 Source of human *Campylobacter* infections

While poultry have been recognised as the main source of infection with *C. jejuni*, recent advances in molecular typing and high-throughput sequencing have allowed the assignment of relative contributions of sources of infection to the burden of human disease. Studies carried out in Scotland (Sheppard *et al*, 2009; Fig. 1.5) and England (Wilson *et al*, 2008) indicated that, second to poultry, sheep and cattle are the next major sources of contamination, with the environment and wild birds playing smaller roles. . Furthermore, even studies in other countries have observed a similar pattern of source attribution of campylobacteriosis, e.g. in New Zealand (Mullner *et al*, 2009) it was shown that chickens are the main source of human infection and, uniquely, it was possible to assign cases to three major retailers due to the particular structure of the poultry industry in the country. More recently Sheppard *et al* (2014) described specialist and generalist lineages of *C. jejuni* and provided evidence of recombination amongst generalist and specialist species of a common lineage but little recombination amongst different generalist lineages. Due to this, they propose a cryptic niche structure that acts as an ecological barrier to recombination. Furthermore, Dearlove *et al* (2015) suggest that high rates of host-switching amongst generalist lineages of *Campylobacter* may hinder source attribution but even so the majority of clinical cases can be attributed to chickens sources.

While sources other than those mentioned above may contribute to human cases, these represent a minor contribution. For example, although campylobacteriosis has been reported in pets, particularly dogs, it has been shown that they are colonised mainly by other species than those commonly pathogenic to humans and that the prevalence of *C. jejuni* was only 1.2% (Parsons *et al*, 2010).



**Figure 1.5 Source attribution of human clinical *Campylobacter* cases.** (Adapted from Pascoe *et al*, 2013) **A.** Sources of infection for human *Campylobacter* cases. These are: chickens (yellow), cattle (blue), sheep (grey), wild birds (brown) and the environment (green) **B.** Probabilistic model of source attribution of human clinical *Campylobacter* cases. Each vertical bar represents a single isolate and the different colours indicate the probability of it originating from each of the sources described in panel A. Up to 70% of human infections in Scotland are predicted to have originated from chickens in the period studied.

### 1.2.5.3 The avian reservoir as a source of human infection

Up to 80% of the total number of infections may be attributable to the avian reservoir as a whole (Wilson *et al*, 2008; Müllner *et al*, 2010; Dearlove *et al*, 2015) and within this category, handling or consumption of contaminated poultry meat is the main source of *C. jejuni* infection in humans. In Scotland, Sheppard *et al* (2009) attributed between 58% and 78% of *C. jejuni* and between 40% and 56% of *C. coli* isolates from humans (depending on the model used for data analysis) to contaminated chicken meat. This is in agreement with earlier findings of Kramer *et al* (2000) who showed that *Campylobacter* isolates in chicken and lamb on retail sale shared the most subtypes with human *Campylobacter* isolates. In this study, chicken meat also showed the highest prevalence of *Campylobacter* contamination (83.3%), followed by lamb (72.9%).

In accounting for the difference between cases attributed directly to contaminated chicken meat and the avian reservoir as a whole, wild birds and pet chickens may also pose a threat to human through indirect transmission via environmental contamination. For example, Thomas *et al* (1998) reviewed the evidence available for *Campylobacter* infection from aquatic sources, including both untreated and contaminated mains water. Although traditionally thought to be a pathogen with specific requirements for survival, Rollins and Colwell (1986) showed the presence of viable but non-culturable stages in water. Furthermore, poultry other than chickens are likely to contribute to human campylobacteriosis as *Campylobacter* has been reported in turkeys (39% of turkey on retail in the USA was found to be contaminated; Loque *et al*, 2003), pheasants (70% of farmed pheasants in the Czech Republic; Nebola *et al*, 2007) and other birds destined for human consumption.

The previous two reports of the UK Food Standards Agency (FSA) showed that between 2005 and 2009 levels of *Campylobacter* contamination of poultry carcasses on sale did not change significantly being approximately 70% in 2005 (FSA, 2005a) and 64.5% in 2009 (FSA, 2009a), despite targets set for a 50% reduction within that period. In another attempt to control human campylobacteriosis, in 2010 the FSA made reduction of food-borne campylobacteriosis a key aim for their 2010-2015 strategic plan and their *Campylobacter* Risk Management Programme aims to reduce the proportion of highly contaminated chickens (>1000 colony-forming units/g faeces) from 27% in 2008 to under 10% in 2015 (FSA, 2010a). Despite this renewed target, the latest FSA survey has indicated no reduction in *Campylobacter* isolated from chicken on sale in the UK. Following a year-long survey, the FSA announced that in 2015 an average of 73% of raw chicken on sale was contaminated and that 19.4% of chickens were contaminated with >1000 CFU/g (FSA, 2015a). While

there has been a reduction in the proportion of the highest contaminated group from 27% in 2008 to 19.4% in 2015, this is still far from achieving the 10% target set by the FSA in 2010.

Post-slaughter processing is an important source of carcass contamination and Allen *et al* (2007) showed that processing practices can lead to high level colonisation of chicken carcasses. Commonly 100% of processed carcasses are contaminated with an average of 5.3 log<sub>10</sub> CFU and even carcasses from flocks with low or no *Campylobacter* colonisation present become contaminated post-processing (Allen *et al*, 2007). The caecal load of *C. jejuni* frequently exceeds 10<sup>8</sup> CFU/g, meaning that even milligram quantities of intestinal contents can present a significant threat of contamination. This is particularly worrying considering that the human infectious dose is very low, thought to be <500 bacterial cells (FSA, 2005b). The same document mentions that studies to determine the exact number of cells associated with contaminated poultry that will cause human infection were inconclusive but examination of a bottle of bird-pecked milk which was of a batch implicated in a nursery outbreak showed contamination levels of under 10 cells of *C. jejuni* per 100 ml, lower than the reported in previous studies (Black *et al*, 1988). When considering the avian reservoir as potential for contamination of other food-sources, evidence suggests that the level of *Campylobacter* transmission between chickens and other farm animals is low (Boes *et al*, 2005).

#### 1.2.5.4 Temporal and spatial epidemiology of *Campylobacter* infection in chickens and humans

Louis *et al* (2005) revealed in a review of human campylobacteriosis cases between 1990 and 1999, spatial and temporal differences in epidemiology. There were regional differences in the number of *Campylobacter* infections in the UK (Wales and the SW of England having higher numbers than the SE of England), differences in seasonality (increases in *Campylobacter* cases correlated with temperature) and differences in gender of infected subjects (males more than females). This study also found a correlation between the seasonality of human infections and temperature. The seasonal trends and gender differences in human infection were replicated in other studies within the UK (Cody *et al*, 2012).

A similar seasonal trend has been described in chickens: Meldrum *et al* (2005) reported that in 2002 in Wales human cases peaked between weeks 22 and 25 (early June) while chicken cases peaked between weeks 24 and 26 (late June) and suggest that the peak in humans is not due to a rise in chicken contamination. In support of this assumption, other studies identified that the seasonality in chickens is related to sunshine hours, temperature and rainfall (Wallace *et al*, 1997; Jorgensen *et al*, 2011). These studies indicate that the



seasonal pattern of human and chicken *Campylobacter* infection is linked to the environment and that successful strategies for control of infections with this pathogen will most likely have to combine interventions addressing both food-borne and environmental infections.

### **1.3 *Campylobacter* infections in chickens**

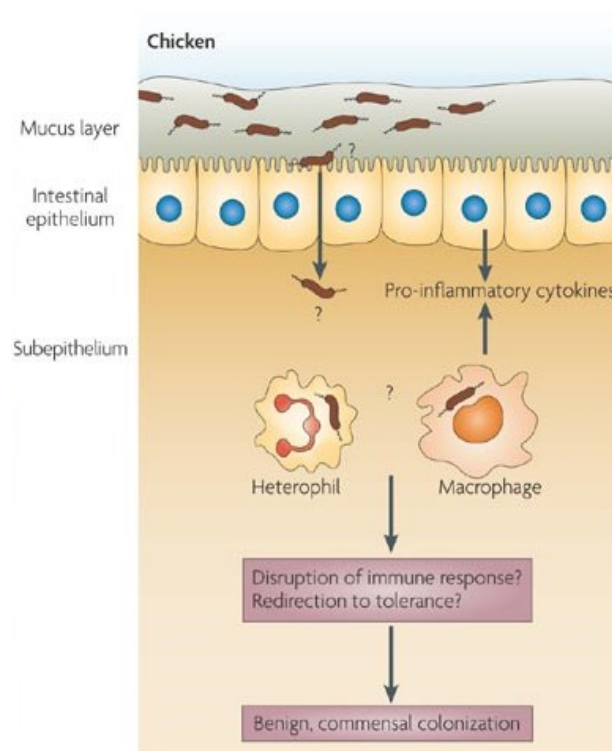
#### *1.3.1 Characteristics of the infection in chickens*

In contrast to human campylobacteriosis, *Campylobacter* infections in chickens are often asymptomatic. In chickens, *Campylobacter* colonises mainly the caeca, large intestine and cloaca, with densely packed *Campylobacter* cells observed in mucous crypts (Beery *et al*, 1988) but it does not typically induce a profound inflammatory response or clinical signs in chickens or turkeys (Lam *et al*, 1992). *Campylobacter* can frequently reach numbers as high as 10<sup>8</sup> CFU/g of caecal contents (Hue *et al*, 2011) or even higher (as observed in our experimental studies) and this high level of colonisation of chickens, combined with the slaughter practices utilised by the poultry industry leads to a high level of carcass contamination.

Even though older studies describe *Campylobacter* as an avian commensal, more recent studies highlighted that *C. jejuni* elicits prolonged inflammatory responses, damage to intestinal mucosa and diarrhoea in some commercial breeds of broiler chicken (Humphrey *et al*, 2014). Moreover, it was recently reported that *C. jejuni* adversely affects villus morphology, epithelial barrier integrity and body mass gain in broilers (Awad *et al*, 2015), with defects in energy retention possibly being due to repression of nutrient uptake systems (Awad *et al*, 2014). Field data supporting these experimental observations has been obtained for the first time in the UK (Nick Sparks, personal communication). *Campylobacter*-positive birds are also more likely to exhibit digital dermatitis and signs of colibacillosis (Bull *et al*, 2008), though causal links have yet to be formally proven. Furthermore, Young *et al* (1999) recovered *Campylobacter* from the liver of day-old Leghorn chicks infected with *C. jejuni* and, even though low level, the invasiveness of *C. jejuni* in chickens further suggests that this bacterium is not a true commensal in this host. The relevance of spread of *C. jejuni* from the gut to liver is evidenced by the outbreaks due to undercooked chicken liver pate and parfait (DEFRA, 2015).

### 1.3.2. Immune responses to *Campylobacter* infection in chickens

Young *et al* (2007) reviewed the immune responses during chicken colonisation and describe differences compared to responses in humans (Fig. 1.6). They describe that, in contrast to the human infection, *C. jejuni* resides primarily in the mucous layer in the chicken, with lower levels of invasion in the intestinal mucosa. Even though heterophils and macrophages are recruited to the site of infection, there is no subsequent strong inflammatory response as in humans. While some host and pathogen factors that may contribute to this apparent tolerance towards *Campylobacter* in the chicken have been described, the exact mechanisms that lead to persistent colonisation in the chicken are yet to be elucidated. Evidence on the nature of the immune response in chickens from experimental and natural infections is reviewed below.



**Figure 1.6. Summary of immune responses to *Campylobacter* in chickens and outcome of infection** (adapted from Young et al, 2007). *Campylobacter* reside mainly in the mucous layer in the chicken and do not invade the intestinal mucosa to a level as high as in human infection. The exact mechanisms that lead to apparent tolerance of *Campylobacter* and its long term persistence in the avian host have not yet been elucidated (denoted by the question marks).

### 1.3.2.1 Humoral immune responses

The nature of the immune response induced by *Campylobacter* following natural infection of broilers has been described extensively, however, the majority of studies focused on humoral responses due to them being relatively easier to describe than the cellular arm of immunity. Even though studies have described the ability of 2-day-old chicks to be as efficiently colonised as 2-weeks-old chicks by *C. jejuni* through direct oral or cloacal challenge (Shanker *et al*, 1988) or in seeder challenge models (Shanker *et al*, 1990), multiple epidemiological studies have described that broiler flocks are commonly negative for *Campylobacter* until 2-3 weeks of age. Smitherman *et al* (1984) describes that chicks housed in sheds in which the previous batch of birds was *Campylobacter* positive only become positive at 12 days of age when the litter is not changed or at 40-46 days of age when the litter is changed. Such findings were reproduced by Genigeorgis *et al* (1986), Lindblom *et al* (1986) and others.

Subsequent studies suggest that maternal anti-*Campylobacter* antibodies transmitted in egg yolk are responsible for this delay in colonisation. Sahin *et al* (2001) measured the levels of anti-*C. jejuni* IgG in sera of chicks originating from *Campylobacter* infected layers and describe that decreasing antibody titres at weeks 3 and 4 of age coincided with the incursion of *Campylobacter* into the flocks. Furthermore, this observation was validated experimentally as Sahin *et al* (2003) showed a significantly lower percentage of chicks colonised by *C. jejuni* following challenge at 3-days-old if they originated from birds raised at specified pathogen-free (SPF) status that had been experimentally infected with *C. jejuni*, compared to chicks originating from non-infected birds. More recently, experimental infection studies have identified individual proteins that are recognised by these maternally-derived antibodies (Shoaf-Sweeney *et al*, 2008) and some of these are discussed later as potential vaccine candidates. Some of these proteins have been independently validated as being immunogenic in adult birds infected with *C. jejuni*: Cawthraw *et al* (1994) showed that flagellin is the immunodominant antigen in experimentally infected day-old chicks, Yeh *et al* (2015) identify FlgE1, FlgK, FlhF, FliG and FliY as the predominantly immunogenic proteins within the flagellum apart from flagellin and Yeh *et al* (2014a) identified Cj0473 of *C. jejuni* D1-39 as the predominantly immunogenic chemotaxis protein from the 15 tested.

An alternative hypothesis that has been proposed for the delay in incursion of *Campylobacter* into broiler flocks is the effect of the intestinal microbiota. However, Shanker *et al* (1990) did not significantly change the susceptibility of chicks to *Campylobacter* colonisation following treatment with adult microflora. Furthermore, Skanseng *et al* (2013) showed that although increasing complexity of feed can reduce

*Campylobacter* spread within a flock, individual diet components did not have an effect on its spread from bird to bird. Even in the face of a reduction in *Campylobacter* transmission between birds, the microbiota only changed significantly in the upper, but not the lower, intestine following introduction of complex feed.

### 1.3.2.2 Cellular immune responses

*In vitro* studies of immune molecular mechanisms induced in the chicken by *Campylobacter* infection are few. Smith *et al.* (2005) showed that INT407 cells and epithelial chicken kidney cells (CKC) were both similarly invaded by *C. jejuni* 11168H. However, in *in vitro* cultures of HD11 chicken macrophage cell line, *C. jejuni* showed decreased survival when these cells were pre-treated with recombinant chicken IFN- $\gamma$ , suggesting a possible role of this type of immune response, which was described as protective in humans, in resistance to colonisation. Furthermore, avian HD11 cells and CKCs increase their production of IL-1 $\beta$ , IL-6 and IL-8 in response to infection with *C. jejuni*, suggesting that the outcome of infection in chickens is not due to their inability to develop a pro-inflammatory response. Another study investigated comparatively the activation of chicken and human immune responses following *Campylobacter* infection (de Zoete *et al.*, 2009). It was shown that both human MM6 monocytic cells and chicken HD11 macrophages secrete similar levels of IL-8 and IL-1 $\beta$ , with higher activation by lysed *Campylobacter* cells compared to live intact cells. Furthermore, in the same study, various Toll-like Receptors (TLRs) were transfected into HeLa 57A or HEK293 cells. Notably, even though both live and lysed *Campylobacter* activated both human and chicken TLR4 similarly as evidenced by similar amounts of NF- $\kappa$ B, there was a marked difference in the expression profile of IFN- $\gamma$  between chicken and human cells stimulated with either live or lysed bacteria. This suggested that signalling through the MyD88-independent pathway via TLR4, with subsequent activation of IFN- $\gamma$  inducible genes, is reduced in the chicken and may partially explain differences in the outcome of infection in these two hosts as it has been shown that there is an association between the level of IFN- $\gamma$  expression in humans and protection from illness (Tribble *et al.*, 2010; Section 1.2.3).

*In vivo*, Smith *et al.* 2008 showed that experimental *Campylobacter* infection in both day-of-hatch and 2-week-old Light Sussex chickens induced a significant, but time- and magnitude-limited, increase in production of pro-inflammatory cytokines. In both age groups, CXCLi1 and CXCLi2 were significantly increased at 12-24h post-infection in caecal tissue while CXCLi1 was also increased in ileal tissue in the 2 week-old group and levels of both cytokines decreased by day 7 in both groups. Kogut (2002) has shown that CXCLi1 and

CXCLi2 are both involved in chemotaxis of phagocytes which is in accordance with the findings of Smith *et al* (2008) that there was a significant increase in the number of heterophils in both caecal and ileal sections (only in the two week-old group).

Furthermore, the pattern of TLR activation has also been characterised *in vivo* and supports the findings of the *in vitro* studies mentioned above. Shaughnessy *et al* (2009) compared activation of TLR gene expression by *Salmonella* and *Campylobacter* infections in chickens and report that TLR4 and TLR21 expression is increased by both genera of bacteria and TLR5 and TLR15 was increased by *Salmonella* only. The same study highlighted also that although both bacteria increased TLR4 expression, expression of IFN- $\gamma$  was only induced in response to *Salmonella* infection, suggesting a role of cell-mediated immunity in clearance of *Salmonella*. This was demonstrated by Beal *et al* (2006), who showed that antibody-deficient surgically-bursectomised chickens clear primary and secondary *Salmonella* infections just as effectively as intact birds. While the contrast between the outcomes of these two infections suggests a possible lack of activation of cell-mediated immunity in *Campylobacter* infections may lead to its long-term persistence, studies to formally test this hypothesis not been performed in chickens to date.

Even though the differences in host immune responses in chickens and humans have been characterised, the extent of evasion or subversion of host responses by *Campylobacter* leading to long-term colonisation in chickens are not fully understood. The mechanisms through which *Campylobacter* appears not to induce adequate Th1-polarised immunity and through which the initial pro-inflammatory immune response is dampened remain to be clearly defined. Apart from mechanisms of bacterial immune subversion described below, Bingham-Ramos *et al* (2008) has shown that *Campylobacter* also colonises the Bursa of Fabricius in chicks of up to 28 days at levels of between  $10^4$  to  $10^7$  CFU/g tissue and suggested that this may play a role in the modulation of the avian immune response to *Campylobacter*, resulting in partial tolerance and a low inflammatory response to its presence.

More recent experiments tried to characterise the changes in gene expression following infection of chickens with *Campylobacter* through RNA sequencing. Connel *et al* (2010) reported that genes involved in innate immune responses, cytokine signalling, B and T cell activation and immunoglobulin production were significantly upregulated in resistant birds compared to susceptible birds. However, due to the high number of genes that change expression and lack of subsequent validation, interpretation of specific immune mechanisms responsible for this difference is difficult.

### 1.3.3. *Campylobacter* factors influencing colonisation in chickens

As reviewed by Hermans *et al* (2011), identification of *Campylobacter* factors that influence colonisation in chickens has been aided by the ability to screen random and defined mutants in experimental animals. As is the case for human infection (Grant *et al*, 1993; Newell *et al*, 1985), the flagellum has been shown to be important in colonisation of the avian host. The *C. jejuni* flagellum is composed of two flagellar subunits, namely FlaA (major flagellin) and FlaB (minor flagellin) and it has been shown that while both are important for a fully functional flagellum, FlaA only is sufficient for motility (Guerry *et al*, 1991). Grant *et al* (1993) showed that mutants in FlaA and FlaB adhere equally well to cells in culture but they exhibited decreased internalisation, suggesting that the presence of both is required for full flagellar function. Although both proteins form a heterodimer along the length of the flagellar filament, it appears that FlaB epitopes are poorly surface-exposed (Guerry *et al*, 1991). *In vivo*, the presence of the major flagellin, FlaA, has been demonstrated to be vital for chicken colonisation as a mutant lacking this gene did not colonise chickens (Wassenaar *et al*, 1993). Independent reports confirmed FlaA as essential for colonisation (Jones *et al*, 2004). A mutant that was able to assemble FlaA-only flagella (it was lacking only FlaB) had 100-fold enhanced colonisation compared to the WT (Wassenaar *et al*, 1993). Furthermore, the same study showed that high and low motility *C. jejuni* mutants expressing FlaA only were both able to colonise chickens, suggesting that the presence of flagella rather than motility *per se* is vital. This hypothesis is also supported by studies indicating the ability of non-motile *C. jejuni* mutants to colonise when given at high doses (Wosten *et al*, 2004). Mutations in other flagellar genes have been shown to result in abolished colonisation, e.g. FlgK (Fernando *et al*, 2007), however such mutations completely abolish the formation of the flagellar filament.

As well as the presence of flagella, their glycosylation may contribute to long-term persistence in the chicken by allowing *C. jejuni* to evade the immune response to flagellin (Szymaski *et al*, 2003). The major modification of *Campylobacter* flagella is based on strain-specific derivatives of pseudaminic acid (Thibault *et al*, 2001) and mutants lacking this flagellar modification have been shown to be attenuated *in vitro* and in a ferret model of diarrhoea (Guerry *et al*, 2006). *Campylobacter* flagella can also contain strain-specific legionaminic acid modifications (McNally *et al*, 2007). Howard *et al* (2009) showed that mutation of the *cj1324* gene in the O-linked flagellin glycosylation island results in the absence of two legionaminic acid glycan modifications and a reduction in the potential to colonise chickens. Furthermore, the same study described the wide spread presence of these

two modifications in chicken isolates, suggesting that certain glycan-modifications of flagellin may be associated with adaptation to particular hosts.

In addition to flagellar presence and glycosylation, numerous other colonisation factors have been described (reviewed by Hermans *et al*, 2011) and these are involved in chemotaxis, bile resistance, immune evasion through glycosylation, temperature regulation and heat-shock responses, oxidative stress defences, iron transport, nutrient uptake and metabolism. Additionally, particular factors involved in adhesion to chicken intestinal cells have been described more recently and have shown promise as vaccine candidates, e.g. the adhesins CadF (Konkel *et al*, 2003) and FlpA (Flanagan *et al*, 2009). Evidence for the role of some of these factors in chicken colonisation is described in Section 4.1 in relation to their use as vaccine candidates against *Campylobacter* colonisation in chickens.

In studies using signature-tagged transposon mutagenesis, Hendrixson and DiRita (2004) reported factors required for colonisation to include flagellar-associated proteins (the flagellar motor proteins MotA and MotB and the flagellar hook protein FlgK), chemotaxis regulating proteins (CheY), proteins involved in glycosylation (PglE, F and H) and proteins proposed to be involved in oxidative defence mechanisms (DocA). Some of these proteins were validated in a high-throughput sequencing study using signature-tagged transposon mutagenesis (Johnson *et al*, 2013). This latter study also described additional factors that may be involved in colonisation, such as additional flagellar proteins, including the flagellar capping protein FliD, methyl-accepting chemotaxis proteins, iron ABC-transporter protein and other transmembrane transporter proteins. The capsule was inferred as important in chicken colonisation in an independent signature-tagged transposon mutagenesis study (Grant *et al*, 2005) as it was consistently absent from colonised birds. However, the same study also revealed the stochastic nature of mutant loss and colonisation when using *Campylobacter* as the identity of the mutants that colonised and were lost was different between experiments and birds within the same experiment. As such, genes found to be involved in colonisation from such studies should be interpreted with caution unless validated through colonisation studies using single mutants. A capsule-deficient mutant ( $\Delta kpsM$ ) was shown to be attenuated in colonisation/disease causation in chicken, ferret and murine models of campylobacteriosis (Jones *et al*, 2004; Bacon *et al*, 2001; Maue *et al*, 2013) and another capsule-deficient mutant ( $\Delta kpsE$ ) showed a  $2\log_{10}$  CFU *C. jejuni*/g caecal contents reduced colonisation in chickens (Bachtiar *et al*, 2006). Some of the above-mentioned genes have also been found to be up-regulated in a transcriptional profiling study of *C. jejuni* 11168H using microarray analysis of genes expressed during chicken infection (Woodall *et al*, 2005). Apart from upregulation of genes involved in electron transport and

central metabolic pathways, suggesting a change in physiological state of *Campylobacter* during adaptation to the chicken intestinal environment compared to growth in broth culture, iron acquisition genes (*chuA* and *chuB*) and oxidative stress defence genes (*cj0020c* and *cj0358*) were highly upregulated.

#### 1.3.4 Risk factors for *Campylobacter* infection in chickens

As mentioned above, there is a summer peak of *Campylobacter* infection in both humans and chickens. A major study investigating risk factors for *Campylobacter* colonisation of farmed broilers identified the months of July, August and September as having an increased risk of chicken colonisation (Ellis-Iversen *et al*, 2009). The presence of cattle on or near the farm also increased the risk of infection. In addition, the practice of thinning or partial depopulation has been demonstrated to have a major impact on flock positivity. Humphrey *et al* (1993) first described that farms that do not practice boot dipping become colonised with *Campylobacter* by day 10, whereas those that used a phenolic disinfectant remained *Campylobacter*-free until slaughter. Subsequently, Hald *et al* (2001) showed in a study of 10 flocks that partial depopulation increases the number of *Campylobacter*-positive flocks and Allen *et al* (2008) demonstrated through the study of 51 flocks within the UK that the practice of thinning is a risk factor for *Campylobacter* colonisation. These findings highlight the importance of adequate disinfection before entering the bird house during production.

Other factors that increase the risk of *Campylobacter*-positive flocks are the type of management, with free-range and organic birds being more likely to be colonised (Heuer *et al*, 2001). Furthermore, the outcome of *Campylobacter* colonisation has been shown to be different in birds with different growth rates, with fast-growing breeds having more watery faeces and an increased incidence of hock marks and pododermatitis (Williams *et al*, 2013). This may be linked to the genotype of the different breeds used in the study. However, under both experimental infection, as used in the aforementioned study, or in natural field infection, fast and slow growing chickens harbour equally high numbers of *Campylobacter* and bird growth rate has not been identified as a risk factor for colonisation (Gormley *et al*, 2014). Interestingly, the use of fly screens in broiler houses was shown to reduce the percentage of *Campylobacter* positive flocks from 51.4% to 15.4% (Hald *et al*, 2007) implying a role for flies and vectors and this is another measure that could easily be implemented in addition to the above at a relatively low cost.



## 1.4 Measures to reduce *Campylobacter* colonisation in chickens

To date, no effective control measures for the reduction of *Campylobacter* in poultry have been widely implemented in spite of the UK government making its control a food safety and public health priority in recent years. A 2 log<sub>10</sub> reduction in chicken carcass contamination levels has been modelled to translate into a reduction in human cases due to fresh chicken of over 12-fold (Lindqvist and Lindblad, 2008). However, such a reduction in carcass contamination is likely to require a more substantial reduction in caecal colonisation levels given the way in which chickens are processed in the slaughterhouse. Furthermore, no control measures for reduction of carcass contamination that are highly effective, easy to implement, cost effective and widely acceptable to the public have yet been found, though topical application of organic acids, bacteriophages and blast chilling can achieve modest reductions of c. 1 log<sub>10</sub> CFU in skin contamination.

In addition to the limitations mentioned above for, topical carcass treatments would not address the issue of *Campylobacter* contamination of organs and deep muscular tissue. An approach that can reduce liver as well as carcass contamination would reduce the most important cause of food-borne *Campylobacter* outbreaks. In a systematic review, Newell *et al* (2011) identified 2621 original research articles on *Campylobacter* in poultry, with at least 173 references on control through bio-security measures alone. Due to the vast amount of literature available, rather than forming an exhaustive review, this section will outline with examples, the measures that have been tested to date for control of *Campylobacter* at various levels within the food chain.

### 1.4.1 On-farm control measures

Following the EU-wide ban of antibiotics in animal feed in January 2006, the number of laboratory-confirmed *Campylobacter* human infection has been on the increase (Fig. 1.4). Since then, on farm control measures for the reduction of *Campylobacter* have had to rely on alternative control strategies. Measures advocated by the FSA include biosecurity measures designed to protect the flock such as washing of hands, wearing protective clothing, dedicated footwear, cleaning and disinfection of equipment, cleaning and disinfection of the house prior to re-stocking, control of visitors, provision of *Campylobacter*-free water, feed and litter (FSA, 2010b). These measures are based on the Norwegian study of Lyngstad *et al* (2008) which showed that water sources, specific houses

on a single farm, hired stockmen, multiple broiler houses on farm and less than 9 days between destocking and repopulation act as risk factors for flock *Campylobacter* positivity.

At least in the UK, biosecurity measures are generally viewed positively by poultry farmers and there is general recognition that, apart from controlling *Campylobacter*, good biosecurity would safeguard against other diseases and protect their future (Ringrose and Hall, 2015). However, the same survey highlighted uncertainty as to which control measures are the most effective and should be implemented. Newell *et al* (2011) highlighted in a systematic review of the literature on biosecurity measures for the control of *Campylobacter* that there is a lack of understanding of the source of this infection in poultry flocks. Consequently, it is difficult to assess which control measures are most likely to result in flocks being *Campylobacter*-negative. The review also concluded that although the rigorous application of biosecurity measures is likely to help the control of *Campylobacter*, effective control leading to negative flocks will most likely require additional measures such as vaccination, probiotics, etc. Even so, biosecurity measures can be used to successfully keep flocks free of *Campylobacter* and lessons can be learnt from Iceland, where the proportion of *Campylobacter*-positive flocks declined from 40% to around 10% following the introduction of strict biosecurity measures and the policy of freezing produce from *Campylobacter*-positive flocks (Fridriksdottir, 2011) in 1999-2000.

An alternative approach for controlling *Campylobacter* in poultry at the farm level is the selective breeding of poultry with improved heritable resistance to *Campylobacter* colonisation. Boyd *et al* (2005) showed that chicks from two inbred layer lines show levels of *Campylobacter* colonisation that are 10 to 100 fold different and the difference is consistent from 24 hours post-challenge to the end of the study (3 weeks). The same study also showed, through backcrossing experiments of the two inbred lines, that these differences were inherited in a way consistent with genetic control through a single autosomal dominant locus and the effect was observed with different *C. jejuni* challenge strains. Fife *et al* (2010) further investigated the heritable resistance against *Campylobacter* and *Salmonella* in the in-bred lines used in the aforementioned study and fine-mapped the quantitative trait loci (QTLs) associated with resistance to *Salmonella*. Ongoing studies at The Roslin Institute will further map QTLs associated with resistance to *Campylobacter* in inbred lines and assess their penetrance in commercial populations of chickens. A survey of the genotype and *Campylobacter* burden of over 3000 broilers in a commercial context is also ongoing to estimate heritability of resistance and identify candidate resistance genes (Kaiser *et al*, unpublished).

Modified diets have also been considered for the control of *Campylobacter* on farm. The use of medium-chain fatty acids has been trialled but intestinal mucus appears to protect against their action even though they are effective *in vitro* (Hermans *et al*, 2010). Organic acids have also been tested in drinking water in experimental flocks but they only reduced colonisation by  $2\log_{10}$  CFU/g caecal contents if the birds were challenged with  $10^3$  CFU of *C. jejuni* (Chaveerah *et al*, 2004). No reductions were observed when birds were challenged with  $10^5$  CFU of *C. jejuni*. As mentioned above, Skanseng *et al* (2013) attempted to alter the intestinal microflora through diet and increase resistance of chickens to *Campylobacter* but their results suggested that the observed modest decrease was due to a direct effect of the feed rather than changes in the microbiota. Other feed additives are reviewed below alongside their potential use for carcass decontamination.

#### 1.4.2 Control at slaughter

Reductions in *Campylobacter* colonisation at farm level could be aided by measures applied at slaughter designed to further reduce carcass contamination. The WHO produced through *Codex Alimentarius*, a document that outlines potential control measures of *Campylobacter* at this stage (*Codex Alimentarius*, [http://www.who.int/foodsafety/micro/jemra/guidelines/cxg\\_078/en/index.html](http://www.who.int/foodsafety/micro/jemra/guidelines/cxg_078/en/index.html)). It details measures such as the use of carcass washing systems, chilling or irradiating carcasses, which have been shown to have variable effects on reducing *Campylobacter* contamination.

##### 1.4.2.1 Thermal control of *Campylobacter* on chicken carcasses

Chilling has also been researched as a means of reducing *Campylobacter* contamination and this may be a measure more widely acceptable to the consumer than others described below, provided that chilling does not induce damage to the surface of the carcass. Immersion chilling in water was shown to reduce *Campylobacter* numbers on chickens by  $2\log_{10}$  CFU/ml of carcass rinse (Northcutt *et al*, 2008). Previously, Northcutt *et al* (2003) achieved a more modest effect of  $1.2\log_{10}$  CFU/ml of carcass rinse after chilling in dilute sodium hypochlorite. More severe cold treatment such as freezing of naturally contaminated carcasses and storage for 31 days at  $-20\text{ }^{\circ}\text{C}$  reduced *Campylobacter* by 0.7 to  $2.9\log_{10}$  CFU/g (Georgsson *et al*, 2006). This is further supported by the work of Maziero and de Oliveira (2010) who found a decrease of  $2.5\log_{10}$  in frozen poultry by direct plating. However, no difference in recovery of *Campylobacter* from fresh, chilled or frozen products was noted following enrichment cultures, indicating potential long-term survival in food.

Historical evidence from Iceland suggests that freezing can be used as a strategy to reduce human campylobacteriosis. Following the re-introduction of fresh chicken meat from contaminated flocks in 1996, the number of cases of campylobacteriosis rose 7-fold in the country by 1999 (Tustin *et al*, 2011; Fridriksdottir, 2011). Recently, the British Oxygen Company (BOC, UK) have described the use of liquid nitrogen for rapid surface chilling to decrease carcass contamination with *Campylobacter* and have demonstrated a 10-fold reduction in industry scale trials, without affecting carcass quality or the ability to sell treated chickens as fresh produce (BOC, 2015). However, the installation of the system is likely to require substantial alterations to the processing lines and significant costs to the slaughterhouse and its efficacy may depend on the contaminating strain.

In addition to chilling, spraying of carcasses with high temperature water can be used. However, due to the thermophilic nature of *Campylobacter* this method is likely to risk of damage to the carcass as washes of higher temperatures than for other bacterial pathogens may need to be used. Li and Swem (2002) showed that an automated inside-outside washer using water at 55 °C only decreased *Campylobacter* contamination by 0.78log<sub>10</sub> and that temperatures higher than 60 °C initiated cooking of the carcass and changed its organoleptic properties.

#### 1.4.2.2. Chemical control of *Campylobacter* on chicken carcasses

A variety of chemical washes for the carcass have been tested, although their use may reduce public acceptance of the product. The use of 1 to 3 washers dispensing water with 25-35ppm total chlorine was shown to reduce *Campylobacter* counts by about 0.5 log<sub>10</sub> CFU/ml of whole carcass rinse sample (Bashor *et al*, 2004). Spraying of carcasses with Acidified Sodium Chlorite (ASC) after the aforementioned wash may further reduce *Campylobacter* levels by 1.3 log<sub>10</sub> CFU/ml of whole carcass rinse sample (Bashor *et al*, 2004). Whyte *et al* (2001) showed that dipping carcasses in 10% sodium triphosphate (TSP) solution, pH12 for 15 seconds followed by a 15s rinse in water reduced *Campylobacter* levels by 1.7 log<sub>10</sub> CFU/g of neck skin. In contrast to their use in broiler flocks, *in vitro*, organic acids can nearly eliminate *C. jejuni* from acidified water within one hour (Chaveerach *et al*, 2002) and as such may be more amenable to use as a topical disinfectant. Lactic acid has also been tested for surface decontamination of carcasses and has been demonstrated to reduce surface contamination by 1 log<sub>10</sub> when a 5% solution was sprayed for one minute and by nearly 4 log<sub>10</sub> after 7 days of storage at 4 °C when the solution was not rinsed off (Chaine *et al*, 2013).

#### 1.4.2.3 Control of *Campylobacter* on chicken carcasses through irradiation

Irradiation was advocated in the WHO guidelines but consumers appear reluctant to accept such treatment. Whilst public acceptance towards irradiation is increasing in the US, in the UK and most of the EU the public still does not widely accept food treated as such (reviewed by Wilcock *et al*, 2004). Yogasundram *et al* (1989) showed that a dose of 1KGy from a Co-60 source completely eliminates *C. jejuni* from chicken carcasses following inoculation at  $10^3$  CFU/cm<sup>2</sup>. Isohanni and Lyhs (2009) describe the use of UV light for the reduction of *Campylobacter* on poultry and obtain more modest reductions of approximately 0.7 log<sub>10</sub> on chicken meat and skin. Furthermore, they show no alteration in organoleptic properties of the carcass. While the latter approach may be more acceptable to the consumer, the modest decrease achieved means that it would have to be combined with other decontamination measures to elicit a significant reduction in carcass contamination.

#### 1.4.2.4. Use of natural antimicrobials for the control of *Campylobacter*

More recently, and in line with the public view on the benefits of control of diseases using natural products, bacteriophages, bacteriocins and other natural antibacterials have been tested. Connerton *et al* (2011) extensively reviewed bacteriophage therapy for control of *Campylobacter* in poultry and for sanitisation of carcasses post-slaughter. Although trials reviewed in the aforementioned paper have proven that bacteriophage therapy is effective in both controlling colonisation levels in chickens and on carcasses it is likely that they are more suited to the latter application. Extensive use of bacteriophages in live chickens can quickly give rise to phage-resistant *Campylobacter* strains during primary production and it would require careful timing before slaughter.  $\alpha$ -Endolysins specific to *Campylobacter* and obtained from *Campylobacter* bacteriophages may also be useful and would act specifically on the target rather than other constituents of the microflora. As an alternative to bacteriophages, Li (2009) reviewed and advocates the use of bacteriocins (natural or artificial anti-bacterial peptides), possibly administered through live commensal vectors, for the control of *Campylobacter*. Such an approach could have the added advantage of competitive exclusion through the use of probiotic bacteria such as *Lactobacilli* (Morishita *et al*, 2000).

#### 1.4.3 Control during retail

In addition to controlling *Campylobacter* contamination on the farm and at slaughter, its survival on the final product could be reduced during retail. Control at this level

can rely on tightly controlling storage temperature, as at the typical storage temperature of < 4 °C *Campylobacter* cannot grow. Additionally, modified atmosphere packaging (MAP) has been tested. Due to the microaerophilic nature of *Campylobacter*, it has been shown that MAPs that contain high levels of oxygen (80%) were most effective in inducing a decline in *Campylobacter* on the surface of chicken meat (Meredith *et al*, 2014). However, these conditions increased the multiplication of other spoilage microbes that are not sensitive to high oxygen concentrations. The same study reported that a MAP containing 40:30:30 CO<sub>2</sub>:O<sub>2</sub>:N<sub>2</sub> offered the best balance between decreased *Campylobacter* survival and decreased growth of spoilage microbes.

Another measure that has been tested during retail would be the application of certain marinades containing naturally occurring antimicrobials. For example, Zakariene *et al* (2015) show that a thyme-based marinade can decrease numbers of *C. jejuni* on fresh chicken wings by 1log<sub>10</sub> and Isohanni *et al* (2010) show that white and red wine used can induce a similar decrease on chicken meat if marinated for 48h. The use of marinades on fresh chicken meat could be combined with the use of ready-to-cook bags that allow whole chickens to be roasted without the need to remove the product from packaging before cooking.

#### 1.4.4. Control at consumer level

Lastly, a reduction in human campylobacteriosis can be achieved by consumer education. A survey published earlier this year in the USA (Kosa *et al*, 2015) indicated that consumer practices are generally unsafe and they increase the risk of campylobacteriosis. The study reported that 70% of consumers still wash poultry meat or carcasses before cooking, a practice now shown to be unsafe due to the likelihood of splashing contaminated water on surfaces surrounding the sink. In addition, only 17% of consumers were found to correctly store fresh poultry in the refrigerator and only 11% thaw frozen poultry correctly.

In the UK, the FSA has recently started a consumer education campaign as part of their Acting on *Campylobacter* Together (ACT) Campaign. It provides advice to consumers (FSA, 2015b), including not to wash raw chicken, to wash used utensils, to cover and chill raw chicken and to cook chicken thoroughly.

Whilst each of the control measures discussed in the above sections can decrease contamination of poultry with *Campylobacter* and the risk of contracting this bacterium, it is likely that efficient control of *Campylobacter* will require a combination of measures. Recognising this, the *Campylobacter* Joint Working Group has been formed in the UK, including members such as The British Poultry Council, DEFRA, FSA, NFU, The British

Retail Consortium and research organisations. Some of the priorities identified by this group (outlined at <http://www.campylobacter.org.uk>) include evaluation of biosecurity measures, financial incentives for *Campylobacter*-free produce, improved advice to consumers and testing of steam, electrolysed water and ultrasound treatments. The *Campylobacter* Joint Working Group recognises that an effective vaccine could be a valuable additional tool for control of this pathogen in broilers but it also recognises that this is a long-term measure as significant additional research is needed (FSA, 2010a).

## **1.5 Vaccines against *Campylobacter* in poultry**

In light of the studies above, it is evident that many measures for control of *Campylobacter* give modest results, are not widely acceptable to the public or are not easy to implement on the scale of modern broiler production (FSA, 2009b). As such, effective vaccines for control of *Campylobacter* in broilers are desirable as they would allow control of this problem at source. The “UK Research and Innovation Strategy for *Campylobacter* in the Food Chain” for 2010-2015 (FSA, 2010a) recognises the need for an effective vaccine in poultry. Moreover, it recognises a need to improve knowledge of the nature of the immune responses conferring protection against colonisation by *C. jejuni* in chickens. Research to date into vaccines for control of this pathogen in poultry is reviewed in this section. Tables 1.1. to 1.4 summarise the literature found in PubMed when searching for “*Campylobacter* (and) vaccines”. Owing to variation in almost every parameter between different studies direct comparison of the protective effect of candidate vaccines is not possible.

### **1.5.1 Whole cell live vaccines**

The use of whole cell live vaccines, attenuated through various strategies was one of the earliest forms of vaccination. The vaccine against smallpox was the first to be used and its safety and efficiency led to this disease becoming the first and only human disease to be eradicated world-wide, in 1980 (WHO; <http://www.who.int/csr/disease/smallpox/en/>). Live vaccines have also been used successfully in animals, including for the control of food-borne zoonoses. In late 1990's, non-typhoidal human salmonellosis acquired from poultry and eggs was the main food-borne zoonosis (DEFRA, 2015). The introduction of live-attenuated vaccines against *Salmonella* in layer birds, in conjunction with biosecurity measures, successfully brought under control this problem as salmonellosis decreased by over 60% between 1997 and 2000 (Cogan and Humphrey, 2003). Studies that focused on the use of live *C. jejuni* strains as vaccines are summarised in Table 1.1.

**Table 1.1 Summary of studies reporting the use of whole cell live vaccines against *Campylobacter***

Reference	Animal model	Campylobacter antigen used	Route of vaccination	Dose of vaccine	Adjuvants	Time of vaccination	Challenge dose and time	Humoral responses	Evidence of protection
Abimiku <i>et al</i> , 1989	Mouse; model of transfer of immunity to progeny	Whole cell heat-killed (65 or 100 °C) or formalin-fixed (0.5% for 2 days) compared to live vaccine	Oral or IP	0.2ml (both IP and oral) of either: inactivated cultures at 5, 10 or 50 International Opacity Units (I.O.U); 50 µg; live culture at 5 I.O.U.	None	Once weekly for 4 weeks; 10e9 CFU for the live vaccine	Progeny challenges at 4-6 days old with 10e7 CFU	IgG induced by all vaccinations, but lower in the live vaccine group; IgA and IgM were undetectable; Bactericidal activity of serum and milk did not correlate with protection.	IP route: 65 °C heat-killed vaccines as protective as formalin-fixed (~65% protection); 100 °C killed non-protective; live vaccine conferred 100% protection. Oral route: live vaccine only tested - ~25% protection. IP live vaccine protection against heterologous challenge highly variable (0-75%). Protection expressed as reduction in numbers colonised.
Guerry <i>et al</i> , 1994	Rabbit	<i>ΔrecA C. jejuni</i> 81-176 or WT	Oral	10e10 CFU	N/A	Age not specified; 1-2 kg	Challenged one month after vaccination via the removable intestinal tie adult rabbit diarrhoea (RITARD) procedure; 10e4 per animal	Not determined	Reduced duration of colonisation in the RITARD model from 7 days for non-vaccinated animals to 1.5 days for animals vaccinated with either <i>ΔrecA C. jejuni</i> 81-176 or WT.
Rollwagen <i>et al</i> , 1993	Mouse	Sonicated whole cell vaccine; compared to live vaccine	Oral	50, 100 or 300 µg of sonicated whole <i>C. coli</i> cells; compared to 10e9 CFU of live cells	Delivered with or without 25µg of heat-labile enterotoxin (HLE) of <i>E. coli</i>	Once weekly for three weeks	10e9 CFU/bird around 4 weeks post-vaccination	Detected IgA in intestinal lavage of all adjuvated groups. In groups receiving sonicated vaccines, the highest titre was in the highest dose (equal to the group receiving the live vaccine). Lower induction in nonadjuvated groups.	Only adjuvated vaccines tested. Sonicated vaccine decreased colonisation by 60% in homologous challenge. Complete protection against homologous challenge from 12 days post-infection by the live vaccine. No protection in heterologous challenge
Ziprin <i>et al</i> , 2002	Chicken	A combination of equal numbers of individual <i>ΔdnaJ</i> , <i>ΔcadF</i> , <i>ΔciaB</i> and <i>ΔpldA</i> of <i>C. jejuni</i> F38011	IM alone or with oral gavage	Each strain at 10e9 CFU/ml; 0.1ml IM and 1ml orally	With or without Ribi's Adjuvant R-700	5th day of hatch	11th day of hatch	Not undertaken	No decreases in caecal <i>Campylobacter</i> colonisation observed



While the use of live non-attenuated vaccines appears to provide protection against colonisation in murine models, there are unlikely to be usable in chickens as *Campylobacter* naturally persists long-term in the avian intestines. In contrast to studies undertaken in rabbits as a model of human disease (Guerry *et al*, 1994), vaccination of chickens with live *C. jejuni* strains with defined attenuating mutations (Ziprin *et al*, 2002) did not induce protection against colonisation. Secondly, the immune response to experimental *Campylobacter* infection of chickens is mainly against flagellin (Cawthraw *et al*, 1994) making such a response likely to allow immune evasion through variable flagellin glycosylation (as reviewed by Szymanski *et al*, 2003) and potentially limit the use of live vaccines against heterologous strains. Lastly, if considered for use in humans or transmitted through the food chain, such vaccines pose an increased risk of inducing the development of GBS and RA in vaccinated patients compared to subunit vaccines and also pose a risk of causing disease if transmitted to immunocompromised patients.

### 1.5.2 Whole cell inactivated vaccines

A second category of vaccines that have been tested, mostly in early studies, is whole cell inactivated vaccines. The literature available on the use of these vaccines against *Campylobacter* is summarised in Table 1.2.

In mice, the studies undertaken suggest that protection using whole cell inactivated or lysed vaccines is less than that conferred by live whole cell vaccines (Abimiku *et al*, 1989; Rollwagen *et al*, 1993). This could be due to the failure of inactivated bacteria to stimulate the immune response as efficiently as live bacteria, suggested by studies describing improved seroconversion and protection using adjuvated inactivated whole-cell vaccines, compared to the same vaccines used without adjuvants (Rollwagen *et al*, 1993; Baqar *et al*, 1995). Furthermore, as for live vaccines, there is a lack of protection conferred against heterologous challenge (Rollwagen *et al*, 1993; Dolby and Newell, 1986).

In chickens, only one study reported a reduction of  $2\log_{10}$  CFU of *C. jejuni* per g of caecal counts upon administration of inactivated *Campylobacter* (Widders *et al*, 1996), but this is unlikely to be sufficient for wide field use of these vaccines and the inadequate description of the study design makes confirmation of such findings difficult. Other studies reported no protection (Okamura *et al*, 2012) or reductions of  $2\log_{10}$  CFU/g of caecal contents, but which were not statistically significant (Rice *et al*, 1997), most likely due to the study being inadequately powered through the use of only three animals per group per time-point.

Interestingly, Hermans *et al* (2014) succeeded in passively immunising chickens by

**Table 1.2 Summary of studies reporting the use of whole-cell inactivated vaccines against *Campylobacter***

Reference	Animal model	Campylobacter antigen used	Route of vaccination	Dose of vaccine	Adjuvants	Time of vaccination	Challenge dose and time	Humoral responses	Evidence of protection
Abimiku <i>et al.</i> , 1989	See Table 1.1								
Baqar <i>et al.</i> , 1995	Mouse	1:1 mixture of heat (60 °C) and formalin (0.02M) killed <i>C. jejuni</i> 81-176	Oral	10e5, 10e7 or 10e9 CFU	With or without 25µg HLE	6 to 8 weeks of age; three doses at 48 hour intervals	4 weeks post-vaccination; 10e8 CFU	All groups apart from highest dose with no adjuvant produced intestinal secretory IgA with lowest titre in the highest dose group (with or without adjuvant); All groups seroconverted and had similar levels of serum IgA and IgG	All adjuvated doses induced protection (60%-100% reduction in number colonised at day 10 after challenge). Only the highest dose non-adjuvated vaccine induced protection.
Dolby and Newell, 1986	Mouse; vaccination of dams and evaluation of transfer of immunity to pups	Whole cell heat-killed (65 or 100 °C) or outer membrane preparation	IP	0.2ml of cultures at 10 or 50 I.O.U.; 50 µg of outer membrane preparation	None	Once weekly for 4 weeks	Progeny challenged at 4-6 days after birth	Detected IgY against flagellar antigens	The 65 °C-killed whole cell vaccine protected progeny against homologous challenge; no protection by the 100 °C-killed vaccine or against heterologous challenge; Higher protection with higher dose vaccine; Outer membrane protein vaccine was non-protective.
Hermans <i>et al.</i> , 2014	Chicken; passive immunisation study using a seeder challenge model	Whole-cell lysate (WCL) compared to hydrophobic protein fraction	Layers: IM	Layers: 50 µg of whole cell-lysate or 25 µg of hydrophobic fraction; Progeny: fed 5% egg yolk (from vaccinated layers) in feed	Layers: 1:1 mixture with Freund's Complete Adjuvant; Progeny: N/A	Layers: First vaccine at 19 weeks; boosters at 21, 23 and 25 weeks; Progeny: feeding started at 6 days of age	Layers: N/A; Progeny: seeder birds challenged with 10e4 at 10 days of age, all birds PMed at 13 days of age	Layers: <i>C. jejuni</i> specific IgY detected in egg yolk but not in white; IgA and IgM undetectable	Layers: N/A; Progeny: WCL decreased colonisation of seeder birds by 5 log <sub>10</sub> and abolished transmission to sentinels; Hydrophobic fraction decrease overall colonisation by 5 log <sub>10</sub> but did not abolish transmission to sentinel birds
Okamura <i>et al.</i> , 2012	Japanese Jidori chickens	Whole cell inactivated (formalin-killed)	SC	Approx. 10e8 CFU/bird	Oil or aluminium hydroxide gels	Day of hatch and 37 days old	10e7 CFU	IgY induced	No reduction in caecal colonisation or spleen/liver count

Table 1.2 Continued

Reference	Animal model	Campylobacter antigen used	Route of vaccination	Dose of vaccine	Adjuvants	Time of vaccination	Challenge dose and time	Serology	Evidence of protection
Rice <i>et al</i> , 1997	Broiler chickens	Whole cell inactivated (formalin-killed)	Oral	10e9 CFU/bird	None	days 2 and 8	Challenged at day 2 with seeder birds that received 10e4 CFU/bird	IgA detected	No reduction at 17 days of age
					25 µg of HLT per dose per bird	Days 2, 9 and 16			2 log <sub>10</sub> CFU/g reduction at 46 days but only three birds used so not statistically significant
					50 µg HLT per dose per bird	Days 2, 4 and 6			0.5 log <sub>10</sub> CFU/g reduction at 31 days but not significant and no reduction at 50 days
Rollwagen <i>et al</i> , 1993	See Table 1.1.								
Widders <i>et al</i> , 1996	Chicken	Killed whole cells (KWC); keyhole limpet haemocyanin (KLH) used as a model antigen	SC, IP or Oral	Unknown	Unknown	Vaccinated with KLH on day of hatch or at 24 days old; Vaccinated with KWC <i>C. jejuni</i> at days 16 and 26 post-hatch	Unknown	Only 5/13 birds responded to KLH when vaccinated on day of hatch; 10/11 responses if vaccinated at 24-days old. No IgA or IgM induction by KWC vaccine. Induction of serum IgY observed with KWC vaccine and intestinal secreted IgY correlated with protection.	Reduction of 2log <sub>10</sub> CFU/g observed in the KWC vaccinated group.

feeding young chicks yolk from eggs laid by hens vaccinated with a whole cell lysate of *C. jejuni*. In a seeder challenge model, this study showed decreases of  $5\log_{10}$  CFU of *C. jejuni* per g of caecal contents in the birds that were directly challenged and abolition of transmission to sentinel birds. However, the model used a low challenge dose of  $10^4$  CFU/bird and this may have contributed to the high level of protection observed. Nevertheless, this model of challenge may mimic natural infection more closely. This study and the study of Dolby and Newell (1986) showing protection of mouse pups following vaccination of dams suggests that vaccines against *Campylobacter* in poultry could be used through immunisation of laying hens and passive transfer to the chicks, similarly to the use of vaccines against *Salmonella*, however, the possibility of this method of immunisation is yet to have been demonstrated experimentally in chickens. Studies using recently described Ig heavy chain deficient transgenic chickens (Schusser *et al*, 2014) will help to formally establish the relevance of antibody in protection.

### 1.5.3. Subunit vaccines

A third category of vaccines that have been tested against *Campylobacter* are recombinant subunit vaccines and the studies describing the use of such vaccines are summarised in Table 1.3.

As the flagellum was shown early in *Campylobacter* research to be vital for intestinal colonisation, early studies focused on using this subunit as a vaccine. While protection was observed using this subunit in both chicken (Khoury and Meinersmann, 2011) and mouse models (Lee *et al*, 1999), it was observed in a dose-dependent manner and in chickens it only achieved protection of the majority of vaccinated animals when doses as high as 1mg of recombinant antigen per animal were used. A recombinant vaccine reliant on high amounts of purified protein would be expensive to administer on a wide scale to the broiler industry and would, most likely, preclude its field use. Other studies that showed high level protection utilising other recombinant antigens have the same drawback: Neal-McKinney *et al* (2014) used 250  $\mu\text{g}$  of recombinant FlpA to induce a 3.5  $\log_{10}$  reduction in CFU/g of caecal contents in chickens, Du *et al* (2008) used 200  $\mu\text{g}$  of 6xHis-Peb1A to induce near complete clearance in mice and Islam *et al* (2010) had to use 300  $\mu\text{g}$  of GST-PorA to induce clearance of *C. jejuni* infection in mice. Other studies using lower amounts of recombinant antigens reported lower levels of protection, e.g. Buckley *et al* (2010) induced a reduction of 2  $\log_{10}$  CFU *C. jejuni*/g caecal contents following vaccination with 14  $\mu\text{g}$  of 6xHis-CjaA, however, the lack of dose titration experiments in each of these studies and their highly variable design does not allow the contribution of specific factors to be assessed.

**Table 1.3 Summary of studies using recombinant antigens as vaccines against *Campylobacter***

Reference	Animal model	Campylobacter antigen used	Route of vaccination	Dose of vaccine	Adjuvants	Time of vaccination	Challenge dose and time	Antigen-specific humoral responses	Evidence of protection
Annamalai <i>et al</i> (2013)	Chicken	Outer membrane proteins	Oral or SC	25, 125 or 250 µg for oral vaccines, 125 µg only for s.c.	With or without poly-lactide-co-glycolide encapsulated nanoparticles	Days 7 and 21	Challenged at 35 days with 10e8 CFU/bird	Detected induction of serum and caecal IgY and IgA in the SC vaccinated group	Protective effect observed only with SC vaccine to below the detectable limit by plating
Baqar <i>et al</i> , 2008	Mice	FlaC, FspA1 and FspA2; all as 6xHis fusions; <i>flaC</i> and <i>fspA1</i> cloned from <i>C. jejuni</i> 81-176; <i>fspA2</i> cloned from <i>C. jejuni</i> GG8486.	Intranasal	5, 25 or 100 µg	Each dose delivered with or without 1 µg of LTR192G	Three vaccinations, two weeks apart	Intra-nasally with 3x10e9 CFU, 28 days after last vaccine dose; with either the <i>C. jejuni</i> GG8486 homologous strain or <i>C. jejuni</i> 81-176 heterologous strain; only the 100 µg groups were challenged	No serum IgG or IgA induced by FlaC alone at 5 and 25 µg but induced at 100µg and in all groups with adjuvant. No faecal IgA with any non-adjuvated dose; increasing response with 25 and 100 µg adjuvated doses. FspA1 induced IgG and IgA in all vaccinated groups; dose-response effect if non-adjuvated. FspA2 did not induce IgG and IgA with 5 and 25 µg non-adjuvated doses; it induced IgG and IgA responses in a dose-dependent manner in all doses when adjuvated.	Protection was only tested in the highest dose vaccine groups. Efficacy given as reduction in clinical score. FlaC did not provide protection against either challenge. FspA2 had lower protective efficacy (38%) than FspA1 (64%) in homologous challenge. FspA1 had 31% protective efficacy against heterologous challenge. No protection against heterologous challenge observed with FspA2.
Buckley <i>et al</i> 2010	Chicken	6His-CjaA	SC injection	14 µg/bird	TiterMax	1 and 14 days or 15 and 29 days	10e7 CFU at day 28 post-hatch	IgY and bile IgA induced	1log <sub>10</sub> CFU/g reduction at week 3 and about 2log <sub>10</sub> at week 4 post-challenge (across 2 biological replicates, each with 3 birds per interval); no difference between the two vaccination times
Du <i>et al</i> , 2008	Mouse	6XHis-Peb1A	IM or SC	25, 50 or 100 µg mentioned in M&Ms	Mentioned as CFA/IFA but not which or if both and no dose mentioned	Mice were 7-9 weeks of age; vaccinated 4 times at 1 weeks intervals	Oral challenge with <i>C. jejuni</i> 81-176; time of challenge not mentioned	IgG induced in groups vaccinated with 50, 100 and 200 µg, both IM and SC;	30% survival at 7 days in control group, 50% in the 50µg group and 85% in the 100 and 200 µg groups; no reduction in clinical score in the 50µg group, 70% reduction in clinical score in the other groups

Table 1.3 Continued

Reference	Animal model	Campylobacter antigen used	Route of vaccination	Dose of vaccine	Adjuvants	Time of vaccination	Challenge dose and time	Antigen-specific humoral responses	Evidence of protection
Islam <i>et al</i> , 2010	Mouse	GST-PorA	Oral	300 µg per bird in 300 µl volume	5 µg of HLT	Age of first vaccination not mentioned; vaccinated twice 1 week apart	Challenged 3 weeks after second vaccination. Given 10e9 CFU	The vaccines induced significant antibody increases (given as an average of serum and intestinal IgA, IgM and IgY)	Homologous strain did not colonise mice. Variable levels of protection against three heterologous strains (reduction by 50-75% in numbers colonised)
Khoury and Meinersmann (2011)	Chicken	Flagellin	Given orally or IM	250, 500, 750 or 1000 µg per bird for oral vaccine and 250 or 1000 for IM	Fused to HLT, given in 4M urea	2 and 4 weeks of age	Challenged at 3 weeks with 2e8 CFU/bird	Both regimens induced secretory intestinal IgA; 1mg oral dose significantly more	Three challenge trials run with the 1000µg dose - in each trial vaccination reduced colonisation to approximately half but only half of the birds were colonised in the control groups
Lee <i>et al</i> , 1999	Mouse	MBP-FlaA from <i>C. jejuni</i> 81-176	Intranasal or oral	3, 6, 12, 25, 50 µg	Each dose delivered with or without 5 µg of HLT	6-8 weeks old; first dose at 7 days post-housing, second dose 7 days later	Homologous challenge. Intranasally with 2x10e9 CFU, 26 days after second vaccination. Also orally challenged with 10e10, 10e9 and 10e8 CFU.	Intranasal challenge: Serum IgG and intestinal lavage IgA were induced in a dose-dependent manner. Adjuvated vaccines induced significantly higher titers than non-adjuvated vaccines for each dose. Oral challenge: not determined	Dose-dependent protection induced, with approximately 80% efficacy in protection against clinical symptoms and reduction in number of animals colonised for the highest dose group, when delivered with the adjuvant. Only half the efficacy when delivered without adjuvant. Oral challenge: protection with all doses at 7 days but complete protection only with the 10e8 dose.
Neal-McKinney <i>et al</i> , 2014.	Chicken	A combination of GST-tagged 90mers of FlpA, CadF and FlaA (1 <sup>st</sup> vaccination) and 6xHis-tagged entire length antigens (2 <sup>nd</sup> vaccination), individually or a combination of the three. Cloned from <i>C. jejuni</i> F38011	IM, in the pectorals	240 µg of GST-tagged 90mer for first immunisation. 240 µg of full length protein for second immunisation 80 µg of each protein for the trivalent vaccine	Montanide ISA 70VG (30% antigen, 70% adjuvant)	First vaccination at 6 days of age. Booster at 16 days of age.	Challenged at 20 days of age with 2x10e8 CFU/ bird with homologous strain.	All vaccines induced serum IgY. No differences noted between groups. For the FlpA-vaccinated group, no correlation between serum IgY induction and caecal <i>Campylobacter</i> counts was observed in individual birds.	Reported as reductions in median caecal counts with no statistical tests: 3 log <sub>10</sub> CFU/g reduction with the FlpA and trivalent vaccines. If mean is calculated and t-test used pairwise with Bonferonni Correction: approx. 3.6 log <sub>10</sub> CFU/g reduction with the trivalent and 2.5 log <sub>10</sub> reduction with FlpA; CadF reduction not significant; trivalent vaccine not a statistical improvement over FlpA alone.

Table 1.3 Continued

Reference	Animal model	Campylobacter antigen used	Route of vaccination	Dose of vaccine	Adjuvants	Time of vaccination	Challenge dose and time	Antigen-specific humoral responses	Evidence of protection
Paul <i>et al</i> , 2014	Chicken	6xHis-tagged CadF, FlpA, PorA, FlaA, CmeC, Peb1A, JlpA. Individually or all together	IM for layers Oral for <i>C. jejuni</i> -challenged chicks	Layers: 100 µg of each protein <i>C. jejuni</i> -challenged chicks: feeding 10% (w/w) egg yolk powder from above layers	Layers: 0.5ml Freund's Complete Adjuvant (FCA) Chicks: N/A	Layers: 35 weeks of age <i>C. jejuni</i> -challenged chicks: feeding from 2 days of age	Layers: None Chicks: challenged at 2 days of age with 10e8 CFU, with concomitant feeding of hyperimmune egg yolk powder	IgY induction measured in egg yolk.	No decreases were observed in groups treated with hyperimmune yolk from hens vaccinated with individual or the combination of antigens.
Prokhorova <i>et al</i> , 2006	Mouse	8 surface-expressed proteins identified through MS; 6xHis-tagged, cloned from <i>C. jejuni</i> ML53	SC	1, 5, 10 and 25 µg/mouse of each protein	100 µg Alum	Age of mice not mentioned; Vaccinated of days 0, 14 and 28 of experiment	2.5-5x10e8 CFU of <i>C. jejuni</i> ML1 or ML53	Serum antibody titres (isotype not mentioned) determined by Western Blot; not seen in the 1 µg group.	Only mice with high antibody titres (not defined) from each dose were challenged. Claimed that two proteins decreased faecal shedding but animals vaccinated with different doses were included in the same group and a t-test was inappropriately used to test multiple treatment groups and time-points.
Zeng <i>et al</i> , 2010	Chicken	6xHis-CmeC	Oral gavage	50 and 200 µg	Modified heat labile <i>E. coli</i> enterotoxin (10 or 70 µg); groups with and groups without	7 and 21 days	10e6 or 10e5 at day 35 post-hatch	IgY and IgA induced only by high dose CmeC plus high dose mL T	No protection seen for up to 12 days post-infection

In passive immunisation studies using CadF, FlpA, PorA, FlaA, CmeC, Peb1A and JlpA as recombinant subunit antigens singly or in combination, feeding of 10% (w/w) egg yolk powder from hyperimmune layers to broilers did not induce protection against homologous challenge when fed either prophylactically or as treatment (Paul *et al*, 2014). These findings are in contrast to those of Hermans *et al* (2014) using inactivated whole cell vaccines to immunise laying hens. Antibodies against a limited spectrum of antigens may not be able to neutralise bacteria as efficiently as antibodies against whole cells, however the differences in study design could also account for the discrepancy between the studies. For example, Paul *et al* (2014) used a direct challenge model, giving  $10^8$  CFU of the challenge strain to each bird whereas Hermans *et al* (2014) used a low dose seeder challenge model.

In view of above, it is likely that an effective vaccine would have to be vectored to have economic benefits on a wide scale in the field. Vectoring in an attenuated heterologous bacterial strain that does not cause pathology in chickens or humans would have the added advantage of potential ease of administration via the oral route. Furthermore, an oral vaccine would also avoid potential reactions at the site of injection due to adjuvants, which could increase the number of carcasses condemned as unfit for human consumption. Depending on the choice of vector it may also simultaneously protect against other avian or zoonotic pathogens.

#### 1.5.4. Subunit vectored vaccines

Recognising the potential benefits of a vectored subunit vaccine over a purified recombinant antigen, studies have also focused on vectored vaccines against *Campylobacter* and such studies are described in Table 1.4. The majority of this research has used attenuated *S. Typhimurium* vectors, with the exception of one study describing vectoring of CjaA in *Eimeria tenella* and reduction of caecal colonisation in chicken by approximately 10-fold (Clark *et al*, 2012) and of a study that described a similar reduction in chickens by vectoring FlaA in a naked DNA vector that induces expression and antigen presentation in the host (Huang *et al*, 2010).

Despite the potential benefits of low production costs and ease of administration the majority of the vectored vaccines tested so far have only elicited reductions of  $2\log_{10}$  CFU in caecal *Campylobacter* counts in chickens and the only study reporting the use of such vaccines in mice failed to induce protection (Sizemore *et al*, 2006). Vectoring *Campylobacter* antigens in live bacterial vectors has the potential advantage of inducing an immune response more likely to mimic natural infection compared to the parenteral administration of recombinant antigens alone. However, the disadvantage may be that



**Table 1.4 Summary of studies describing vectored subunit vaccines against *Campylobacter***

Reference	Animal model	Campylobacter antigen used	Vector	Localisation within vector	Route of vaccination	Dose of vaccine	Adjuvants	Time of vaccination	Challenge dose and time	Ag-specific humoral responses	Evidence of protection
Buckley <i>et al</i> , 2010	Chicken	CjaA	Live attenuated <i>Salmonella</i> - <i>aroA</i> mutant	Cytoplasmic	Oral	1e7 CFU/bird	TiterMax	1 and 14 days	10e7 CFU at day 28 post-hatch	IgY and bile IgA induced	1.4 log <sub>10</sub> CFU/g reduction at weeks 3 and 4 post-challenge (across 6 biological replicates - 18 birds in total per time interval)
			Live attenuated <i>Salmonella</i> - <i>aroA</i> mutant	Cytoplasmic – expression from low copy plasmid				Given at 1, 4 or 8 days post-hatch as primary vaccination and two weeks later as secondary			Decreases induced at week 3 post hatch by the 1day group (only statistical significance) and at week 4 by the 1 and 4 day group - the latter two likely not significant due to insufficient bird numbers
			WT and live attenuated <i>Salmonella</i> mutants - <i>aroA</i> , <i>fliM</i> , <i>spaS</i> , <i>ssaU</i>	Cytoplasmic - expression from low copy plasmid				Given at 1 and 14 days post-hatch			1.5 log <sub>10</sub> CFU/g reduction with $\Delta$ <i>aroA</i> and a further 1.5log <sub>10</sub> reduction with WT, $\Delta$ <i>spaS</i> and $\Delta$ <i>ssaU</i> ; $\Delta$ <i>ssaU</i> ; more invasive WT strain induced better protection
		CjaA, Peb1A, GlnH, ChuA	Live attenuated <i>Salmonella</i> - <i>aroA</i> mutant	Cytoplasmic - expression from low copy plasmid				Given at 1 and 14 days post-hatch			Only CjaA and Peb1A induced 1.8 and 1.5 log <sub>10</sub> CFU/g reductions
Clark <i>et al</i> , 2012	Chicken	CjaA	<i>Eimeria tenella</i>	Intracellular	Oral	300 sporozoites once or successive infections of 100, 500, 3000 or 5000	None	days 1, 3, 7 and 20	10e5 CFU/bird	Serum IgY induction shown by Western blotting	Both vaccination regimens induced 0.8log <sub>10</sub> CFU/g reduction

Table 1.4 Continued

Reference	Animal model	Campylobacter antigen used	Vector	Localisation within vector	Route of vaccination	Dose of vaccine	Adjuvants	Time of vaccination	Challenge dose and time	Ag-specific humoral responses	Evidence of protection
Huang <i>et al</i> , 2010	Chicken	FlaA	pCAGGS conjugated to chitosan as DNA vaccine	Transgenic expression in nasopharynx and upper trachea	Intranasal	75 µg in each nostril	None	Days 1, 15 and 29	day 42 with 5e7	IgY and IgA induced	2log <sub>10</sub> CFU/g reduction in caecal counts
Laniewski <i>et al</i> , 2012	Chicken	CjaD	Live attenuated <i>Salmonella</i> ; balanced lethal system using pYA3341 Asd+ plasmid	cytoplasm	Oral	10e8 CFU/bird	None	1 and 21 days	10e6 at day 28 post-hatch with heterologous strain collected off chicken carcasses	IgY and IgA induced	0.8 log <sub>10</sub> CFU/g statistically significant at week 1; 0.5 log <sub>10</sub> CFU/g but not significant at week 2; used 5 birds per time point per group
Laniewski <i>et al</i> , 2014a	Chicken	CjaA	<i>Salmonella</i> Typhimurium	Inner membrane/ periplasm (demonstrated by cellular fractionation of <i>Salmonella</i> vector)	Oral	10e8 CFU/bird	None	Days 1 and 14	Challenged at 28 days with 10e5 CFU/bird	Detected serum IgY and caecal IgA. FACS analysis - induction of B cell responses but lack of T cell responses	No protection observed
Laniewski <i>et al</i> , 2014b	Chicken	CjaD	<i>Salmonella</i> Typhimurium	Inner membrane/ periplasm(demonstrated by cellular fractionation of <i>Salmonella</i> vector)	Oral	10e8 CFU/bird	None	Days 1 and 14	Challenged at 28 days with 10e6 CFU/bird	IgA induced	Reduced colonisation by 1log <sub>10</sub> CFU/g week 1 after challenge (significant) and 0.5 log <sub>10</sub> CFU/g two weeks after challenged (non-significant)
Layton <i>et al</i> , 2011	Chicken	CjaD, CjaA, ACE393	Live <i>Salmonella</i> , attenuated	Outer membrane, within Lamb	Oral	10e8 CFU/bird	CD154	On day of hatch	day 21, with 1e7 CFU	IgY and IgA induced	Day 32: all three decreased caecal <i>Campylobacter</i> counts; CjaD reduced by 6log <sub>10</sub> CFU/g (below limit detectable by qPCR)

Table 1.4 Continued

Reference	Animal model	Campylobacter antigen used	Vector	Localisation within vector	Route of vaccination	Dose of vaccine	Adjuvants	Time of vaccination	Challenge dose and time	Ag-specific humoral responses	Evidence of protection
Sizemore <i>et al</i> , 2006	Mouse	Peb1A fom <i>C. jejuni</i> MGN4735	<i>Salmonella</i> Typhimurium; expressed from pYA3342 (a pBR-based Asd+ plasmid)	Cytoplasmic; expressed at high level	Oral	1-2.5 10e8 CFU/mouse	None	Days 1 and 12 of experiments; female BALB/c mice, 7-9 weeks old	Challenged with <i>C. jejuni</i> 81-176;	IgG induced against vector and Peb1A in 100% of animals; no IgA detected against the vector or Peb1A	No protection observed - no reduction in bacterial loads or intensity of clinical symptoms
	Mouse	Peb1A fom <i>C. jejuni</i> MGN4735	<i>Salmonella</i> Typhimurium; expressed from an arabinose regulated runaway plasmid, Asd+-based	Cytolasmic; expressed at lower level than above	Oral	1-2.5 10e8 CFU/mouse	None	Days 1 and 12 of experiments; female BALB/c mice, 7-9 weeks old	Challenged with <i>C. jejuni</i> 81-176;	IgG induced against vector and Peb1A in 90% of animals; no IgA detected against the vector or Peb1A	No protection observed - no reduction in bacterial loads of intensity of clinical symptoms
	Mouse	Peb1A fom <i>C. jejuni</i> MGN4735	<i>Salmonella</i> Typhimurium; expressed from as an Hly fusion in an Asd+ - based plasmid, pUC ori	Extracellular secretion; low level secretion	Oral	1-2.5 10e8 CFU/mouse	None	Days 1 and 12 of experiments; female BALB/c mice, 7-9 weeks old	Challenged with <i>C. jejuni</i> 81-176;	IgG induced against vector in all animals and against Peb1A in 20% of animals; no IgA detected against the vector or Peb1A	No protection observed - no reduction in bacterial loads of intensity of clinical symptoms
Wyszynska <i>et al</i> , 2004	Chicken	CjaA	Live attenuated <i>Salmonella</i>	Cytoplasmic – expressed from high copy number plasmid	Oral	10e8 CFU/bird	None	1 day and 14 days post-hatch	2x10e8 CFU at day 28 in PBS/gelatine - heterologous challenge with broiler carcass isolated strain	IgY and IgA induced	About 6log <sub>10</sub> CFU/g reduction but other authors have been unable to replicate the results, albeit using vectors with different properties

antigens and epitopes from the vector may become immunodominant over the *Campylobacter* antigen(s) used and reduce the immune response to it. One study that reported a 6log<sub>10</sub> reduction in CFU of *C. jejuni* per g of caecal contents in chickens (Layton *et al*, 2011) presented the antigen (CjaD) on the surface of the bacterium, in tandem with the immunostimulatory molecule CD154 (CD40 ligand). This may have resulted in an enhanced binding of the antigen-CD154 doublet to antigen-presenting cells and enhanced activation of other immune cells (macrophages and/or T-cell independent B cell activation), however, the effects of using this immunostimulatory molecule in the vaccine were not compared to a vaccine that vectored CjaD alone. Another study reported a 6 log<sub>10</sub> CFU/g reduction (Wyszynska *et al*, 2005) using vectored CjaA, however the results were not replicated by other independent studies. Buckley *et al* (2010) obtained a 1.4 log<sub>10</sub> reduction in CFU/g of caecal contents using the same vectored antigen and Laniewski *et al* (2014a) did not observe protection using CjaA, albeit the properties of the *Salmonella* vectors were different in these two later studies.

Further knowledge of the nature of the immune response induced by protective vectored vaccines or mounted by chickens that are naturally resistant to colonisation would allow the rational development of vaccines that induce the appropriate type(s) of immune response more strongly.

### 1.5.5 Other strategies for immunisation of chickens

In addition to the studies described above, glycoconjugate vaccines have been tested in models of human campylobacteriosis. Monteiro *et al* (2009) reported significant reduction in clinical scores in mice following vaccination with a capsular polysaccharide conjugated to a mutant diphtheria toxin. No studies using glycoconjugate vaccines in chickens have been reported to date. However, the characterisation of the PglB enzyme from *C. jejuni* and its ability to glycosylate carrier proteins in recombinant *E. coli* strains (Cuccui and Wren, 2013) opens the door to cost-effective production of glycoconjugate vaccines for use in chickens.

Another reported strategy was similar to the studies investigating the passive transfer of immunity through feeding of anti-*Campylobacter* antibodies to chickens, however, in this case, the antibodies were generated in a llama (Riazi *et al*, 2013). Following inoculation of the llama with a *C. jejuni* flagella, two flagella-specific single-domain antibodies were selected and pentamerised. When 1mg of the pentamer was fed daily to two-day-old chicks challenged with 10<sup>8</sup> CFU *C. jejuni*, the caecal colonisation levels were decreased by 2.5 log<sub>10</sub> CFU *C. jejuni*/g. The study showed that the pentamer was able to bind flagella, agglutinate bacteria and inhibit motility in soft agar, providing a likely explanation for the observed

decrease in colonisation. Even though potentially useful, such antibodies are likely to be expensive to produce on a suitable scale for field use.

## **1.6 Aims of this study**

This study aimed to refine a previously described live-attenuated *Salmonella*-vectored vaccine against *Campylobacter* in poultry, based on the *Campylobacter* antigen CjaA (Buckley *et al*, 2010), through vectoring this antigen in PoulVac® *E. coli*, an attenuated avian pathogenic *E. coli* vaccine. The effect of codon-optimisation of protein expression from the PoulVac *E. coli* was also assessed.

In addition, this study also aimed to evaluate novel *Campylobacter* antigens as recombinant subunit vaccine candidates in chickens. This required the rational selection, cloning, purification and testing of candidate antigens in chicken vaccination and challenge models. Where protection was observed, we also sought to define the nature, magnitude and timing of avian immune responses and to associate them with protection. The capacity of induced antibodies to recognise their *Campylobacter* targets was also investigated.

## 2. Materials and Methods

### 2.1 Bacterial strains and plasmids

The bacterial strains used in this study are detailed below:

- *E. coli* XL1 Blue (Stratagene, USA) was used for molecular cloning and plasmid propagation and maintenance. Genotype:  $\Delta recA1 \Delta endA1 \Delta gyrA96 \Delta thi-1 \Delta hsdR17 \Delta supE44 \Delta relA1 \Delta lac$  [F' *proAB lacI<sup>q</sup>ZΔM15 Tn10* (Tet<sup>r</sup>)].
- *E. coli* Rosetta (DE3) pLysS (Novagen, Germany) was used for protein expression as it contains additional tRNA genes that overcome rare codons in *E. coli* and allow universal expression. Genotype: F<sup>-</sup> *ompT hsdS<sub>B</sub>(r<sub>B</sub><sup>-</sup> m<sub>B</sub><sup>-</sup>) gal dcm* (DE3) pLysSRARE (Cam<sup>R</sup>).
- The *C. jejuni* M1 strain (Grant *et al.*, 2005) was used as a source of DNA for cloning of *Campylobacter* antigens and as a model strain in the challenge and vaccination studies conducted in chickens. This was chosen in order to permit comparison with previous studies published in our group (Buckley *et al.*, 2010).
- The *C. jejuni* 11168H strain (Karlyshev *et al.*, 2001) was used as a model organism for the study of *cj0424* and as a heterologous challenge for *Campylobacter* antigens cloned from the M1 strain. This strain was chosen for testing of the role of *cj0424* in colonisation as it is the only commonly used laboratory strain that contains this gene.
- PoulVac EC<sup>®</sup>, an O78 strain of APEC that carries a  $\Delta aroA$  mutation (La Ragione *et al.*, 2013), originating from an intact vaccine vial received from Zoetis, was used for construction of live-attenuated vectored vaccines.
- ST4/74 strain of *S. Typhimurium* that carries the  $\Delta aroA$  attenuating mutation was also used for the construction of live-attenuated vectored vaccines and has been described previously (Buckley *et al.*, 2010).

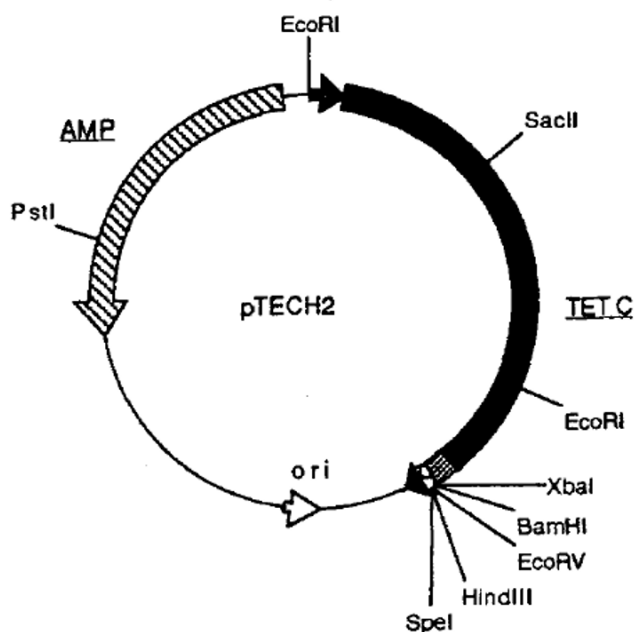
The plasmids used for cloning in this study were:

- pTECH2 (Khan *et al.*, 1994) for the expression of *Campylobacter* antigens in live-attenuated *Salmonella* or APEC vaccines (Figure 2.1). Genotype: E1 origin of replication giving 15-20 copies per cell; under the control of a *nirB* promoter activated by growth under anaerobic conditions; ampicillin resistance gene for selection; multiple cloning site (MCS) at 3' end of *tetC*.
- pGEX-4T1 (GE Lifesciences, UK) for the cloning and expression of *Campylobacter* antigens as fusions to the C-terminus of GST; these were utilised as recombinant purified anti-*Campylobacter* vaccines. Genotype: origin of replication from pBR322

giving approximately 50 copies/cell; *tac* promoter for chemical induction; contains a *lacI<sup>q</sup>* gene to control basal level of expression; ampicillin resistance gene for selection; MCS at 3' end of *gst*.

- pMal-p2X (New England Biolabs [NEB], UK) for the cloning and expression of *Campylobacter* antigens as fusions to the C-terminus of MBP; these were utilised as recombinant purified proteins in ELISAs measuring humoral responses following vaccination. Genotype: origin of replication from pBR322 giving approximately 50 copies/cell; also contains an M13 origin of replication; *tac* promoter for chemical induction; contains a *lacI<sup>q</sup>* gene to control basal level of expression; ampicillin resistance gene for selection; multiple cloning site (MCS) at 3' end of *malE*.

The plasmids constructed in this study are given in Table 2.1. Bacterial strains produced in this study included *E. coli* XL1 Blue and *E. coli* Rosetta strains containing each of the pGEX-4T1 plasmid constructs given in Table 2.1 and *E. coli* XL1 strains containing each of the pMal-p2X plasmid constructs given in Table 2.1. Further strains constructed included PoulVac<sup>®</sup> *E. coli* containing the empty pTECH2 plasmid and both of the pTECH2-*cjaA* constructs given in Table 2.1 below and *S. Typhimurium* ST4/74 strains containing the empty pTECH2 and the pTECH2-*cjaA* constructs.



**Figure 2.1 Map of the pTECH2 plasmid.** Genes encoding *Campylobacter* antigens were cloned 3' of *tetC* as in-frame fusions in the MCS using the *BamHI* and *HindIII* enzymes.

**Table 2.1. Plasmids constructed in this study.**

<b>Plasmid</b>	<b>Description</b>
pTECH2- <i>cjaA</i>	<i>C. jejuni</i> M1 <i>cjaA</i> fused 3' to <i>tetC</i> in pTECH2
pTECH2- <i>cjaA</i> <sub>co.</sub>	<i>C. jejuni</i> M1 <i>cjaA</i> codon optimised for expression in <i>E. coli</i> fused 3' to <i>tetC</i> in pTECH2
pGEX-4T1- <i>cjaA</i>	<i>C. jejuni</i> M1 <i>cjaA</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>tetC</i>	<i>tetC</i> gene from pTECH2 fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>fliD</i>	<i>C. jejuni</i> M1 <i>fliD</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>fspA</i>	<i>C. jejuni</i> M1 <i>fspA</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>sodB</i>	<i>C. jejuni</i> M1 <i>sodB</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>porA</i>	<i>C. jejuni</i> M1 <i>porA</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1-(PorA <sub>L7</sub> *)	<i>C. jejuni</i> M1 loop7 of PorA fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>tpx</i>	<i>C. jejuni</i> M1 <i>tpx</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>accD</i>	<i>C. jejuni</i> M1 <i>accD</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>cmeC</i>	<i>C. jejuni</i> M1 <i>cmeC</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>flgE2</i>	<i>C. jejuni</i> M1 <i>flgE2</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>flgK</i>	<i>C. jejuni</i> M1 <i>flgK</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>cj0124</i>	<i>C. jejuni</i> M1 <i>cj0124</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>cj0125</i>	<i>C. jejuni</i> M1 <i>cj0125</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>cj0126</i>	<i>C. jejuni</i> M1 <i>cj0126</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>cadF</i>	<i>C. jejuni</i> M1 <i>cadF</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>flpA</i>	<i>C. jejuni</i> M1 <i>flpA</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>cj0586</i>	<i>C. jejuni</i> M1 <i>cj0586</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>cjaC</i>	<i>C. jejuni</i> M1 <i>cjaC</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>flgL</i>	<i>C. jejuni</i> M1 <i>flgL</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>cjaD</i>	<i>C. jejuni</i> M1 <i>cjaD</i> fused 3' to <i>gst</i> in pGEX-4T1
pGEX-4T1- <i>ggT</i>	<i>C. jejuni</i> M1 <i>ggT</i> fused 3' to <i>gst</i> in pGEX-4T1
pMAL-p2X- <i>cjaA</i>	<i>C. jejuni</i> M1 <i>cjaA</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>fliD</i>	<i>C. jejuni</i> M1 <i>fliD</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>fspA</i>	<i>C. jejuni</i> M1 <i>fspA</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>sodB</i>	<i>C. jejuni</i> M1 <i>sodB</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>porA</i>	<i>C. jejuni</i> M1 <i>porA</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X-(PorA <sub>L7</sub> *)	<i>C. jejuni</i> M1 loop7 of PorA fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>tpx</i>	<i>C. jejuni</i> M1 <i>tpx</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>accD</i>	<i>C. jejuni</i> M1 <i>accD</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>cmeC</i>	<i>C. jejuni</i> M1 <i>cmeC</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>flgE2</i>	<i>C. jejuni</i> M1 <i>flgE2</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>flgK</i>	<i>C. jejuni</i> M1 <i>flgK</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>cj0124</i>	<i>C. jejuni</i> M1 <i>cj0124</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>cj0126</i>	<i>C. jejuni</i> M1 <i>cj0126</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>cadF</i>	<i>C. jejuni</i> M1 <i>cadF</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>flpA</i>	<i>C. jejuni</i> M1 <i>flpA</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>cj0586</i>	<i>C. jejuni</i> M1 <i>cj0586</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>cjaC</i>	<i>C. jejuni</i> M1 <i>cjaC</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>flgL</i>	<i>C. jejuni</i> M1 <i>flgL</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>cjaD</i>	<i>C. jejuni</i> M1 <i>cjaD</i> fused 3' to <i>malE</i> in pMal-p2X
pMAL-p2X- <i>ggT</i>	<i>C. jejuni</i> M1 <i>ggT</i> fused 3' to <i>malE</i> in pMal-p2X

\* Base pairs 897 to 1020 of *porA* encoding for protein residues 299 to 340 were cloned.



## 2.2 Culture conditions

*E. coli* and *Salmonella* were grown in Luria Bertani (LB; Oxoid, UK) broth or agar at 37 °C, unless otherwise indicated, with shaking at 200 rpm for liquid cultures. *C. jejuni* was grown on modified charcoal cephaloperazone deoxycholate agar (mCCDA; Oxoid, UK) or in Mueller-Hinton Broth (MH; Oxoid, UK), at 37 °C in a microaerophilic workstation (Don Whitley Scientific, UK) in a low oxygen atmosphere (5% O<sub>2</sub>, 5% CO<sub>2</sub> and 90% N<sub>2</sub>). Liquid cultures of *Campylobacter* were grown with shaking at 400 rpm using a table top shaker (IKA, Germany) under low oxygen conditions as above. Antibiotics were used at the final concentrations of 100µg/ml ampicillin, 50µg/ml kanamycin, 20µg/ml nalidixic acid and 34 µg/ml chloramphenicol where appropriate.

## 2.3 DNA manipulation

### 2.3.1 Plasmid DNA extraction

Plasmid DNA was extracted from bacterial strains using the QiaPrep Spin Miniprep Kit (Qiagen, UK) or the Qiagen Plasmid Midi Kit, following the manufacturer's instructions. The Qiagen kits work by selectively binding different sizes of DNA in high salt solutions to silica membranes, followed by washing and elution of the DNA of interest in low salt solutions or water.

### 2.3.2 Genomic DNA extraction

Genomic DNA (gDNA) was extracted using the Wizard Genomic DNA Purification Kit (Promega, UK), following the manufacturer's instructions. The kit works by lysing the cells, eliminating proteins via precipitation in salt, eliminating RNA via RNase digestion and then concentrating and washing high molecular weight DNA by isopropanol precipitation.

### 2.3.3 DNA purification

PCR products were purified using the QIAquick PCR (Qiagen) purification kit and gel extraction of PCR amplicons using the QIAquick Gel Extraction Kit (Qiagen), according to the manufacturer's instructions. Elutions were in 50 µl of water. This kit works on the same principles that the plasmid DNA extraction kit uses.

## 2.4 Polymerase chain reaction

Diagnostic polymerase chain reactions (PCRs) were run using the *Taq* DNA Polymerase (Invitrogen), in a reaction of 25µl or 50 µl final volume comprising of the following: 0.2mM each dNTP, 1.5mM MgCl<sub>2</sub>, 0.5µM each primer, 0.05% (v/v) buffer W1 (included in the kit), PCR buffer without MgCl<sub>2</sub> diluted 10-fold and 1-2µl of template DNA solution (containing various amounts of template DNA) or bacterial suspensions in water, at various concentrations. The cycling conditions were as follows:

- Initial denaturation for 45s at 94 °C
- Thirty cycles of denaturation for 45s at 94 °C, annealing for 30s at 55 °C and extension for 90s at 72 °C
- Final extension for 10 minutes at 72 °C
- Holding of the reaction at 4 °C until use.

Agarose gel electrophoresis was undertaken in 1% (w/v) agarose gels, with 1:10,000 dilution of SybrSafe (Invitrogen, UK) and run at 80-100V for 45-90 minutes.

## 2.5 DNA Quantitation

DNA quantity and concentration were measured using a Nanodrop 1000 machine (Thermo, UK). This was done according to the manufacturer's instructions, by adding 1 µl of the DNA sample to the pedestal of the machine.

## 2.6 Codon optimisation

Codon optimisation of *C. jejuni cjaA* for expression in *E. coli* was carried out by Dundee Cell Products (Dundee, UK) in order to assess whether it would offer increased expression via the pTECH2 plasmid in the APEC vector. In the process of codon optimisation, codons of a heterologous gene that are rarely used in the host in which the gene is to be expressed are replaced with highly used synonymous codons. The codon-optimised variant of *cjaA* was synthesised *de novo* and inserted into the pBluescript plasmid. The codon optimised sequence of *cjaA* will be referred to as *cjaA<sub>c.o.</sub>* and was verified by double-stranded dideoxy chain-termination sequencing by the manufacturer. The sequence of the codon-optimised variant in alignment with the wild-type gene is given in Section 3.2.

## 2.7 Molecular cloning

### 2.7.1 PCRs for molecular cloning

PCR amplicons for cloning were obtained using Phusion proof-reading polymerase (Finnzymes, Finland), with reactions containing reagents as directed by the manufacturer's instructions. Cycling conditions were either as above (Section 2.4) or used a two-stage cycling step in the PCR protocol if the melting temperature ( $T_m$ ) of the primers was above 72 °C. The two-step protocol was as above but missed the annealing stage during the cycling step. Primer sequences used for amplification of genes for cloning are given in Sections 3.2 and 4.2.

### 2.7.2 Restriction enzyme digests

All DNA digestion reactions contained the following: 1 µl of each restriction enzyme supplied by NEB and containing 20 units (U) for *Bam*HI and *Sal*I or 10 U for *Not*I, 2 µl of an appropriate React buffer, 0.2 µl of acetylated bovine serum albumin (BSA), between 1 µl and 16.8 µl of DNA in water containing a maximum of 1.5 µg of DNA and water to a total of 20 µl. All DNA digestion reactions were incubated at 37 °C for 2h. For cloning of the *Campylobacter* antigens into the pGEX-4T1 and the pMal-p2X plasmids, 10 U (in 1 µl) of calf intestinal phosphatase (CIP; NEB, UK) was added to the digestion reactions of the plasmids during the last hour of incubation. Digest reactions were passed through a PCR purification column to remove enzymes and obtain DNA eluted in water.

### 2.7.3 Ligation

All ligations reactions contained the following: 2 U (in 1 µl) of T4 DNA Ligase (Promega, UK), 1 µl of T4 DNA ligase buffer (Promega), DNA insert and vector in ratios of 3:1, 1:1 or 1:3 (up to a total volume of 8 µl and a total amount of DNA vector of 100 ng) and water to 10 µl. The reactions were incubated overnight on ice in water and for a following hour at room temperature (RT) the following morning, before being transformed into an appropriate bacterial strain.

### 2.7.4 Transformation of bacterial cells

Plasmid transformations into chemically-competent *E. coli* XL1 were done according to the manufacturer's instructions. A fast version of the electroporation protocol (Choi *et al.*, 2006) was used for electroporation into the *E. coli* Rosetta strain or the APEC or

*Salmonella* strains for construction of live-attenuated vaccines. For this, the cells of 6 ml of overnight culture of the strain to be used were collected by centrifugation in a 1.5ml microcentrifuge tube at 16,060 g for 5 minutes. The cells were washed twice in 1 ml 20% (w/v) sucrose in water and re-suspended in a final volume of 100 µl of 20% sucrose (w/v). Finally, 50 µl of the cells were mixed with 1 µl of DNA containing between 100ng and 1 µg of DNA and pulsed in a 0.1 cm cuvette (BioRad) at 2.5kV, 25mF, 200 Ω using the GenePulser machine (Biorad). Immediately, 450 µl of super optimal broth with catabolite repression (SOC; Oxoid) warmed at 37 °C was added and the cells were then incubated for 1h at 37 °C with shaking at 200 rpm before being plated on the appropriate selective media and incubated at 37 °C for 18-24h.

## **2.8 DNA sequencing**

Sequencing of plasmid DNA or PCR amplicons was performed using the dideoxy chain-termination (Sanger) method and was contracted to Dundee DNA Sequencing and Services (University of Dundee, UK), to Source Bioscience (Nottingham, UK) or to GATC Biotech (Germany) and the samples were prepared according to their instructions. DNA sequences were analysed in Lasergene 10 (DNASTAR). Chromatograms were manually trimmed at the 5' and 3' end to good signal. The sequence obtained using the reverse primer was reverse complemented and a contig was formed between this and the sequence obtained from the forward primer. The consensus sequence was aligned in Clustal W2 with the expected sequence extracted from the *C. jejuni* M1 genome available in the Nucleotide database of the National Centre for Biotechnology Information (NCBI).

## **2.9 Bacterial growth curves**

Bacterial growth curves were measured in order to determine whether the introduction of the pTECH2 plasmids had an inhibitory effect on the growth of the parent strains. For these assays overnight cultures were diluted 1:10,000 in LB and 100 microliters of this dilution added to a 96 well-plate in triplicate. A further 10-fold dilution of this culture ( $10^{-5}$ ) was plated on selective media for retrospective enumeration of viable bacteria in the inoculum. The 96 well plate was incubated at 37 °C with shaking every 30 minutes, followed by an OD<sub>595</sub> reading, using a Multiskan Ascent spectrophotometer (Thermo Scientific). This cycle was continued for 18 hours.

## 2.10 Cell invasion assays

Cell invasion and proliferation assays were used to assess whether the introduction of the pTECH2 plasmid in the PoulVac® *E. coli* and *Salmonella* strains had an inhibitory effect on the invasiveness and net intracellular survival of the parent strains. These assays were done in HeLa cells (ACTC CCL-2), which were grown and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 200mM L-glutamine (Life Technologies, UK), in appropriately sized tissue culture flasks (Corning, USA). Cells were passaged when they reached 90% confluence by treatment with 3ml of 0.5% (w/v) trypsin-EDTA solution in PBS (Life Technologies) for 5 minutes followed by inactivation with an equal volume of heat-inactivated bovine serum (HIBS). The cells were then pelleted by centrifugation at 400g for 5 minutes and washed in Hank's balanced salt solution (HBSS, Invitrogen, UK). The cells were then pelleted as above, the supernatant discarded and the cells seeded in a tissue culture flask of increased size and incubated as above.

The invasion and proliferation assays were undertaken in 24 well tissue culture plates (Corning) that were seeded with  $5 \times 10^5$  HeLa cells 24 hours before infection. Cells were incubated and maintained in the media described above. Before infection, the supernatant was removed and the cells were washed in HBSS once. Bacterial cultures were prepared by inoculation of a 10ml volume of LB medium from an overnight culture and grown to a minimum OD<sub>600nm</sub> of 0.5. These cultures were then diluted 1:15 in DMEM and a 1ml volume was added to each well to give a multiplicity of infection (MOI) of 50. Inocula were determined by retrospective plating of serial ten-fold dilutions. The 24 wells plates were then incubated as above for 1 hour and at the end of the incubation period 1ml of 100 µg/ml gentamicin (Invitrogen, UK) in DMEM was added to each well and incubated for a further hour. At the end of the incubation period, the HeLa cells were lysed in 0.1% (w/v) sodium-deoxycholate (Thermo, UK) and 10-fold serial dilutions in PBS plated on LB agar.

## 2.11 Protein expression and purification

### 2.11.1 Purification of GST and GST-fusion proteins

Before large scale purification of recombinant GST-tagged *Campylobacter* antigens to be used in vaccination trials, small scale induction and purification studies were performed to assess the ease of purification of each protein (solubility, yield, purity, expression of truncated variants and ease of elution were taken into consideration) and the likelihood of successful large scale preparation. For these, cultures of 25ml of the *E. coli*

Rosetta cells containing the GST–*Campylobacter* antigen fusion constructs were inoculated at a 1:100 dilution from a stationary phase overnight LB culture with antibiotics as appropriate and incubated for 3h, with shaking at 200 rpm, at either 28 °C or 37 °C and induced with either 0.1 mM or 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Thermo Scientific, UK). Following induction, the cultures were incubated for a further 3h at either 28 °C or 37 °C and then bacterial cells were harvested by centrifugation for 15 minutes at 3200g. Bacterial pellets were lysed in 5ml/g wet cell paste of BugBuster MasterMix (Novagen, UK) and the lysates centrifuged at 3200g for 10 minutes to pellet the insoluble fraction. GST fusion proteins were purified from the supernatant using glutathione sepharose beads (GE Healthcare, UK), in batch format, following the manufacturer’s recommended protocol. Bound GST fusion proteins were eluted for one hour (50mM TrisHCl, 40mM glutathione pH 8), in a volume double that of the beads. Beads were eluted three times and fusion protein-containing eluates confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below. Only antigens for which purification of sufficient quantities within these conditions was possible were taken forward to be purified in larger quantities and tested as anti-*Campylobacter* vaccines in chickens.

For large scale vaccine production, individual cultures of 500ml to 2 litres of the *E. coli* Rosetta cells containing the GST-tagged *Campylobacter* antigen of interest were grown as described above for small scale protein inductions. For poorly soluble antigens, multiple 2 litre cultures, up to a total of 6 litres of culture, were each grown in 5 litre flasks. Bacterial cells were harvested by centrifugation in a KR4i ultracentrifuge (Thermo, UK) at 15,075g for 15 minutes. Protein purification was in batch format, on glutathione sepharose beads, as described above.

### 2.11.2 Purification of MBP-fusion proteins

Each of the *Campylobacter* antigens tested as a recombinant GST-tagged anti-*Campylobacter* vaccine was also purified as an MBP-tagged recombinant protein in order to be used in ELISAs measuring the humoral responses induced by vaccination. This approach was chosen in order to avoid detection of antibodies against the GST fraction of the recombinant purified vaccines. Their purification was run in batch format, as described for the large scale production of the GST-tagged proteins above. However, the pMal-p2X constructs were expressed in the *E. coli* XL1 Blue cells in which they were originally cloned. This approach was chosen as the quantities of purified recombinant protein required for ELISAs were smaller than those required for vaccination and sufficient expression was

obtained in this strain. Conditions for incubation temperature and IPTG concentration were as described for the GST-tagged constructs.

### 2.11.3 6xHis-tagged protein purification

A 6xHis-tagged CjaA recombinant protein was purified in order to be tested in parallel with GST-CjaA, as used in previous studies utilising 6xHis-CjaA (Buckley *et al*, 2010). The culture and induction conditions were as described for the large scale purification of GST-CjaA and purification was run in batch format utilising Ni-NTA His-Bind resin (Novagen, UK), according to the manufacturer's recommended protocol.

## 2.12 Protein quantitation

Protein quantitation of the GST-fused *Campylobacter* antigens was done using the Bradford Assay, using the QuickStart™ Bradford 1x dye reagent (Biorad) and the 2mg/ml liquid BSA standard (Biorad), following the manufacturer's instructions for the 96-well plate microassay protocol. This measurement assay works on the basis of a colorimetric change induced by the binding of Coomassie brilliant blue G250 dye to primarily basic and aromatic amino acid residues. This method was chosen for the GST-fused antigens as the method used for quantitation of the MBP-fusion constructs is inaccurate in the presence of the glutathione present in the elution buffer for the GST-tagged proteins.

Quantitation of the 6xHis- and MBP-tagged antigens was via the Direct Detect system (Merck-Millipore, USA), following manufacturer's instruction. This system works by directly measuring protein amounts in a set volume of 2µl by measuring the absorbance of infrared light of amide bonds present in proteins.

## 2.13 SDS-PAGE

Protein preparations were separated via SDS-PAGE on 10% Mini-TGX gels (Biorad, UK), ran in SDS buffer (Scientific Laboratory Supplies [SLS], UK) in a Protean Tetra Mini cell (Biorad, UK). All gels were run at 300V for 18 minutes. The Dual Colour Precision Plus protein standards (Biorad, UK) were used to estimate the molecular weight of separated proteins.

Gels were stained either with Coomassie stain, using the Biosafe Coomassie stain (Biorad, UK), according to the manufacturer's instructions or silver stained using the Pierce Silver Stain Kit (Thermo, UK), following manufacturer's instructions.

## 2.14 Western blotting

For Western blotting, SDS-PAGE gels were transferred to polyvinylidene fluoride PVDF membrane using the TransBlot Turbo system and transfer packs (Biorad, UK), following the manufacturer's recommended conditions for rapid transfer (up to 25V, 2.5 A, 3min). Membranes were blocked for one hour, at room temperature, in 5% (w/v) BSA in phosphate-buffered saline (PBS) containing 0.01% (w/v) Tween-20 (PBS-T). Membranes were washed three times in PBS-T for 15 minutes each and then incubated for one hour with the primary antibody diluted in PBS-T. Washing was performed for 15 minutes once, followed by three washes of 5 minutes each. Membranes were then incubated for 1 hour with secondary antibody diluted in PBS-T containing 1% (w/v) non-fat dry milk powder (Blotto, Santa-Cruz, USA). Blots were washed as above and developed using Clarity enhanced chemiluminescence (ECL) (BioRad, UK) and autoradiography (Amersham Hyperfilm ECL, GE Lifesciences, UK). The ECL substrate was allowed to incubate on the membrane for 5 minutes. Table 2.2 lists the antibodies used in this study.

**Table 2.1 Antibodies used in this study.**

Manufacturer	Batch number	Type of antibody	Antigen recognised	Species raised in	Isotype	Procedure used in	Dilution used at
Sigma Aldrich	051M4887	Secondary: HRP conjugated	Chicken IgY	Rabbit	Polyclonal	Western blotting; ELISA	1:3,000 for both
Santa Cruz	K1903	Primary	GST	Rabbit	Polyclonal IgG	Western blot	1:10,000
AbD Serotec	010312	Secondary: HRP conjugated	Chicken IgA	Goat	Polyclonal IgG	ELISA	1:10,000
Abcam	GR110283-1	Secondary: Alexa Fluor® 488 conjugated	Chicken IgY	Goat	Polyclonal IgG	Immunofluorescence (IF)	1:500
AbD Serotec	1006	Primary	Tetanus exotoxin	Goat	Polyclonal IgG	Western blotting	1:10,000
Sigma Aldrich	1212156A	Secondary: HRP conjugated	Rabbit IgG	Goat	Polyclonal	Western blotting	1:10,000
AbCam		Primary	MBP	Rabbit	IgG1	Western blotting	1:10,000
This study	N/A	Primary	SodB	Chicken	Polyclonal IgY	IF	1:500
This study	N/A	Primary	FluID	Chicken	Polyclonal IgY	IF	1:500



## 2.15 Vaccination trials

### 2.15.1 Experimental animals

All procedures were conducted under Home Office PPL 60-4420, according to the requirements of the Animal (Scientific Procedures) Act 1986, and with the approval of the local ethical review committee (Moredun Research Institute (MRI), Edinburgh, UK). Institutional guidelines of the MRI for the care of animals and for animal procedures were also followed. A total of 800 White Leghorn chickens, obtained on the day of hatch from the National Avian Research Facility (NARF) at The Roslin Institute (Edinburgh, UK) were used. Eggs were incubated and hatched under specified pathogen-free conditions either at MRI or at the NARF and transported to the MRI on the day of hatch. Animals were housed in groups of up to 20 birds, in colony cages of 1.5 m<sup>2</sup>, in a single room (up to 6 cages per room). The birds were on wire mesh floors, partially covered with cardboard to provide support for young birds. Groups were of mixed sex and were wing-tagged for individual identification following assignment to a cage. Water was given *ad libitum* and the feed consisted of a fine-ground starter vegetable-protein-based sterile irradiated feed for the first two weeks of life and (DBM Ltd., UK). The birds were then weaned on a grower pellet diet for the remainder of the experiment (DBM Ltd). Birds were sourced from *Campylobacter*-free layer flocks and confirmed to be free of *Campylobacter* by plating of cloacal swabs on mCCDA, followed by microaerophilic incubation.

### 2.15.2 Experimental design

To maintain consistency in experimental design with previous vaccination trials undertaken by our laboratory, the vaccination and challenge protocol described in Buckley *et al* (2010) was followed. Briefly, between 12 and 24 birds were used per experiment per group. A mechanical dispenser and high accuracy syringes (Hamilton-Bonaduz, Switzerland) were used for both vaccinations and challenge to deliver an accurate dose. Vaccination was subcutaneous on each side of the thorax, in a volume of 50 µl per side. At each vaccination, the antigen preparations were mixed in a 1:1 ratio with TiterMax Gold (Sigma-Aldrich, UK) and each bird received  $4.3 \times 10^{-10}$  moles of entire recombinant protein. Birds were given the primary vaccination on the day of hatch and a booster 14 days later.

Birds were challenged at 28 days post hatch (dph) through oral gavage, with c.  $10^7$  CFU of *C. jejuni* M1 or 11168H per bird, in a volume of 50 µl. Challenge cultures were prepared by first streaking the M1 strain on mCCDA and then, following incubation as described above, inoculating single colonies into MH broth and incubating the liquid cultures

for 24 hours. The liquid cultures were then standardised to an OD<sub>600</sub> of 0.15, corresponding to 2x10<sup>9</sup> CFU/ml. They were confirmed to have spiral morphology and rapid motility and then diluted a further 10-fold in PBS to a final working concentration of 2x10<sup>8</sup> CFU/ml.

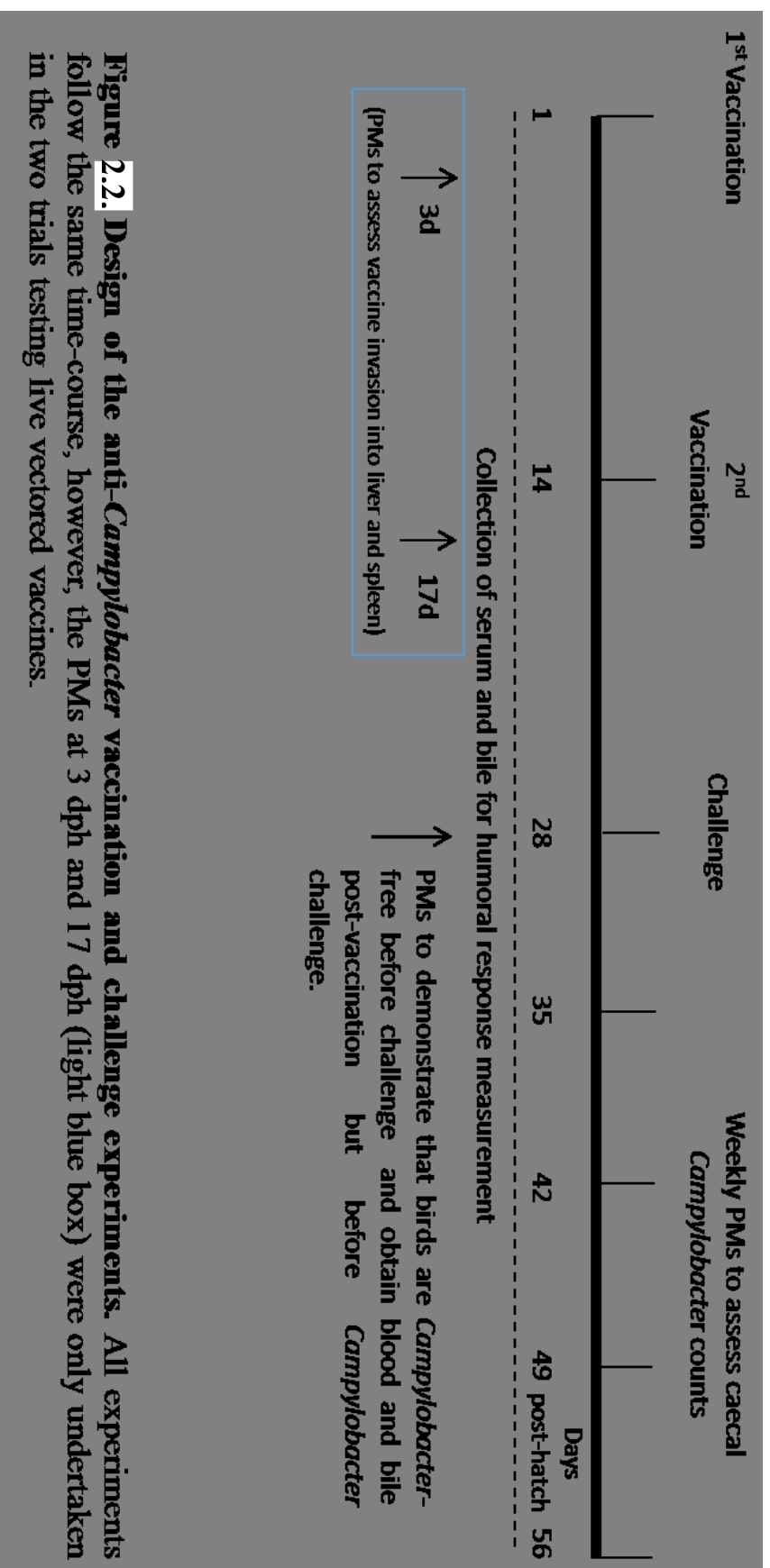
Starting one week after challenge, between 4 and 6 birds were removed at weekly intervals for *Campylobacter* enumeration and at the same time samples of blood and bile were collected for the measurement of humoral responses at *post-mortem* examination. Blood was collected through cardiac puncture after the death of the birds and stored at 4 °C overnight to allow coagulation. The following morning blood cells were pelleted by centrifugation at 3200g for 10 minutes. Serum was collected and stored at -80 °C until use. A schematic representation of the time-course of the vaccination and challenge experiments is given in Figure 2.2.

### 2.15.3 Assessment of caecal colonisation

Enumeration of caecal *Campylobacter* was performed by weighing 1g of caecal contents for each of the samples available and diluting this 1:10 with 9ml of Bolton Broth (Oxoid, UK) containing Bolton Broth selective supplement (Oxoid, UK). From this, a further six 10-fold serial dilutions in PBS were made in 48 well plates, in a total volume of 1ml for each dilution. From each dilution, 100µl was plated on mCCDA plates, which were incubated in a microaerophilic atmosphere for 48 hours, as described above. The 10<sup>-1</sup> dilution in Bolton Broth was incubated for 24 hours as described above, following which it was plated by streaking with a 10 µl sterile plastic loop on mCCDA plates, which was then incubated for 48 hours as described above. Colonies were counted on each of the mCCDA plates and only counts between 10 and 400 were used in calculating an average CFU/ml of caecal contents. Given a 1 gram sample, the theoretical limit of detection was 100 CFU/g by direct plating and 1 CFU/g following enrichment culture.

### 2.15.4 ELISAs

ELISAs were performed to measure antigen-specific serum IgY and secretory bile IgA (sIgA) against each of the recombinant antigens tested as vaccine candidates. The assays were done essentially as previously described by Buckley *et al* (2010), however, no blocking step was used for the measurement of serum IgY. Plates were coated with an appropriate amount of antigen diluted in carbonate-bicarbonate buffer in a total volume of 100 µl in each well and incubated at 37 °C for 1h. They were then washed five times in PBS containing 0.1% (w/v) Tween-20 (PBS-T) by submerging the plate in solution, flicking the liquid out of



**Figure 2.2. Design of the anti-*Campylobacter* vaccination and challenge experiments.** All experiments follow the same time-course, however, the PMs at 3 dph and 17 dph (light blue box) were only undertaken in the two trials testing live vectored vaccines.

the wells, inverting and tapping on paper. One hundred  $\mu\text{l}$  of serum or bile samples diluted in PBS-T were then added to each well, using an appropriate dilution for each antigen and the plates were incubated at 37 °C for 1h. Plates were then washed as described above, 100  $\mu\text{l}$  of secondary antibody added to each well and then incubated at 37 °C for 1h. Secondary antibodies were an HRP-conjugated rabbit anti-chicken IgY antibody (Sigma, UK) used at a 1:3000 dilution to detect bound serum IgY or an HRP-conjugated goat anti-chicken IgA antibody (AbD Serotec, UK) used at a 1:10,000 dilution to detect bound sIgA. Coating conditions and dilutions of the samples were optimised for each antigen using chequerboard analyses for IgY and sIgA individually. Chequerboard analyses are used to determine appropriate antigen coating concentration and sample dilution to ensure the readings are obtained within the linear range of the assay. For this, ELISAs with three-fold serial dilutions of representative samples were performed on halving concentrations of coating antigen. Plates were developed using tetramethylbenzidine (TMB) substrate (Biolegend, UK), for five minutes, as described by the manufacturer, reactions stopped by addition of 1M sulphuric acid and then absorbance was measured at 450nm on a Multiskan Ascent plate reader (Thermo, UK).

## **2.16 Immunofluorescent staining of *C. jejuni* cells**

Assessment of subcellular localisation of SodB within *C. jejuni* cells was carried out by immunofluorescent staining of whole *Campylobacter* cells. Serum from GST-SodB-vaccinated chickens collected before challenge was used as primary antibody. Poly-L-lysine treated glass cover slips were coated with 500  $\mu\text{l}$  of an overnight culture of *C. jejuni* 11168H, and fixed in 4% (w/v) paraformaldehyde for 30 minutes. Cover slips were washed three times with 1ml of PBS. Where indicated, cells were permeabilised with 10% (v/v) Triton X-100 for 30 minutes and non-specific sites blocked for 30 minutes in 0.5% (w/v) bovine serum albumin (BSA) in PBS (BSA-PBS). Cover slips were as above. Pooled serum from GST-SodB vaccinated birds was applied at 1:500 in BSA-PBS and a goat anti-chicken IgY conjugated to AlexaFluor-488 secondary antibody (Abcam, UK) was used for detection at a 1:500 dilution in BSA-PBS. Cover slips were as above after incubation with each of the primary and secondary antibodies. Cover slips were dipped in water once to remove salts in PBS and then mounted on glass slides using Prolong Gold (Life Technologies), according to manufacturer's directions. Images were captured using fluorescent and light microscopy on a DML microscope (Leica, Germany).

## 2.17 Generation of subcellular fractions of *C. jejuni*

Subcellular fractions of *C. jejuni* 11168H were generated by Halah Al-Haideri, University of Sheffield. For the preparation of the periplasmic fraction, an osmotic shock procedure was used. *C. jejuni* 11168H cultures in early stationary phase were harvested by centrifugation (8,000g, 10 min, 4 °C) and resuspended in 20 ml buffer containing 20% (w/v) sucrose, 30 mM Tris-HCl pH8, 1mM EDTA (STE) and incubated with gentle shaking for 30 min. The cells were pelleted by centrifugation at 10,000g, for 10 min at room temperature and then re-suspended in 10 ml ice-cold 10 mM Tris-HCl pH8. Following incubation with gentle shaking (20 rpm, 2 hr, 4°C), cells were pelleted by centrifugation at 15,000g for 25 min at 4 °C, and the supernatant containing the periplasmic proteins was carefully removed and stored at -20 °C.

To prepare the inner and outer membrane fractions, *C. jejuni* 11168H cells were cultured to early stationary phase and harvested by centrifugation (15,000g, 20 min, 4 °C) then re-suspended in 10 ml 10 mM HEPES pH 7.4. The cells were broken by sonication with 6 x 15 sec pulses at an amplitude of 16 microns using a Soniprep 150 ultrasonic disintegrator (SANYO) and then pelleted by centrifugation (15,000g, 20 min, 4°C) to remove unbroken cells and debris. The supernatant was carefully transferred to a pre-cooled tube. In order to isolate the outer and inner membrane, total membranes were first pelleted by ultracentrifugation (100,000g, 1 hr, 4 °C) in a Ti70.1 rotor (Beckman Coulter, UK) and the pellet washed twice with 5 ml of 10 mM HEPES pH 7.4. The membrane pellet was finally re-suspended in 1 ml 10 mM HEPES pH 7.4. To solubilise the inner membrane, an equal volume of 2% (v/v) Sarcosyl (Sodium N-Lauryl sarcosinate) dissolved in 10 mM HEPES buffer was added with gentle mixing. Following incubation at 37 °C for 30 min, the mixture was pelleted by centrifugation (15,000g, 30 min, 4 °C). The supernatant (solubilized inner membrane) was collected and stored at -20 °C and the outer membrane pellets were resuspended in 10 mM HEPES buffer pH 7.4, washed and re-pelleted twice. Finally, the pellet was resuspended and dispersed in 0.5-1 ml of 10 mM HEPES buffer pH 7.4 and stored at -20 °C.

The purity of the fractions was demonstrated by Halah Al-Haideri through Western blotting with  $\alpha$ CapA (an outer membrane auto-transported adhesin, Ashgar *et al*, 2007) or  $\alpha$ MfrA (a periplasmic fumarate reductase subunit associated with an inner membrane complex, Guccione *et al*, 2010).

## **2.18 Model for testing the effect of *C. jejuni* mutations in *Galleria mellonella***

A model to assess bacterial virulence in wax moth (*Galleria mellonella*) larvae was developed in order to assess whether *cj0424* may play a role in colonisation or virulence. The model has been reported to detect the attenuating effect of mutations in factors known to influence virulence in mammalian models and chicken colonisation (Champion *et al*, 2010). For this model, the *C. jejuni* 11168H wild-type,  $\Delta cj0424$  mutant and a  $\Delta cj0424::cj0424$  complemented strain provided by Halah Al-Haideri were inoculated on mCCDA by streaking with a 10  $\mu$ l sterile plastic loop. After incubation of the plates for 48 hours, multiple liquid cultures of each strain were inoculated in MH broth and grown for 24 hours as described above. In contrast to the vaccination experiments, liquid cultures were inoculated using a sweep of multiple colonies from the mCCDA plates. At the end of this incubation period, each culture was assessed visually, under an inverted light microscope, at 400x magnification, for morphology and motility of the *C. jejuni* cells. Only the three cultures with the most similar morphology and motility were taken forward to be tested in wax moth larvae.

For testing in wax moth larvae, cultures of each strain were standardised to an OD<sub>600</sub> of 0.15 and then diluted a further 10-fold in PBS. For each culture, 10 larvae were injected with 10  $\mu$ l each, on the caudo-dorsal aspect, using a LT1200 syringe (Hamilton-Bonaduz, Switzerland). Each larva received a total dose of c. 10<sup>6</sup> CFU of *C. jejuni* determined by retrospective plating of serial ten-fold dilutions of inocula on mCCDA. Larvae were then incubated microaerophilically, in inverted 90mm Petri dishes, on a disc of filter paper. The number of live/dead larvae was recorded for each of the groups at three hour intervals and differences in the survival of each of the groups assessed as detailed below.

## **2.19 Model for testing the effects of *C. jejuni* mutations in chickens**

A model of *Campylobacter* challenge and persistence was developed in chickens in order to assess the effect of the  $\Delta cj0424$  mutation on the colonisation potential of *C. jejuni* 11168H. These experiments were run under the same Home Office project and personal licence as described above for the vaccination experiments. Ethical approval was obtained from the MRI. White Leghorn eggs were sourced from and incubated in the NARF as described above. When hatched, day-old chicks were transferred to MRI and grown under SPF conditions as described above, in groups of 20 birds. Cultures of the three *C. jejuni*

11168H strains described in the wax moth larvae model were prepared as described above. Cultures of identical motility and morphology were used to separately challenge each of the birds by oral gavage with 50  $\mu$ l of culture diluted in PBS containing  $10^8$ ,  $10^6$  or  $10^4$  CFU. Each group received only one of the three strains utilised. Caeca were collected from 4-6 birds at *post-mortem* examination at weekly intervals for three weeks following challenge. *Campylobacter* numbers present in caecal contents were determined by plating serial dilutions on mCCDA as described above for the vaccination experiments.

## 2.20 Statistical analysis

All statistical analyses were performed using Minitab 17 (Minitab, UK), with the exception of the survival curves for the *Galleria mellonella* model, which were done in Prism 6 (GraphPad, USA).

Individual caecal *Campylobacter* counts were logarithmically transformed and the arithmetic mean was calculated. Significant reductions compared to control groups were determined using *post-hoc* Dunnett tests following fitting of a third order hierarchical general linear model (GLM) that took into account interactions between experiment, time of collection and treatment group. A separate model was fitted for each of the datasets presented subsequently.

As the main purpose of serum IgY measurement was to demonstrate successful vaccine delivery, only significant inductions in each vaccine group relative to control birds were assessed, without comparisons between different treatment groups. Significant changes in serum IgY were determined through a two-tailed Student's t-test on the raw OD<sub>450nm</sub> data.

Antigen-specific fold changes in OD<sub>450</sub> of serum IgY in individual birds were calculated by dividing the OD<sub>450</sub> measures in each vaccinated bird by the average of the GST-vaccinated control group calculated at each sampling time-point. Correlations between serum IgY levels and caecal *Campylobacter* counts in individual birds were assessed by fitting of a linear regression to the data. P values of  $\leq 0.05$  were considered significant.

Differences in survival curves of *Galleria melonella* were assessed using the Survival Analysis function in Prism 6. The logrank test was utilised and p values  $\leq 0.05$  were considered significant.

### 3. Live vectored vaccines for the control of *Campylobacter* in poultry

#### 3.1 Introduction

##### 3.1.1 The use of CjaA in vectored vaccines against *Campylobacter* in poultry

Vaccines for the control of *Campylobacter*, including subunit vaccines vectored in attenuated bacterial strains were discussed in Section 1.5. Previous research within our laboratory described the use of attenuated *S. Typhimurium* strains vectoring CjaA as vaccines for the control of *Campylobacter* infection in poultry (Buckley *et al*, 2010). The study describes the use of  $\Delta$ aroA *S. Typhimurium* 4/74 carrying a pTECH2 plasmid that expressed CjaA as a fusion to the C-fragment of tetanus toxin (TetC), under the control of the *nirB* promoter, which is believed to be induced *in vivo* by anaerobic conditions. Following oral vaccination of Light Sussex birds with  $10^7$  CFU of the vaccine per bird, a reduction of approximately  $1.5\log_{10}$  CFU *C. jejuni*/g in caecal colonisation of the homologous *C. jejuni* M1 strain was observed at 48 and 56 days post-hatch. While the protection was statistically significant, it reflected six biological replicates, each using 3 birds per group per time-point and variation was observed between trials. Furthermore, protection was observed late in the life of commercially reared birds as most broilers are slaughtered around 6 weeks of age.

The same study investigated the use of different attenuating mutations and reported that strains of *Salmonella* with different mutations vary in the protection induced when vectoring CjaA. *S. Typhimurium* attenuated through  $\Delta$ spaS or  $\Delta$ ssaU induced significantly larger decreases in caecal *Campylobacter* loads (approximately  $3.5\log_{10}$  CFU/g) compared to the  $\Delta$ aroA attenuated strain (approximately  $1.5\log_{10}$  CFU/g when tested in parallel, similar to the initial experiment across six biological replicates). This was observed across two biological replicates, each using 3 birds per time point per group. In *Salmonella*, *spaS* and *ssaU* encode components of the *Salmonella* pathogenicity island (SPI) 1 and 2 Type III secretion systems respectively and they have been shown to be important for colonisation of the chicken gut and systemic disease depending on the serovar and age of bird at inoculation. In *S. Typhimurium*, which causes age-dependent systemic disease in chickens with only the very young being affected, both SPI-1 and SPI-2 secretion systems are important in day old chicks as mutants in both aforementioned genes showed reduced gut colonisation in chickens and reduced clinical signs (Jones *et al*, 2007). Dieye *et al* (2009) showed that SPI-1 is



essential for spleen and gut colonisation of week-old chicks by *S. Typhimurium* but a SPI-2 mutant was only defective in colonisation of the spleen. In contrast, in *S. Gallinarum*, which causes overt pathology and clinical signs in chickens of all ages, only SPI-2 appears to be essential for virulence and colonisation as only the  $\Delta$ *ssaU* mutant had a severely attenuated phenotype (Jones *et al*, 2001).

Other independent reports describe the use of CjaA-based vaccines for control of *Campylobacter* infection in chickens. Wyszynska *et al* (2004) reported a  $6\log_{10}$  CFU/g reduction in caecal colonisation following vaccination of chickens with  $10^8$  CFU of an *asda* balanced-lethal system attenuated *S. Typhimurium*  $\chi^{3987}$ , vectoring CjaA from the high copy number plasmid PYA334. The reductions were observed at days 3 to 12 following challenge with  $10^8$  CFU of *C. jejuni* 72Dz/92 strain carrying a plasmid conferring chloramphenicol resistance. Another study reports a more modest reduction of  $2\log_{10}$  CFU/g when vectoring CjaA in a  $\Delta$ *aroA* mutant of *S. Typhimurium* that constitutively expressed a 35aa fragment of CjaA on the surface of the bacterium, from a chromosomal insertion of the *cjaA* fragment in a region of the *lamB* gene that gives rise to surface expressed protein (Layton *et al.*, 2010). Interestingly, the vector strain presented the CjaA fragment in concert with a 10aa fragment of CD145, predicted to contain the binding motif for activation of CD40, though the requirement for this modification in protection was not tested. In addition to the above studies demonstrating the successful use of CjaA in bacterial vectors, one study reported a  $1\log_{10}$  CFU/g reduction in caecal *Campylobacter* numbers following vaccination of chickens with transgenic *Eimeria tenella* expressing CjaA (Clark *et al*, 2012). Properties of the vector system are evidently significant as the same authors that claimed a  $6\log_{10}$  CFU/g reduction in caecal colonisation after vaccination of chickens with *S. Typhimurium*  $\Delta$ *aroA* expressing CjaA (Wyszynska *et al*, 2005) later observed no protection when using a different strain for delayed regulated expression (Laniewski *et al*, 2014a).

### 3.1.2 Avian pathogenic *E. coli* as vectors for CjaA-based vaccines

Given the previous research in our group described above and the reports of independent groups on the successful use of CjaA in vectored vaccines against *Campylobacter* in poultry, this project initially aimed to construct and test refined bacterial vectors expressing this antigen. Towards this aim, we developed and tested a  $\Delta$ *aroA* avian pathogenic *E. coli* (APEC) vaccine expressing CjaA.

APEC are a group of highly heterogeneous bacteria (reviewed by Guabiraba and Schouler, 2015) that cause a complex of respiratory and systemic diseases generically named avian collibacillosis with clinical signs including airsacculitis, polyserositis, perihepatitis,

pericarditis, septicaemia and other mainly extra-intestinal presentations (reviewed by Dho-Moulin and Fairbrother, 1999). Dziva and Stevens (2008) discuss the various virulence factors present in APEC, but emphasise that these factors do not universally occur in all APEC, which suggests disease is likely to be induced through different molecular mechanisms by highly different strains. In view of the pathogenic potential of APEC, we hypothesised that strains engineered to express CjaA may induce improved protection against *C. jejuni* given that more invasive strains of *Salmonella* were shown to provide better protection (Buckley *et al*, 2010).

Furthermore, using an APEC-based vaccine would allow poultry farmers to control both colibacillosis and *Campylobacter* colonisation concomitantly. This could potentially act as a financial incentive to control a seemingly innocuous infection in chickens due to reduced mortality and improved productivity conferred by vaccination against APEC. A further advantage of using APEC as a vector is that there is a genetically modified  $\Delta$ *aroA* APEC vaccine licensed for use in poultry in Europe and the United States. This vaccine is marketed by Zoetis as PoulVac<sup>®</sup> *E. coli* and La Ragione *et al* (2013) showed that vaccinated birds did not show mortality when challenged with the wild-type fully virulent APEC O78:H9 strain and that the signs of colibacillosis were reduced from 100% in non-vaccinated bird to approximately 40% of vaccinated birds. The associated patent also claims heterologous protection against serogroup O1 and O2 strains. Using PoulVac<sup>®</sup> *E. coli* as a vector creates a precedent for the licensing of genetically modified APEC-based vaccines for field use and may have the added advantage of ease of commercialisation and reduced development costs for Zoetis. Other advantages of using PoulVac<sup>®</sup> *E. coli* are its ease of use and safety. The vaccine is typically delivered as a spray directed at the heads of the birds on the day of hatch and the safety profile of this vaccine has been determined during its licensing. Although it can colonise the gut of chickens it does not cause clinical signs in vaccinated birds (La Ragione *et al*, 2013). Lastly, the vaccine has been shown to be protective against colibacillosis in turkeys and as such may allow the concomitant control of *Campylobacter* in other commercially important birds. Our laboratory has been studying the immune responses induced by PoulVac<sup>®</sup> *E. coli* in turkeys and chickens and their association with protection (Sadeyen *et al*, 2015a; Sadeyen *et al*, 2015b).

Lastly, APEC may pose a lower risk of zoonosis than attenuated *Salmonella* vaccines. Even though they have been shown to contain many of the virulence associated genes found in human uropathogenic *E. coli* (UPEC) strains (Rodriguez-Siek *et al*, 2005) and human neonatal meningitis strains (Johnson *et al*, 2012), the latter study also showed that APEC have a markedly lower potential of causing systemic disease and meningitis in

murine models of this disease. In contrast, Nolan *et al* (2010) found serogroup O18 strains of APEC and neonatal meningitis *E. coli* (NMEC) to have a similar disease outcome in a rat model of human disease. As such, the potential of APEC to cause human disease remains to be elucidated.

## 3.2 Materials and Methods

This section will specifically describe the construction, validation and testing of *S. Typhimurium* and APEC-based attenuated vaccines vectoring CjaA, using the general methods described in Section 2.

### 3.2.1. Construction of *Salmonella* vectored vaccines

The  $\Delta$ *aroA* *S. Typhimurium* 4/74-based vaccine strains described previously by Buckley *et al* (2010) served as positive controls in the vaccination and challenge experiments testing the APEC-based vaccines. To construct the pTECH2-*cjaA* plasmid, the *cjaA* gene was amplified from *C. jejuni* M1 using the primers given in Table 3.1 and ligated into pTECH2 following digestion of both the insert and plasmid with *Bam*HI and *Hind*III. Ligation reactions were transformed into chemically-competent *E. coli* XL1 cells and individual colonies screened for positive clones via a colony PCR, using the pTECH2<sub>seq primer forward</sub> and the pTECH2<sub>seq primer reverse</sub> (Table 3.1). At the time of screening, each colony was also re-streaked on LB agar supplemented with ampicillin and incubated for a further 8h, followed by a second round of diagnostic PCR. Clones positive in both PCR rounds were inoculated into liquid media and grown overnight. Plasmid DNA was extracted from each liquid culture and validated through sequencing by Dundee DNA Sequencing and Services.

The  $\Delta$ *aroA* *Salmonella* Typhimurium ST4/74 strain (Buckley *et al*, 2010) was transformed through the fast electroporation method with validated the pTECH2 or pTECH2-*cjaA* plasmids. Following electroporation, individual colonies were re-streaked on LB agar containing ampicillin and nalidixic acid (the parent  $\Delta$ *aroA* *S. Typhimurium* 4/74 strain is nalidixic resistant) and the colonies that grew were screened by PCR for the presence of the expected plasmid. The presence of the  $\Delta$ *aroA* mutation in *S. Typhimurium* ST4/74 vaccines was confirmed via PCR using the primers  $\Delta$ *aroA*-ST<sub>fwd</sub> and  $\Delta$ *aroA*-ST<sub>rev</sub> (Table 3.1). Colonies that were confirmed positive for pTECH2 and pTECH2-*cjaA* were inoculated in 10ml of LB broth and incubated overnight. The following day, they were stored in 16.6% (v/v) glycerol at -70 °C until further use. In addition, these liquid broth

cultures were used to extract plasmid DNA from each strain and plasmids validated via a restriction enzyme digest utilising the *Bam*HI and *Not*I enzymes (data not shown).

### 3.2.2 Construction of APEC vectored vaccines

#### 3.2.2.1 Codon optimisation of *cjaA*

Due to *C. jejuni cjaA* containing 20% of codons that are rarely used in *E. coli*, we tested whether codon optimisation increases the expression level of CjaA within the APEC-based vaccines. Codon optimisation was undertaken by Dundee DNA and Sequencing Services, using a table of codon usage for APEC based on codons used in highly expressed genes. The codon optimised variant was synthesised *de novo* with the *Bam*HI and *Not*I restriction enzyme recognition sequences at the 5' and 3' ends. The newly synthesised gene was introduced in pBluescript to be used as a vector for propagation of the construct. Figure 3.1 shows the sequence of the codon optimised *cjaA* compared to the wild-type variant.

#### 3.2.2.2 Cloning of wild-type and codon optimised *cjaA* into the expression vector and transformation into the vaccine strain

The APEC strain used for the construction of these vaccines originated from an intact vial of PoulVac<sup>®</sup> *E. coli* received directly from Zoetis. This vial was reconstituted according to the manufacturer's recommendations and cells were stored in 16.6% (v/v) glycerol at -70 °C both in their original reconstituted form and after two passages (growth on LB agar plates with subsequent inoculation of a single colony into LB broth). The passaged culture originating from a single colony was used for the construction of the APEC vaccines vectoring CjaA.

To create the pTECH2 plasmids vectoring codon optimised *cjaA* (denoted hereafter as pTECH2-*cjaA*<sub>c.o.</sub>), the pBluescript plasmid containing the codon optimised variant of *cjaA* was transformed into *E. coli* XL1 for propagation and amplification. This strain was grown and *cjaA*<sub>c.o.</sub> was digested from extracted plasmid DNA using *Bam*HI and *Not*I. The digested product was ligated into pTECH2 digested with the same enzymes and the ligation transformed into *E. coli* XL1. Validation of the transformants was undertaken as described above for the construction of pTECH2-*cjaA*, including double strand sequencing of the insert.

The pTECH2, pTECH2-*cjaA* and pTECH2-*cjaA*<sub>c.o.</sub> plasmids were electroporated

```

Wild-type_cjaA      ATGAAAAAAAAATACTTCTAAGTGTTTTAAACGGCCTTTGTTGTAGTAGTATTGGCTGCTTGT 60
Codon_optimised_cjaA ATGAAGAAAATCCTGCTGAGCGTCTGACAGCGTTTGTGCGTGGTGGTCTGGCAGCTTGC 60
***** ***** ** ** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Wild-type_cjaA      GGAGGAAATTCTGACTCTAAAACTTTAAATTCTCTTGATAAGATCAAGCAAAATGGAGTT 120
Codon_optimised_cjaA GGCGGGAATAGCGACAGCAAGACTCTGAATAGTCTGGATAAGATCAAAACAAACGGCGTG 120
** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Wild-type_cjaA      ATTAGGATTGGGGTATTTGGCGATAAACACCTTTTGGTTATGTGGATGAAAAAGGAAAC 180
Codon_optimised_cjaA ATTCGTATCGGTGTGTTGCGGTGACAAACCTCCGTTTCGGGTATGTGGACGAGAAAGGTAAT 180
*** * ** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Wild-type_cjaA      AATCAAGGCTATGATATAGCTTTAGCTAAACGCATAGCAAAAGAACTTTTGGCGATGAA 240
Codon_optimised_cjaA AACCAAGGCTATGACATTGCCCTGGCCAAGCGCATCGCGAAAGAACGTTTGGGGATGAA 240
** ***** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Wild-type_cjaA      AATAAGGTGCAATTTGTTCTTGTGAAGCTGCAAATAGGGTTGAGTTTTTAAATCAAAT 300
Codon_optimised_cjaA AATAAAGTACAGTTTGTGCTGGTAGAAGCTGCCAACCGTGTGAGTTTCTGAAAAGCAAT 300
***** ** ** ***** ** ** ***** ** * ** ***** * ** * ** **

Wild-type_cjaA      AAAGTAGATATTATTTGGCTAATTTTACTCAAACCTCCGCAAAGAGCCGAGCAGGTTGAT 360
Codon_optimised_cjaA AAAGTGACATCATCTGGCAAATTTACGCAGACGCCGAACGCGCCGAACAGGTTGAC 360
***** ** ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Wild-type_cjaA      TTTTGCTTGCCTTATATGAAGGTAGCTTTAGGCGTAGCTGTACCAAAGGATAGTAATATA 420
Codon_optimised_cjaA TTCTGTCTGCCGTATATGAAAGTTGCGCTGGGTGTTGCTGTGCCAAAGGACAGCAATATC 420
** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Wild-type_cjaA      ACTAGCGTAGAAGATTTAAAAGATAAAACCTTGCTTTTAAACAAAGGCACAACAGCAGAT 480
Codon_optimised_cjaA ACGAGTGTGAAAGACCTGAAAGACAAAACGCTGCTGCTGAACAAAGGACCCTGCCGAT 480
** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** * ** *

Wild-type_cjaA      GCGTATTTTACGCAAAATTATCCTGATATTTAAACCTTTAAATACGATCAAAATACCGAA 540
Codon_optimised_cjaA GCATACTTTACCCAGAACTATCCGGACATTAAGACCTGAAATACGACCAGAACACAGAA 540
** * ** ***** ** * ** ***** ** ***** ** * ** ***** ** * ** * ** *

Wild-type_cjaA      ACTTTTGCCGCTTTGATGGATAAAAGAGGCGATGCTTTAAGTCATGATAATACCTTGCTT 600
Codon_optimised_cjaA ACCTTTGCGGCACCTGATGGATAAACGCGGCGATGCACTGAGTCATGATAACACCTTGCTG 600
** ***** ** ***** ***** * ***** * ***** ***** ** * ** *

Wild-type_cjaA      TTTGCTTGGGTGAAAGATCATCTGATTTTAAATGGCTATCAAAGAGTTAGGCAACAAA 660
Codon_optimised_cjaA TTTGCGTGGGTCAAAGATCACCCGATTTCAAATGGCGATTAAAGAACTGGGTAACAAA 660
***** ***** ***** ** ***** ***** ** ***** * ** *****

Wild-type_cjaA      GATGTATCGCACCAGCAGTTAAAAAGGCGATAAAGAAGCTTAAAGAATTTATCGATAAT 720
Codon_optimised_cjaA GATGTAATTGCCCGCGGTAAAGAAAGGTGATAAAGAGCTGAAAGAGTTTATTGATAAC 720
***** ** ** ***** ** ** ***** ***** ** ***** ***** *****

Wild-type_cjaA      TTGATCATTAAACTAGGCCAAGAGCAGTTTTTTTCCACAAGGCTTATGATGAAACTTTAAA 780
Codon_optimised_cjaA CTGATTTAACTGGGCCAGGAACAGTTCTTTTCATAAAGCGTACGATGAAACCTGAAAG 780
**** ***** ***** ** ***** ***** ** * ** ***** * **

Wild-type_cjaA      GCTCATTTTGGAGATGATGTTAAGGCTGATGATGTAGTGATTGAAGGTGGAAAAATTTAA 840
Codon_optimised_cjaA GCGCACTTCGGCGATGATGTTAAGGCTGATGATGTGGTTATTGAGGCGGCAAGATCTAA 840
** * ** * ** ***** ***** ***** ** ***** ** * ** * ** * ** *

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**Figure 3.1 Alignment of the codon optimised variant of *cjaA* with the wild-type variant.** The alignment was performed in ClustalW2. The nucleotide sequence of codon-optimised CjaA in pTECH2 was confirmed to be as presented (data not shown)

into PoulVac<sup>®</sup> *E. coli* and transformed colonies validated as described above for the *Salmonella* vaccines. The primers used for the validation of the  $\Delta$ *aroA* mutation in the parent and vaccine strains are given in Table 3.1. The pTECH2 diagnostic primers were used to ensure each vaccine contained the expected plasmid.

**Table 3.1 Primers used for cloning and validation of the *Salmonella*- and APEC- based vaccines vectoring CjaA.** Underlined sequence represent recognition sites of enzymes used for cloning.

Primer	Sequence (5' to 3')
Primers for cloning of <i>cjaA</i> into pTECH2	
<i>cjaA</i> fwd	CCCCCC <u>GGATCC</u> ATGAAAAAATA
<i>cjaA</i> rev	CCCCCC <u>AAGCTI</u> TTAAATTTTCCACCTTCAATC
Diagnostic primers	
pTECH2 seq primer forward	GGTTGCGACTGGTACTTCGT
pTECH2 seq primer reverse	TTACCGCCTTTGAGTGAGCT
$\Delta$ <i>aroA</i> -STfwd	CCGATTGAAGGGGTAAAAGC
$\Delta$ <i>aroA</i> -STrev	GCCACCGCCAGTAGTAATGA
$\Delta$ <i>aroA</i> -APECfwd	TTGAGTTCGAACGTCGTCAC
$\Delta$ <i>aroA</i> -APECrev	ATACCCCAAGGTCCGAAA

### 3.2.3. Assessment of antigen expression from the live vectored vaccines

The TetC-CjaA and TetC antigens were expressed in the *Salmonella* and APEC vectors from the pTECH2 plasmid, which contains the *nirB* promoter reported to be induced by anaerobic conditions, but found in this study to express under aerobic conditions also. Expression of CjaA from the vaccines described above was assessed through Western blotting of aerobic and anaerobic cultures. For this, the vaccine strains were streaked on LB agar for individual colonies from glycerol stocks and a single colony was inoculated either in 6ml of LB broth in a 20ml universal or in 7 ml of LB broth in a 7ml universal. The cultures were incubated at 32 °C for 16 hours, either with the lid loosened in the case of the 20ml universals or with the lid tightly closed in the case of the 7ml universals, to create aerobic and anerobic growth conditions respectively. The following day, 1 ml of each culture was pelleted by centrifugation at 16,060 g for 5 minutes and the pellet lysed in 100  $\mu$ l of BugBuster. An equal volume of Sample Treatment Buffer (STB) was added to each tube and 20  $\mu$ l of each sample resolved by SDS-PAGE and transferred to PVDF membrane. A Western blot using an anti-TetC antibody (AbCam, UK) used at a 1:10,000 dilution was run

to assess expression of TetC or the TetC-CjaA fusion from the pTECH2 and pTECH2-*cjaA* plasmids.

#### **3.2.4. Comparison of growth curves, invasion and replication of the vaccine strains and the parent strains**

In order to demonstrate that the introduction of the pTECH2 plasmids did not lead to attenuation of the APEC strains used as vectors, their growth curves, invasion and replication phenotypes were tested *in vitro*. Growth curves were determined for each vaccine strain using the method described in Section 2.10 and compared to that of the parent strain. The invasion and net replication profiles of each of the constructed strains were compared to that of the parent strain through the use of a gentamicin protection assay, which was run as described in Section 2.11. The retention of the endogenous plasmids of PoulVac<sup>®</sup> *E. coli* following introduction of the pTECH2 plasmids was also tested. For this, the parent PoulVac<sup>®</sup> *E. coli* and each of the vaccine strains were grown overnight in LB broth. Five ml of culture were pelleted by centrifugation at 16,060 g for 5 minutes and plasmid DNA extracted from each of the strains. One µg of each DNA preparation was run on a 1% agarose gel at 100V for one hour to assess the plasmids present within each preparation. As the use of *S. Typhimurium* successfully vectoring heterologous *Campylobacter* antigens from the pTECH2 plasmid has been described in the literature previously (Buckley *et al*, 2010; Pogonka *et al*, 2003; Somner *et al*, 1999), no such tests were run for the *Salmonella*-vectored vaccines.

#### **3.2.5. Testing of the above vaccines in chickens**

Once the expression of TetC and TetC-CjaA from the *Salmonella* and APEC vectored vaccines was confirmed, they were tested for their protective efficacy against homologous challenge in chickens. CjaA-vectoring vaccines were tested in parallel with vectors containing the empty pTECH2 plasmid and with a PBS only group. Two vaccination and challenge trials were run using the protocol described in Section 2. Subcutaneous vaccination was used to ensure delivery of a standard inoculum. Each trial used between 2 and 5 birds per group per time-point. All of the groups were housed within the same room in the first experiment, whilst in the second experiment control groups (vaccinated with PBS or with the empty vectors) were housed in a separate room. This was due to low level transmission of the vaccine strains to the control groups during the first trial as a result of one bird being erroneously returned to a wrong cage after they escaped their cage during

feeding. This error occurred due to the birds not being wing-tagged during the first experiment by a member of Moredun Animal Services. Subsequent experiments used double wing-tagging to avoid such errors.

### 3.2.6. Dissemination and persistence of the live-vectored vaccines

To assess the dissemination of the vaccines following subcutaneous delivery, liver and spleen samples were collected at three days after each vaccination from two birds in each group. The entire spleen and the central lobe of the liver were sampled for each bird. These were weighed, diluted 1:10 (w/v) in PBS and homogenised using 7mm stainless steel beads in 2ml SafeLock<sup>®</sup> tubes (Eppendorf, UK) in the TissueLyser<sup>®</sup> II machine (Qiagen, UK). Samples were homogenised twice, at 25Hz, for two minutes, with the block being turned between runs in order to ensure equal path length for all samples. Three further 10-fold dilutions of these homogenates were made and 100 µl of each dilution were plated on MacConkey agar plates containing ampicillin. Also, 1ml of the original culture was inoculated into 10ml of LB broth with ampicillin as an enrichment culture. To assess persistence of the vaccines, the first three serial dilutions used for enumeration of *Campylobacter* in caecal contents were also plated on MacConkey agar supplemented with ampicillin.

### 3.2.7. Measurement of serum IgY responses induced by vaccination

#### 3.2.7.1 Production of the coating antigen to be used in ELISAs

In order to measure antigen-specific humoral responses in vaccinated birds, TetC and CjaA were purified as GST-tagged recombinant proteins, expressed from the pGEX-4T1 plasmid. The *tetC* gene was amplified from the pTECH2 plasmid using the primers described in Table 3.2 and digested with *Bam*HI and *Not*I restriction enzymes. The *cjaA<sub>c.o.</sub>* gene was digested with the same restriction enzymes from pTECH2-*cjaA<sub>c.o.</sub>*. Both genes were separately ligated in pGEX-4T1 digested with *Bam*HI and *Not*I. Ligations were transformed into *E. coli* XL1 and 100 µl plated onto LB agar with ampicillin. After overnight incubation, individual colonies were inoculated on LB agar with ampicillin and screened for the expected insert by PCR, using the primers described in Table 3.2. Positive colonies were inoculated in 10ml of LB broth with ampicillin and verified by PCR a second time. Cultures from colonies positive for both PCRs were chosen for plasmid DNA extraction. Plasmids were validated by sequencing on both strands at Dundee DNA and



Sequencing Services and correct plasmids were electroporated into *E. coli* Rosetta using the fast electroporation method.

GST-TetC and GST-CjaA were purified from *E. coli* Rosetta as described in Section 2.11.1, using 500ml cultures. The purity of each of the preparations was assessed via a Western blot using a mouse monoclonal IgG1 anti-GST antibody (Santa-Cruz Biotech, USA), at a 1:10,000 dilution. Before use in ELISAs, protein concentration in each preparation was assessed using the QuickStart Bradford Assay (Biorad, UK).

**Table 3.2 Primers used in construction and validation of the pGEX-4T1 plasmids expressing TetC and CjaA as recombinant GST-tagged proteins.** Underlined sequences represent the recognition sites for restriction enzymes used during cloning.

Primer	Sequence (5'-3')
Primers for <i>cjaA</i> and <i>tetC</i> cloning into pGEX-4T1	
pGEX-4T1- <i>tetC</i> fwd	CCGGCC <u>GGATCC</u> CTGATGAAAAACCTTGATTGTTG
pGEX-4T1- <i>tetC</i> rev	GGGGCC <u>GCCGCGC</u> GTCGTTGGTCCAACCTTCAT
Diagnostic primers for the pGEX-4T1 plasmids	
pGEXfwd	GGGCTGGCAAGCCACGTTTGGTG
pGEXrev	CCGGGAGCTGCATGTGTCAGAGG

### 3.2.7.2 Measurement of serum IgY responses by ELISAs

Antigen-specific serum IgY and bile secretory IgA (sIgA) induction against CjaA and TetC were measured by ELISAs using the GST-TetC and GST-CjaA preparations described above as coating antigens. Checkerboard analyses were run for serum and bile for each antigen separately to determine appropriate coating concentrations and sample dilution. For coating ELISA plates, GST-CjaA was used at a concentration of 0.5 µg/ml and GST-TetC was used at a concentration of 1 µg/ml for measurement of both serum and bile humoral responses.

For birds vaccinated during the first experiment only, induction of serum IgY against CjaA was measured by determining titres of antibodies for each bird. For this, 3-fold serial dilutions were used for each serum sample and the titre was calculated as the dilution of serum which would give the OD<sub>450nm</sub> reading corresponding to the point half way between the high and low plateaus.

For subsequent experiments, serum and bile antibody induction due to vaccination was determined through the use of a single sample dilution that fell within the linear range of

typical curves obtained in checkerboard analyses. To assess CjaA-specific induction, sera and bile were diluted 1:500 and to assess TetC-specific induction they were diluted 1:250. To assess induction of antibodies induced specifically by vaccination, fold-changes in serum IgY compared to the PBS-control group were calculated for each treatment group. At each sampling interval, these were expressed as average fold-change in OD<sub>450nm</sub> reading of individual birds, obtained by dividing the OD<sub>450nm</sub> for individual vaccinated birds to the average OD<sub>450nm</sub> of the PBS-injected control group at that particular sampling interval.

### 3.3 Results

#### 3.3.1 Construction of the live attenuated vaccines vectoring CjaA

Sequencing of the pTECH2-*cjaA* and pTECH2-*cjaA<sub>c.o.</sub>* plasmids demonstrated them to have the expected sequence (as shown in Figure 3.1). The validated plasmids pTECH2-*cjaA* and pTECH2 were transformed into the  $\Delta$ *aroA* *S. Typhimurium* ST4/74 strain. The pTECH2-*cjaA*, pTECH2-*cjaA<sub>c.o.</sub>* and the pTECH2 plasmids were transformed into the  $\Delta$ *aroA* PoulVac<sup>®</sup> *E. coli* strain received from Zoetis. Each vaccine strain was validated by PCR with the pTECH2<sub>(seq primer forward)</sub> and the pTECH2<sub>(seq primer reverse)</sub> primers flanking the cloning site and found to contain the expected plasmids (Figure 3.2, panels A and C). Each vaccine also contained the 100bp deletion in the *aroA* gene, as demonstrated by PCR using the primers given in Table 3.1 (Figure 3.2, panels B and D).

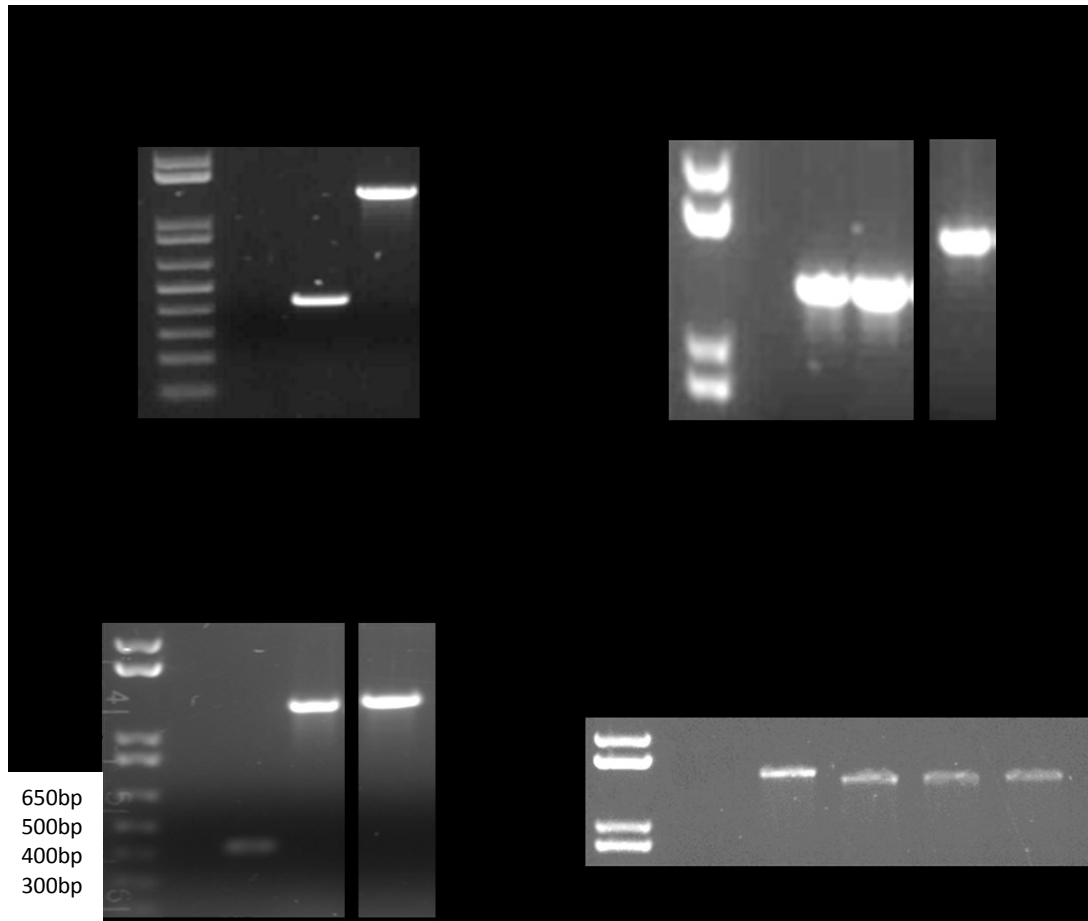
#### 3.3.2 Demonstration of antigen expression in the vaccine strains constructed

Following validation of each of the vaccine strain genotypes as described above, the expression of TetC or TetC-CjaA from the constructed strains was demonstrated via Western blot analysis of total protein from aerobic and anaerobic cultures, using an anti-TetC antibody. All vaccine strains expressed the expected antigens under both aerobic and anaerobic conditions (Figure 3.3). No increase in antigen expression was observed under anaerobic conditions and no improvement in expression was conferred by codon optimisation of *cjaA*.

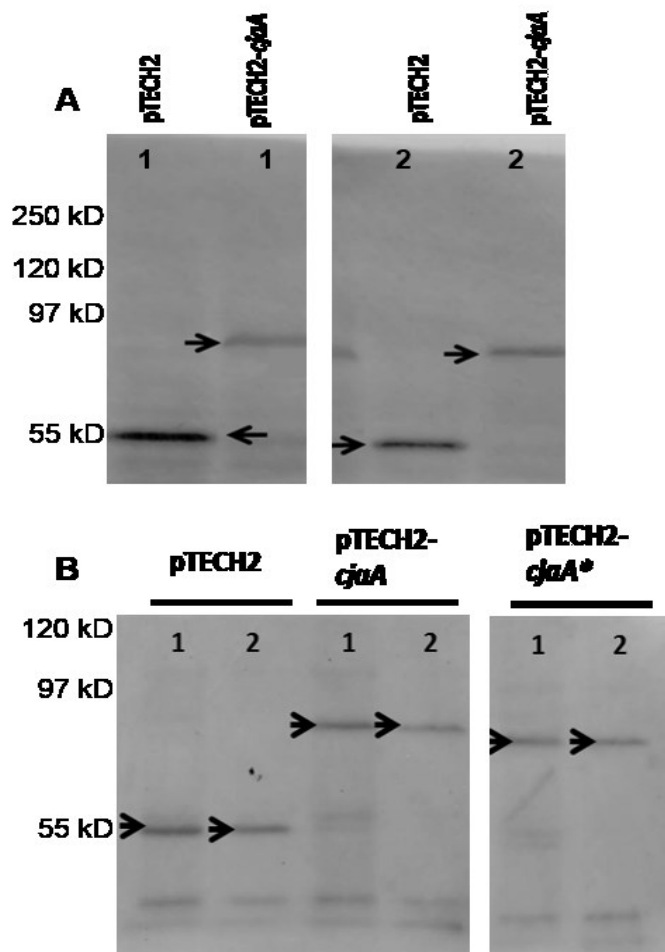
### 3.3.3 Phenotypic validation of the PoulVac<sup>®</sup> *E. coli* vaccine strains

#### 3.3.3.1 Growth curves

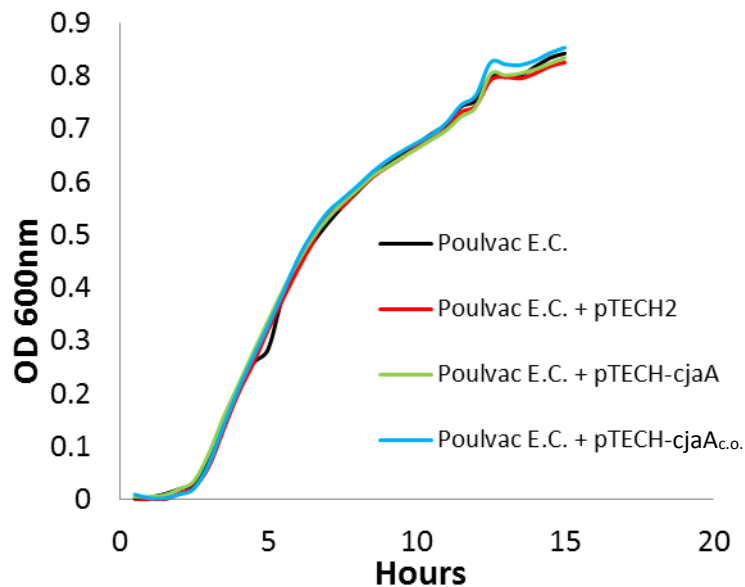
Growth curves were determined in order to assess whether the introduction of the heterologous expression plasmid impaired the growth of the PoulVac<sup>®</sup> *E. coli* strain. Under aerobic conditions in rich medium (LB broth), none of the pTECH2-containing vaccine strains exhibited decreased growth compared to the parent strain not containing the pTECH2 plasmid (Figure 3.4).



**Figure 3.2. Validation of *S. Typhimurium* 4/74 and PoulVac<sup>®</sup> *E. coli*-based live attenuated anti-*Campylobacter* vaccines. A.** pTECH2 and pTECH2-*cjaA* constructs in *S. Typhimurium* ST4/74  $\Delta$ *aroA* vaccines. Expected size of the PCR products are: 450bp for pTECH2; 1290bp for pTECH2-*cjaA* and pTECH2-*cjaA*<sub>c.o.</sub> **B.** Validation of the  $\Delta$ *aroA* attenuating mutation in *S. Typhimurium* vaccines – the mutation was made through the replacement of *aroA* with the *aph* cassette from pACYC177 (1122bp). Expected size of wild-type *aroA* amplicon is 1468bp. **C.** pTECH2, pTECH2-*cjaA* and pTECH2-*cjaA*<sub>c.o.</sub> constructs in the PoulVac<sup>®</sup> *E. coli* vectored vaccines. Expected size of the PCR products are as for panel A. **D.** Validation of the  $\Delta$ *aroA* attenuating mutation in the PoulVac<sup>®</sup> *E. coli* vaccines – the mutation was made through the deletion of a 100bp region of the gene. Expected size of wild-type *aroA* amplicon is 1591bp.



**Figure 3.3. Expression of TetC and TetC-CjaA in the live attenuated anti-*Campylobacter* vaccines.** **A.** Expression of TetC and TetC-CjaA in the  $\Delta$ *aroA* *S. Typhimurium* vaccines under aerobic (1) and anaerobic conditions (2). **B.** Expression of TetC and TetC-CjaA in the  $\Delta$ *aroA* PoulVac<sup>®</sup> *E. coli* vaccines under aerobic (1) and anaerobic conditions (2). CjaA and CjaA<sub>c.o.</sub> were tested in the same Western blot and the separation between them denotes the removal of a lane testing another antigen not described in this study. Arrows indicate the expected size of the antigen in each construct: TetC 55kD, TetC-CjaA 90kD. All cultures were standardised to the OD<sub>450nm</sub> of the culture with the lowest reading and the lysate of 1 ml of each culture was used.



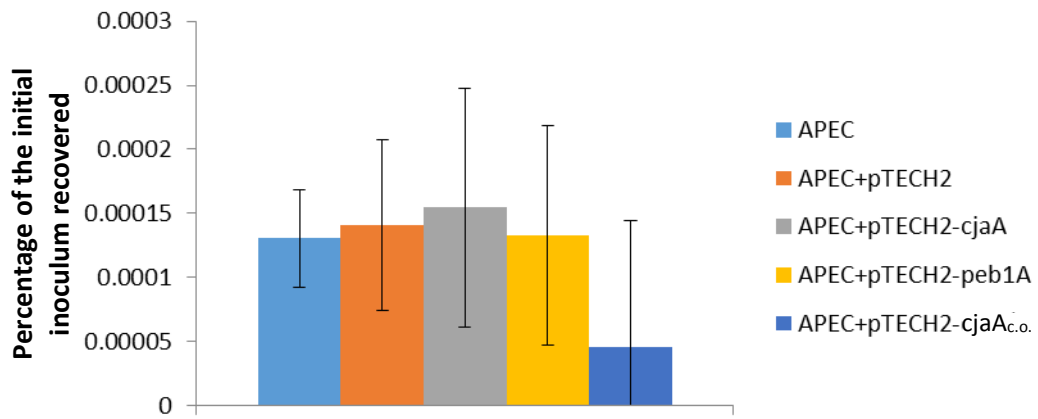
**Figure 3.4. Growth curves of the PoulVac<sup>®</sup> *E. coli*-based anti-*Campylobacter* vaccines.** Growth curves were determined in triplicate for each of three biological replicates by growing in 96 well plates at 37 °C degrees with shaking every 30 minutes, followed by automatic measurement of OD<sub>600nm</sub> after each shaking. Data presented are the average of all replicates.

### 3.3.3.2. Cellular invasion and net replication *in vitro*

In order to further validate that the introduction of the heterologous expression vector did not alter the phenotype of the vaccine strains compared to their parent strain, a cell invasion and proliferation assay was run. This assay is more likely to be relevant to *in vivo* growth conditions than growth in rich medium. Using HeLa cells, no differences in invasiveness and net replication were noted between the vaccine and the parent strains in the case of the PoulVac<sup>®</sup> *E. coli* vectored vaccines (Figure 3.5). The wild-type *S. Typhimurium* ST4/74 strain was also tested in this assay but it is not shown in the figure due to a much higher level of the inoculum being recovered (3% mean recovery across three replicates).

### 3.3.3.3. pTECH2 plasmid compatibility in PoulVac<sup>®</sup> *E. coli*

The retention of the endogenous plasmids in PoulVac<sup>®</sup> *E. coli* was assessed by agarose gel electrophoresis of plasmid DNA extractions from each of the vaccines strains and the parent strain. The plasmid species that were present in the parent strain were also present in all of the vaccine strains (data not shown).



**Figure 3.5 Invasion and net replication of the PoulVac® *E. coli* strains in HeLa cells.** The data represents the average of three biological replicates, each run in triplicate. Bacteria were allowed to invade for 1 hour and the HeLa cells incubated in the presence of gentamicin for a further hour before being lysed. The error bars represent the SEM.

### 3.3.4. *In vivo* testing of the live attenuated vaccines vectoring *CjaA*

#### 3.3.4.1 Safety profile of the vaccines

In each treatment group, a minimum of 32 White Leghorn birds were vaccinated subcutaneously on the day of hatch and at 14 dph with  $10^7$  CFU of the vaccine strains across the two experiments run. The subcutaneous route of vaccination was chosen for reliability of delivery and due to a single study run previously had suggested that the *S. Typhimurium* 4/74  $\Delta$ *aroA* vaccine vectoring *CjaA* was more protective when given via this route than when administered orally (unpublished data cited in Buckley *et al*, 2010) None of the vaccinated birds exhibited clinical signs typical of salmonellosis or colibacillosis following vaccination.

#### 3.3.4.2 Protection against caecal colonisation

The live attenuated *Salmonella* and APEC vaccines vectoring TetC-*CjaA* were tested for their protective efficacy against homologous challenge with the *C. jejuni* M1 strain in chickens in two independent experiments. A third order hierarchical general linear ( $R^2 = 0.48$ ) model taking into account the time of sampling, treatment group and experiment was fitted to the average data of the two experiments. *Post-hoc* Dunnet's tests indicated a significant difference between the group vaccinated with *S. Typhimurium* ST4/74  $\Delta$ *aroA* vectoring *CjaA* and the PBS-injected group ( $p = 0.016$ ) when considering the entire course of colonisation. However, the course of colonisation in this group was not significantly

different to the empty *Salmonella* vector ( $p = 0.7$ ) and neither was there a difference between the empty *Salmonella* group and the PBS-injected group ( $p = 0.2$ ). Furthermore, *post-hoc* Dunnet's test taking into account treatment group and time of vaccination could not distinguish individual time points that were significantly different. As such, it can be concluded that overall no reduction in caecal colonisation was observed with either of the vaccines when compared to vaccines expressing TetC only or with a PBS-injected control group (Figure 3.6C). Data obtained in individual replicates is shown in Panels A and B of Figure 3.6.

#### 3.3.4.3 Recovery of the vaccine strains from liver, spleen and gut

During the first experiment, three birds were sampled at post-mortem examination three days after the first vaccination to assess the invasion of the vaccine strains and dissemination to the liver and spleen. No birds were culled for this purpose after the second vaccination due to poor hatching rates and fewer birds than originally planned being available for the experiment. During the second experiment, three birds were culled for post-mortem examination three days following each vaccination. In each vaccination experiment, both the *Salmonella* and the APEC strains were recovered from the liver and spleen of vaccinated birds.

Bacterial recovery from vaccinated birds, averaged across both experiments, is given in Figure 3.7. No statistically significant differences between primary vaccination and booster or between vaccine strains were observed.

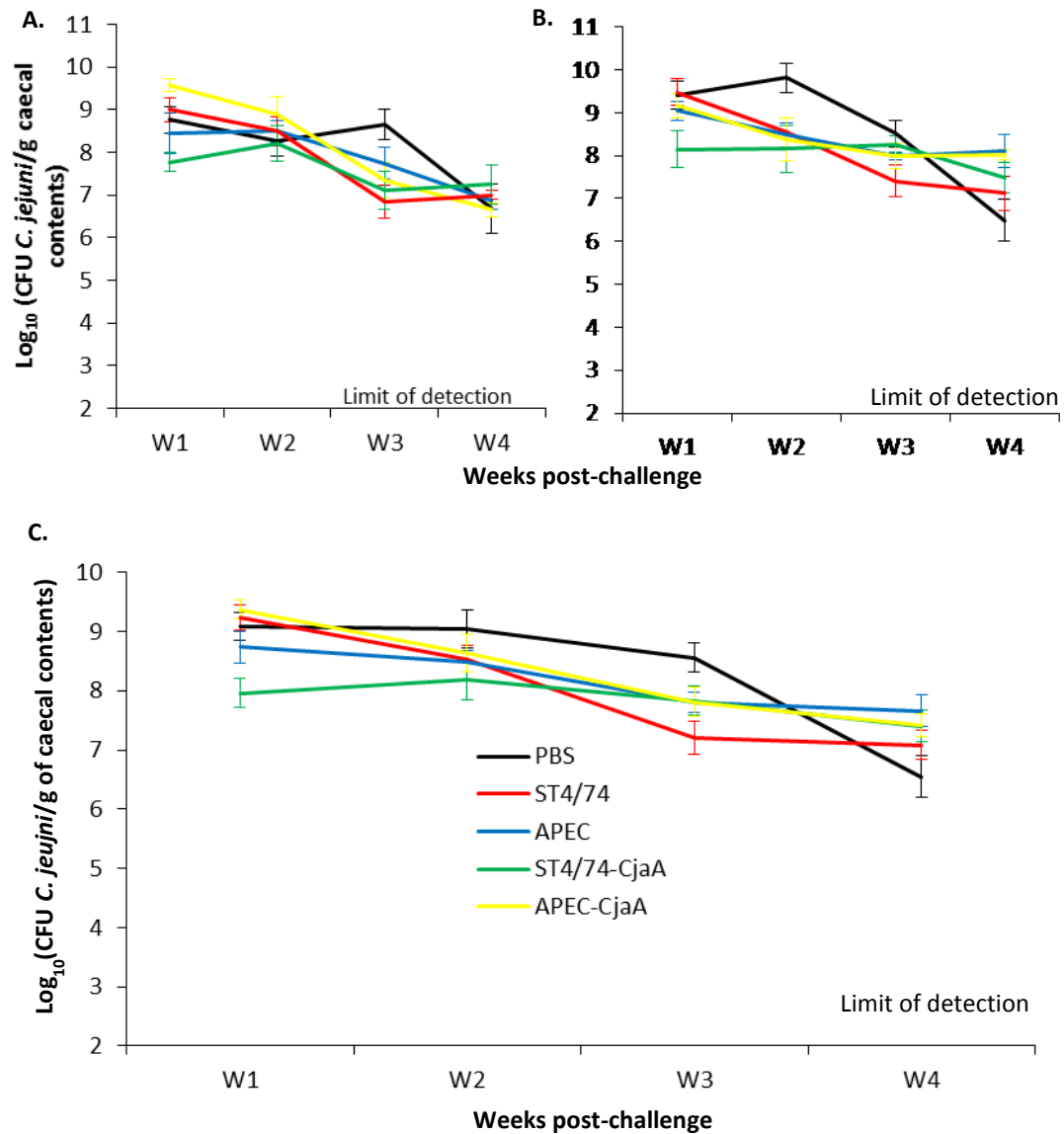
To investigate the duration of colonisation of the gut by the vaccine strains, the first serial dilution used for assessment of *Campylobacter* colonisation was streaked onto MacConkey agar supplemented with ampicillin. While not quantified, this demonstrated the presence of the vaccine in the gut of vaccinated birds strains throughout the duration of the study (data not shown), raising concern that if the vaccines were ever used as described they may persist to the point of entry into the food chain.

### 3.3.5 Induction of humoral responses following vaccination

#### 3.3.5.1 Production of GST-tagged recombinant CjaA and TetC

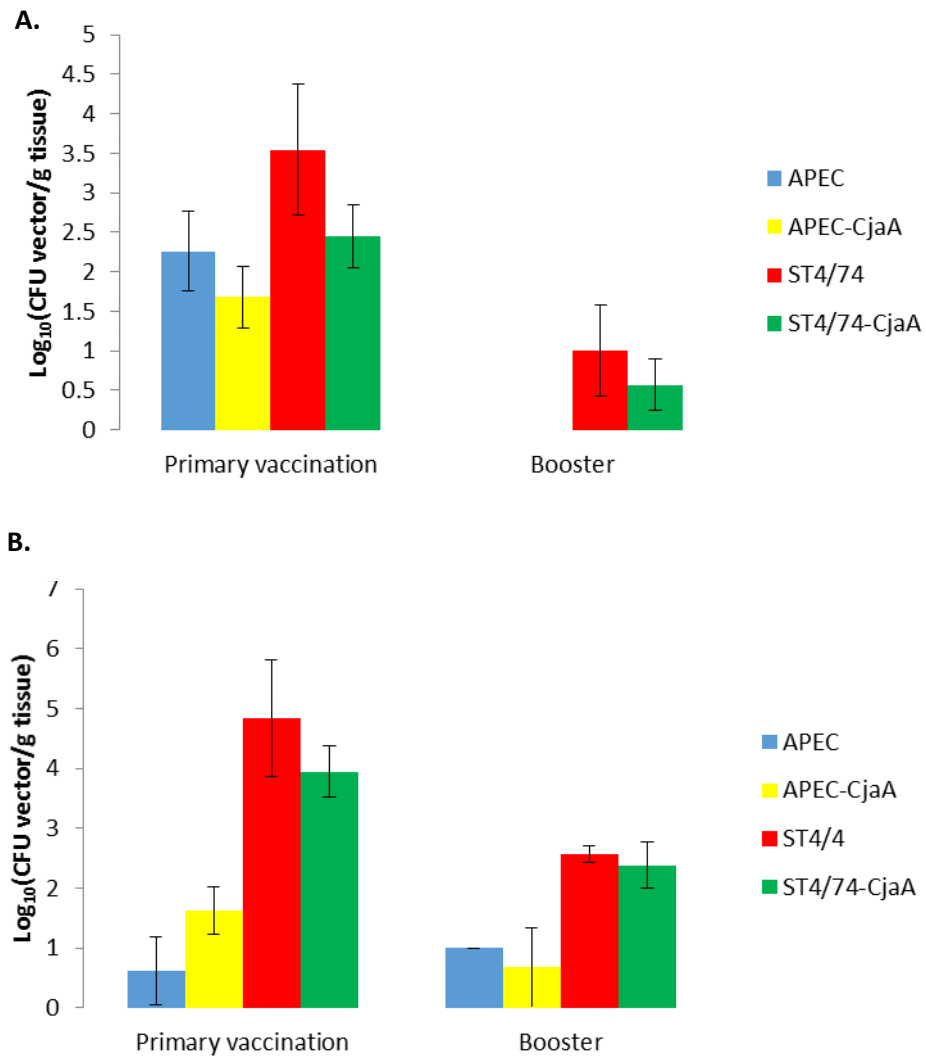
In order to develop ELISAs to measure humoral responses induced by vaccination, TetC and CjaA were cloned into pGEX-4T1 and expressed as recombinant GST-tagged proteins. In small scale preliminary experiments testing induction conditions, CjaA expression gave the optimal balance of quantity and solubility when induced at 28 °C with

0.1 mM IPTG, while TetC produce soluble protein when expressed at 37 °C with 1 mM IPTG (data not shown). Under these conditions, larger scale purifications of GST-TetC and



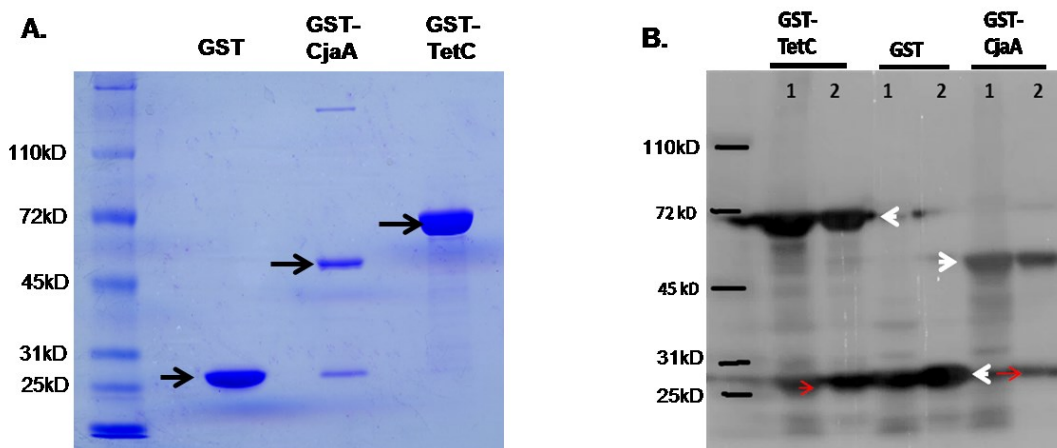
**Figure 3.6. Protective efficacy of the live vaccines vectoring CjaA against caecal colonisation by the homologous *C. jejuni* M1 strain.** **A.** Colonisation kinetics during the first vaccination and challenge experiment. **B.** Colonisation kinetics within the second vaccination and challenge experiment. **C.** Average colonisation kinetics from both vaccination and challenge experiments. In all panels, data is given as average of log-transformed caecal *C. jejuni* M1 counts in vaccinated birds. Caecal *C. jejuni* counts were determined through serial dilution of caecal contents collected at post-mortem examination. Between 3 and 6 birds were sampled at each time-point in each group. Error bars represent the standard error of the mean (SEM). Between time-points, lines infer the course of colonisation and should not be interpreted as longitudinal caecal counts.





**Figure 3.7. Dissemination of the vaccine strains to liver (A) and spleen (B) at three days after each vaccination.** Data is given as average of log-transformed bacterial counts per gram of tissue in each organ. Error bars represent SEM. In total, between 4 and 6 birds were sampled at primary vaccination and 3 birds were sampled at the time of the booster. The entire spleen was collected and as such the limit of detection for this organ was 1 CFU/g.

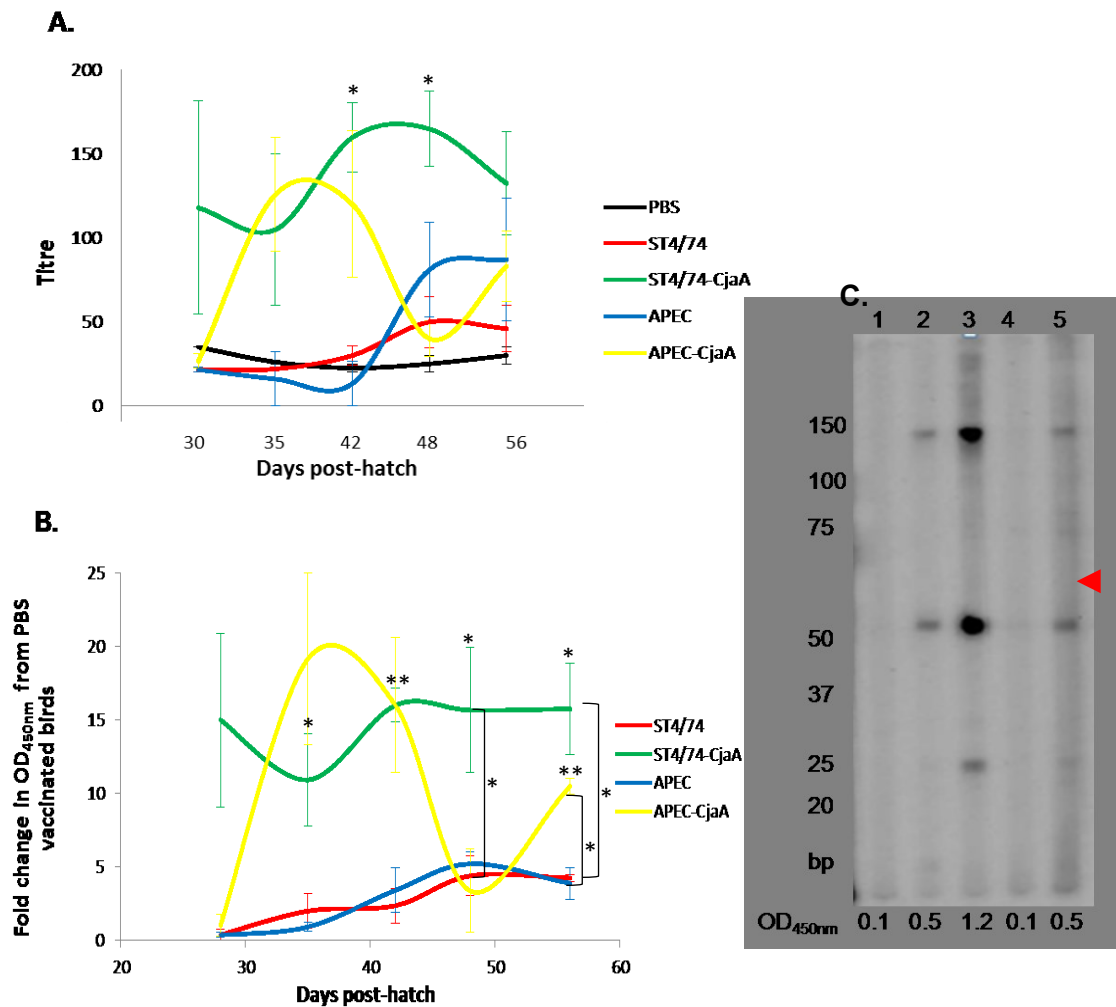
GST-CjaA were run. The final protein preparations used in ELISAs resolved through SDS-PAGE are shown in Figure 3.8A. The purity of the preparations and the identity of the bands observed through Coomassie staining were assessed by a Western blot using an anti-GST antibody. This highlighted the presence of GST-only truncations in both the GST-TetC and the GST-CjaA preparations (Figure 3.8B). Passage of the protein preparation through a concentrator column with a molecular weight cut-off of 50kD did not eliminate the presence of the GST only truncation (Figure 3.8B).



**Figure 3.8. Purification of GST-CjaA and GST-TetC for use in ELISAs measuring antigen-specific humoral responses.** **A.** Coomassie stain of SDS-PAGE resolved GST-TetC and GST-CjaA protein preparations. Black arrows indicate the expected size of each of the constructs (GST: 25kD, GST-CjaA: 50kD and GST-TetC: 75kD). **B.** Purity of the protein preparations in panel A indicated by a Western blot with an anti-GST antibody. 1 and 2 are the same protein preparation before and after passage through a concentrator column with a 50kD molecular cut-off. White arrow heads indicate the expected size of each of the constructs. Red arrows indicate truncation of the constructs with a size equal to that of GST alone that reacts with an anti-GST antibody.

### 3.3.5.2 Induction of serum IgY within the first vaccination trial

Humoral immune responses to vaccination were determined in order to establish that the lack of protection against caecal colonisation by *Campylobacter* was not due to failure to adequately deliver the vaccines. Antigen-specific serum IgY induction was measured by ELISA using the GST-TetC and GST-CjaA coating antigens described above. The average titre obtained in each vaccinated group at each sampling point is given in Figure 3.9A. Significant inductions of antigen-specific serum IgY were observed only in the group that received *S. Typhimurium* ST4/74  $\Delta$ *aroA* vectoring CjaA, at 42 ( $p = 0.01$ ) and 48 ( $p = 0.02$ ) dph. In order to reduce work for subsequent experiments, vaccine-specific induction of serum IgY against CjaA was also calculated as fold-change in OD<sub>450</sub> of each treatment group compared to the control PBS-injected group using a single reading within the linear range of the assay. The data is shown in Figure 3.9B. Significant changes were calculated on the raw OD<sub>450nm</sub> and are observed at the same time-points as in panel A. Additional time-points of significant induction are observed compared to both the PBS-injected controls and the empty vector controls. These are due to the smaller range of values for OD<sub>450nm</sub> compared to titres and as such lower variability of the data obtained in this way. The data suggest that vaccination with *Salmonella* ST4/74  $\Delta$ *aroA* vectoring TetC-CjaA induced a more stable



**Figure 3.9. Induction of CjaA-specific serum IgY in the first vaccination experiment.**

**A.** Specific anti-CjaA serum IgY induction expressed as titre, calculated as the dilution required to give an absorbance reading half-way between the maximum and minimum absorbance obtained in a three-fold serial dilution. Error bars represent the SEM. Significant changes are denoted by \* ( $p < 0.05$ ).

**B.** Specific anti-CjaA serum IgY induction due to vaccination expressed as fold change in OD<sub>450nm</sub> of individual vaccinated birds over the average OD<sub>450nm</sub> of the PBS-injected control group. Only one reading in the linear range was used for each bird. Error bars represent the SEM. Significant changes are denoted by \* ( $p < 0.05$ ) and \*\* for ( $p < 0.01$ ) and brackets indicate differences to the empty vector control groups.

**C.** Validation of ELISAs measuring CjaA-specific serum IgY through Western blotting. The Western blot shows the reactivity to GST-CjaA of sera collected from one bird at each time-point from the *Salmonella* vectoring CjaA vaccine group during the first vaccination experiment. Lanes 1 to 6 represent individual birds at the following time-points: (1) challenge; (2) to (5) weeks 1, 2, 3 and 4 post-challenge. The OD<sub>450nm</sub> given below the lanes are the reading obtained in ELISAs. The lower reactive band is a GST truncation, the middle band is the GST-CjaA species (red arrowhead) and the top band is likely to be GST-CjaA dimer as GST can dimerise.

serum IgY response compared to vectoring in PoulVac<sup>®</sup> *E. coli*, as evidenced by significant induction induced by the latter vaccine only at 56 dph and stable induction of the former throughout the study. However, no significant difference was found between these groups. While a marked induction in serum IgY against CjaA was observed in the PoulVac<sup>®</sup> *E. coli* vectored group at 35 dph, this was not statistically significant due to high variation in the data observed at this time-point.

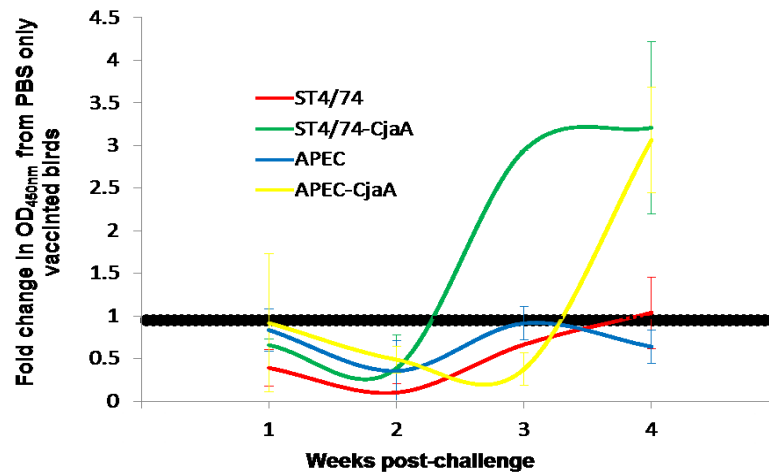
Validation of the specificity of the developed ELISA was undertaken through Western blotting. The GST-CjaA preparation was probed with sera containing different antibody levels obtained at different time-points, using a multi-lane block. Bound serum IgY was detected with the same anti-chicken IgY HRP-conjugated antibody that was used in the ELISA, used at the same dilution. The reactivity of each serum sample towards GST-CjaA is shown in Figure 3.9C and the intensity of the specific reactivity obtained through Western blotting varies in line with the OD<sub>450nm</sub> obtained in ELISAs.

#### 3.3.5.3 Induction of CjaA-specific bile sIgA in the first vaccination trial

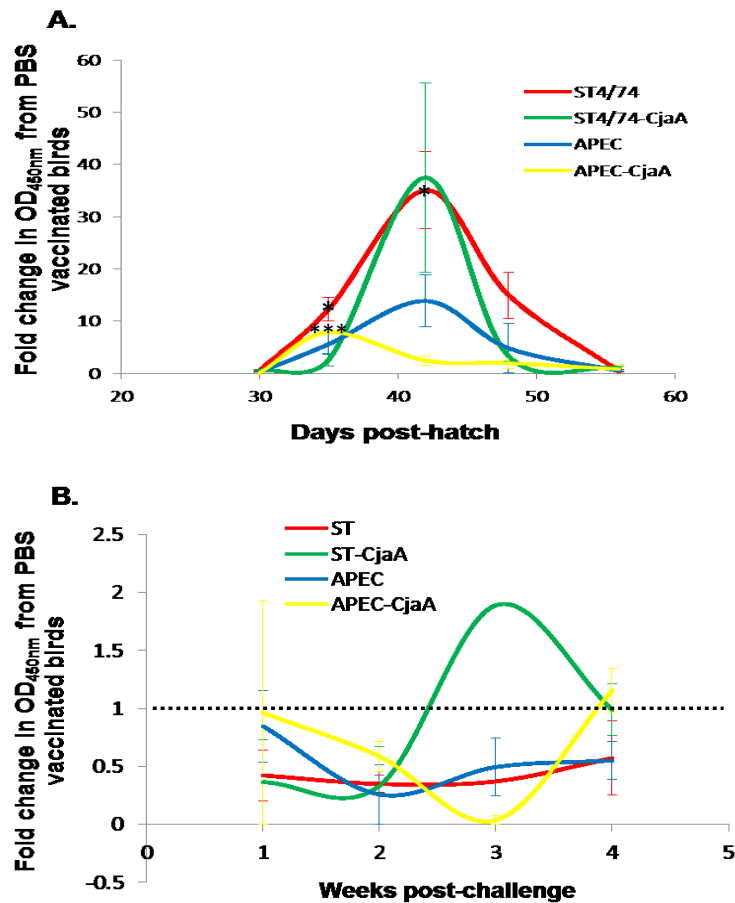
The induction of CjaA-specific bile sIgA during the first vaccination trial was determined as fold-change in OD<sub>450nm</sub> in each treatment group over the PBS-injected control group. This was done in order to assess whether vaccination induced other antibody types than serum IgY alone. The average fold-change in each treatment group is shown in Figure 3.10. Due to variable availability of samples (certain birds had no bile in the gall bladder), statistical significance could not be assessed at all time-points for all groups. Within the groups and time-points at which it was possible to assess statistical significance, no significant changes in bile IgA were found.

#### 3.3.5.4 Induction of TetC-specific humoral responses in the first vaccination trial

Induction of TetC-specific serum IgY and bile sIgA was determined in vaccinated birds as fold changes in OD<sub>450nm</sub> over the control group, as described above. Figure 3.11 shows the average fold-changes in TetC-specific antibodies in both serum (Panel A) and bile (Panel B). No significant induction of bile IgA was observed against TetC in any of the vaccinated groups. Significant induction of TetC antibodies were detected in the group vaccinated with *S. Typhimurium* 4/74  $\Delta$ *aroA* vectoring empty pTECH2 at 35 (p = 0.01) and 42 (p = 0.03) dph and in the group vaccinated with PoulVac<sup>®</sup> *E. coli* vectoring pTECH2-*cjaA* at 35 dph (p = 0.0008).



**Figure 3.10. Induction of CjaA-specific bile sIgA in vaccinated birds within the first experiment.** Data is expressed as average fold-change in OD<sub>450nm</sub> over PBS only vaccinated birds. Error bars represent the SEM.



**Figure 3.11. TetC-specific antibody inductions in within the first experiment.** **A.** TetC specific serum IgY expressed as fold-change in OD<sub>450nm</sub> over PBS vaccinated birds. **B.** TetC specific bile sIgA expressed as fold-change in OD<sub>450nm</sub> over PBS vaccinated birds. Error bars represent the SEM. Significant inductions are denoted by \* ( $p < 0.05$ ) and \*\*\* ( $p < 0.001$ ) and are superimposed on the time-points at which they were observed.

### 3.3.5.5. Induction of CjaA-specific serum IgY in the second vaccination trial

In order to assess whether the vaccines were delivered successfully in the second vaccination experiment, induction of serum IgY was determined as fold increase in OD<sub>450nm</sub> in vaccinated birds compared to the PBS-injected control group. However, loss of sample labelling during storage resulted in approximately half of the samples being discarded. As such, the majority of time points had either missing data or a single value and statistical significance could not be assessed. Out of the time-points at which statistical significance was assessed, there was a significant 2.7 fold increase in the group vaccinated with *S. Typhimurium* ST4/74  $\Delta$ *aroA* vectoring CjaA ( $p = 0.006$ ) at 3 weeks post-challenge and a 1.5-fold increase in the group vaccinated with the PoulVac<sup>®</sup> *E. coli* vectoring CjaA vaccine ( $p = 0.01$ ) at 2 weeks post-challenge. These may indicate successful delivery of the vaccine although caution should be exercised in interpretation of these results in view of the missing data.

## 3.4 Discussion

Vaccines vectoring CjaA as a TetC fusion in  $\Delta$ *aroA* *S. Typhimurium* 4/74 and  $\Delta$ *aroA* APEC O78:H9 (PoulVac<sup>®</sup> *E. coli*) were constructed and shown to express the TetC-CjaA fusion from pTECH2, under the control of the *nirB* promoter. The *nirB* gene of *E. coli* encodes an NADH-dependent nitrite reductase and its promoter was described initially in *E. coli* by Jayaraman *et al* (1987). Furthermore, it was shown that the promoter is partially induced by anaerobic conditions, through the action of the FNR protein, but that optimal activation required the addition of nitrite to the culture medium. Jayaraman *et al* (1988) mapped the nucleotide sequences of the promoter region required for induction by anaerobic conditions and by nitrite. Expression of TetC in *E. coli* driven by the *nirB* promoter and its induction by anaerobic conditions, with an increase in TetC of approximately two orders of magnitude, was demonstrated by Oxer *et al* (1991). The *nirB* promoter was also used for plasmid-driven expression of TetC in a  $\Delta$ *aroA*  $\Delta$ *aroD* *S. Typhimurium* (Chatfield *et al*, 1992), with stable expression and propagation of the plasmid in mice vaccinated with this strain.

In contrast to the tight regulation of the *nirB* promoter claimed by Oxer *et al* (1991) in *E. coli* and by Chatfield *et al* (1992) in *Salmonella*, all of the vaccines constructed in this study expressed the TetC and TetC-CjaA antigens at a similar level under both the aerobic and anaerobic culture conditions used here. Both this study and the studies described above used the same method of induction – completely filling 7ml bijoux and tightening the lid.

However, the difference in oxygen availability within the two culture conditions was not assessed. Alternatively, addition of nitrite to the culture medium may be required for maximal induction that may allow a difference to be observed between culture conditions. This may mimic natural infection conditions as it has been shown that the *nirB* promoter can induce high levels of expression following *Salmonella* invasion in macrophages (Everest *et al*, 1995), which use nitrite production (Iyengar *et al*, 1987) as a defence mechanism against bacterial pathogens, including *Campylobacter* (Iovine *et al*, 2008).

In the case of the APEC-based vaccines, codon optimisation of *cjaA* did not appear to increase TetC-CjaA expression. While codon optimisation has been shown to generally confer increased *in vitro* expression over native genes, there is no universally accepted method of codon optimisation and particular methods may not be broadly applicable as some genes appear refractory to improved expression following codon optimisation (Burgess-Brown *et al*, 2008). This could be the case with *cjaA* expression and suboptimal codon optimisation may account for the premature termination (Goldman *et al*, 1995) observed within this study and also reported by Buckley *et al* (2010). Furthermore, it has been shown that, at least in yeast, the codon usage bias may vary with the phase of growth and codon optimisation may have to take this into account (Lanza *et al*, 2014). This aspect is not widely studied for individual bacteria and is not a factor that most codon optimisation programmes take into account. Another aspect that could contribute to the lack of improvement in TetC-CjaA expression is that the *tetC* gene was not codon optimised. Given that the antigen expressed was a continuous fusion of CjaA to TetC, with TetC being translated first, it is possible that the expression of TetC acted as a limiting factor. Lastly, it is possible that even though not observed *in vitro*, codon optimisation of *cjaA* conferred an expression advantage *in vivo*. While it was attempted to grow the vaccine strains in minimal medium supplemented with aromatic amino acids and assess antigen expression, the level of growth was too low to detect the antigen through a Western blot (data not shown).

When tested *in vivo*, although both of the attenuated *Salmonella* and APEC vectoring TetC-CjaA vaccines induced significant increases in CjaA-specific serum and bile IgA in vaccinated White Leghorn birds, neither of the vaccines induced protection against caecal colonisation by the homologous *C. jejuni* M1 strain. A number of deviations from the study design reported by Buckley *et al* (2010) could have accounted for this difference. Firstly, the breed of chickens used in this study was White Leghorn and it is possible that the nature and timing of immune responses in this breed differs compared to the Light Sussex birds used previously. Secondly, it is possible that through running only two biological replicates with a total of 8-10 birds per time-point, this study was underpowered. The

previous study reported data from six replicates with a total of 18 birds per time-point to yield a statistically significant reduction of  $1.4\log_{10}$  CFU/g. Individual caecal *Campylobacter* counts from the previous trials were not available to assess the variance in those experiments and calculate sample sizes for the current experiments that would ensure adequate statistical power. In addition, subcutaneous vaccination was used in this study while the study reported by Buckley *et al* (2010) used oral vaccination. This approach was chosen as a single experiment previously carried out using subcutaneous delivery of the live-attenuated *Salmonella* vaccines resulted in improved protection (Anthony Buckley, unpublished data). It is possible that oral vaccination may induce a local immune response more appropriate for clearing of the infection and delivery of the vaccines developed within this trial by a different route may result in protection. Lastly, while the previous experiments report that the *Salmonella* vaccine strain was cleared from the intestines of vaccinated birds by 38 dph, in the current experiments all vaccine strains were detected throughout the experiment. The long-term presence of the live *Salmonella* or APEC within the birds, even though attenuated, may have dominated the immune response at the later time-point in the experiment and decreased the magnitude of the immune response towards *Campylobacter*. Additionally, it has been shown that the presence of lesions consistent with *E. coli* infections in broilers is a significant risk factor for *Campylobacter* infection (Bull *et al*, 2008; FSA, 2015b) although mechanisms behind this effect have not yet been elucidated.

In spite of the absence of protection, the induction of CjaA-specific serum IgY was detected in both groups vaccinated with *Salmonella* ST4/47  $\Delta$ *aroA* or PoulVac<sup>®</sup> *E. coli* vectoring TetC-CjaA. Using a single OD<sub>450nm</sub> reading within the linear range of the assay (as determined through checkerboard analysis) proved to be a more sensitive method of detection than the use of titres. Due to the high variability in bird to bird response to vaccination, the lower range of the OD<sub>450nm</sub> measurements compared to titres resulted in less overall variation and an increased power of the assay. Using this method, significant increases were detected compared to both the PBS-only vaccinated group and the empty vector control groups. While the data also suggest a more stable induction of antigen-specific antibody by the *Salmonella* vectored CjaA vaccines, with higher inductions of serum IgY at the time of challenge, no significant differences were observed between these groups. Assessment of differences between these groups would likely require the use of a higher number of birds per time-point due to high variability of the response to vaccination observed in individual birds.

To assess the safety of the live-attenuated vaccines in vaccinated birds, the experimental animals were monitored for clinical signs throughout the duration of the



experiment. None of the vaccinated birds showed clinical signs following vaccination, however, a small number of birds died unexpectedly. These birds did not exhibit gross organ pathology at *post-mortem* examination and the cause of death was unidentified. In addition, three birds from each vaccinated group were culled three days after each vaccination. Bacteria were recovered at low levels from the spleen and liver in the majority of the birds. Recovery of the *Salmonella* vaccine strain from these organs was also observed within the previous studies undertaken in our group. In line with efficient attenuation of both the *Salmonella* and the APEC vaccine, none of the birds culled throughout the duration of the study exhibited gross internal pathology. However, no detailed histopathology was undertaken and organ invasion of the vaccine strains was not measured longitudinally within these experiments. Even though subcutaneous vaccination was used, these findings are in line with those described by La Ragione *et al* (2013) for PoulVac<sup>®</sup> *E. coli* and by Buckley *et al* (2010) for the *S. Typhimurium* vaccines using oral delivery.

To assess the risk of vaccine transmission to the consumer, vaccine presence within the gut was assessed at each of the time-points used in this study. In contrast to the findings of Buckley *et al* (2010), which reported clearance of the vaccine strain at 38 dph after oral vaccination, both the *Salmonella* and the APEC vaccine strains were recovered from the caecal contents of the vaccinated birds throughout the duration of this study. The presence of the vector strains within the caeca of vaccinated birds at time of slaughter poses the risk of carcass contamination with these vaccines and the transmission of these vaccines to consumers should the products not be handled or cooked appropriately. These findings are in line with experiments carried out during PoulVac<sup>®</sup> *E. coli*'s European Medicines Agency (EMA) licensing procedures that describe the persistence of the vaccine strain in cloacal swabs of vaccinated birds for 4 weeks following vaccination through coarse spray (EMA, 2012). While a risk of transmission to consumers was highlighted by the EMA, they also deemed that due to the safety and stability profiles of this vaccine, no withdrawal period needed to be imposed. The position taken by the EMA indicates that, provided the safety and stability of a genetically modified vaccine can be clearly proven, there is scope for their use within the food chain even though they may persist in the animal at the point of consumption.

In order to develop ELISAs for the measurement of serum IgY and bile sIgA, the TetC and CjaA proteins were cloned into the pGEX-4T1 vector and expressed as GST-tagged recombinant proteins. Western blotting with an anti-GST antibody indicated the presence of GST only truncations. As discussed above for the expression of TetC-CjaA within the vaccine strains, it is possible that rare codons in the heterologous genes for TetC

and CjaA resulted in premature termination of translation (Goldman *et al*, 1995). The presence of the GST only truncation after passage through a concentrator column with a molecular weight cut-off of 50kD may be associated with the ability of GST to dimerise (Fabrini *et al*, 2009), resulting in complexes with a molecular weight of 50kD.

Using the GST-CjaA and GST-TetC protein preparations described above, the inductions of CjaA- and TetC-specific antibodies in serum and bile was measured through ELISAs. Significant induction of antigen-specific serum IgY in both vaccination trials indicate that the vaccines were delivered successfully in both experiments. The patterns of induction of CjaA-specific serum IgY and sIgA following vaccination with the *Salmonella*-based vaccine in the first experiment were similar to that described by Buckley *et al* (2010), albeit a different line of bird and route of delivery were used. Within both these experiments and the previously reported ones the peak of serum IgY levels did not correspond with any reduction in caecal *C. jejuni* levels: the peak in OD<sub>450nm</sub> reading was at 49dph, whereas in previous trials significant reductions were observed at 56dph (Buckley *et al*, 2010). However, this measure takes into account the total antibody induction due to both vaccination and challenge. If vaccination-specific induction is calculated as fold-change in CjaA-specific antibodies compared to *Campylobacter* challenged but non-vaccinated birds, the largest fold-change in serum IgY was observed from 42 days onwards and in bile sIgA at 56 dph, which was the time when significant reduction in caecal *Campylobacter* colonisation were observed within the previous experiments (Buckley *et al*, 2010). Similar inductions of antigen-specific bile sIgA were observed in CjaA-vaccinated birds, whether they were vectored in *Salmonella* or APEC. However, the fold-increase compared to control birds was approximately an order of magnitude lower than the increase observed for serum IgY. Furthermore, the levels of *Campylobacter* in the caeca of individual birds did not correlate with antibody induction for either antibody in those birds (data not shown).

In conclusion, whilst no protection was observed with either of the vectors expressing CjaA, both vaccines induced humoral immune responses. This study demonstrated induction of immunity through the use of APEC vaccines vectoring heterologous antigens. As such, the possibility of using APEC as a vector for vaccines against *Campylobacter* remains open but further studies are needed to optimise the vaccine. Due to the lack of a protective effect observed with CjaA-based vaccines the rest of the work described in this thesis focused on the search for an antigen or a combination of antigens that may confer improved magnitude or replicability of protection.

## 4. Production of recombinant subunit vaccines

### 4.1 Introduction

The previous chapter described that whilst both the *Salmonella* and APEC attenuated live vaccines vectoring CjaA induced antigen-specific humoral responses, they did not reduce caecal colonisation by *C. jejuni*. Furthermore, the *Salmonella*-based vaccine vectoring CjaA exhibited a variable protective effect in previous studies in our group (Buckley *et al*, 2010). Lastly, low level cross-transmission of the live vaccine strains between different treatment groups was observed within the first vaccination and challenge experiment. Due to these limitations, further development of live vectored vaccines would have required a high number of replicates to test each condition, becoming labour and resource intensive for a doctoral project.

As such, the remainder of this project focused on the search for an antigen or combination of antigens that could confer improved protection against *C. jejuni* colonisation over CjaA. Towards this aim, novel antigens were tested as recombinant subunit vaccines in order to avoid the cross-contamination risk posed by using live vectored vaccines. Rational antigen selection has gained more ground in recent years: a search for “rational (and) vaccine development” in PubMed returned 11 articles for the year 1994, 23 articles for the year 2004 and 80 articles for the year 2014, highlighting the recent focus on this aspect of vaccine development. Morefield (2011) points out that in light of rapidly evolving diseases, such as avian influenza, the traditional empirical approach to vaccine development would be too slow to allow adequate responses in the face of new strains. In addition, Rueckert and Guzman (2012) review rational development approaches for subunit recombinant vaccines and highlight that in their view a rational approach is not only desirable but necessary for the delivery of tailored vaccines in a cost and time-effective manner for pathogens for which the traditional empirical approach has failed to date. De Gregorio and Rappuoli (2014) also review rational vaccine development but focus on recent technological developments that can aid this approach. One such approach is reverse vaccinology, which mines genome sequence data to identify potential vaccine candidates which are then tested for their protective efficacy as recombinant subunit vaccines. This approach was successfully used for the production of a vaccine against *Neisseria meningitidis* group B: Pizza *et al* (2000) identified candidate antigens from whole genome sequencing data and subsequent rational development of the vaccine led to a recently licensed commercial product (Bai *et al*, 2011).

In view of above, the approach of informed antigen selection was adopted for this project. A literature survey was carried out to select candidate antigens to be tested as purified recombinant subunit vaccines against *Campylobacter* in poultry. Promising candidate antigens were selected from original research articles describing evidence of a role in colonisation *in vivo* in chickens, evidence of efficacy as an anti-*Campylobacter* vaccine in an animal model of vaccination or evidence of the antigen being recognised by antibodies during primary infection or by maternal antibodies. The antigens identified and the studies describing them are summarised in Table 4.1.

Following identification of the above vaccine candidates, other characteristics of the antigens were assessed in order to decide whether they would be likely to constitute an effective vaccine. These characteristics included the presence of the antigen in strains that commonly infect humans and chickens and the sequence conservation of the antigen within these strains. An antigen with high sequence conservation and that is present within the majority of field isolates would increase the likelihood of adequate cross-protection. Furthermore, hydrophobicity was assessed as a predictor of ease of purification of recombinant proteins. Lastly, while consideration was given to the subcellular localisation (as shown in Table 4.1), antigens were not restricted to surface-expressed proteins as the exclusive role of neutralising antibodies in vaccine-mediated protection against *Campylobacter* has not been demonstrated and recombinant vaccines utilising antigens that may not be surface exposed have given promising results (e.g. CjaA, SodB in *Helicobacter pylori*). Gene identity within the annotated *C. jejuni* M1 genome, gene size, protein molecular weight and protein sequence conservation within full-length entries available on NCBI are given for each antigen in Table 4.2.

To obtain the information detailed in Table 4.2, the DNA sequences encoding each of the antigens was extracted from the genome sequence of *C. jejuni* M1 available in NCBI's Nucleotide database. To assess antigen conservation, the DNA sequence given in NCBI's gene information was searched against the non-redundant nucleotide collection (nr/nt) in NCBI's Basic Local Alignment Search Tool (BLAST), utilising the blastn function, with *C. jejuni* subsp. *jejuni* (taxid: 32022) set as the organism. The obtained alignments were screened in 20 *C. jejuni* strains to identify the lowest percentage conservation of the gene within available sequences and this score is given in Table 4.2 with the detailed data in individual strains provided in Appendix I. Protein molecular weight was estimated using the ExPASy ProtParam tool (<http://web.expasy.org/protparam>). Presence of the identified proteins within different *C. jejuni* strains was not formally assessed. However,

**Table 4.1. Characteristics of candidate antigens for use as recombinant vaccines against *C. jejuni* in chickens, identified through a literature search.**

Functional group	Antigen name	Predicted subcellular localisation	Function	Role of gene in chicken colonisation	Other aspects to support as vaccine candidate	Other comments
Adhesins	CadF	Outer membrane, on extracellular surface (Konkel <i>et al</i> , 2005)	Fibronectin binding protein (Konkel <i>et al</i> , 2005)	Reduced colonisation of chickens by a $\Delta cadF$ mutant (Flanagan <i>et al</i> , 2009)	Protective as recombinant protein against homologous challenge in chickens (Neal-McKinney <i>et al</i> , 2014)	N/A
	FlpA	Outer membrane, on extracellular surface (Konkel <i>et al</i> , 2010)	Fibronectin binding protein (Konkel <i>et al</i> , 2010)	Required for maximal host cell adherence (Konkel <i>et al</i> , 2010); Reduced colonisation of chickens by a $\Delta flpA$ mutant (Flanagan <i>et al</i> , 2009)	Highly protective as recombinant protein against homologous challenge in chickens (Neal-McKinney <i>et al</i> , 2014)	Heavily glycosylated: a glycoprotein of ~250 kD in native form (Konkel <i>et al</i> , 2010)
Hypothetical proteins (identified in OM proteomics screen at MRI)	Cj0088	Outer membrane (Cj81176_0124 in Watson <i>et al</i> , 2014)	Part of the operon <i>cj0088-cj0089</i> involved in synthesis of Cj0089 (Paek <i>et al</i> , 2012)	Unknown	None at time of writing	N/A
	Cj0089	Outer membrane (Cj81176_0125 in Watson <i>et al</i> , 2014)	Possible adhesin (Paek <i>et al</i> , 2012)	Unknown	Crystal structure resolved: immunoglobulin-like $\beta$ -sandwich; sequence similarity to fibronectin/fimbriae protein – also contains an immunoglobulin-like $\beta$ -sandwich (Paek <i>et al</i> , 2012)	N/A
	Cj0090	Outer membrane (Cj81176_0126 in Watson <i>et al</i> , 2014)	Part of the operon <i>cj0088-cj0090</i> involved in synthesis of Cj0089 (Paek <i>et al</i> , 2012)	Unknown	None at time of writing	N/A
	Cj0536	Outer membrane (Watson <i>et al</i> , 2014)	Unknown	Unknown	Structural prediction with Phyre 2 (Kelley <i>et al</i> , 2015): Transmembrane beta-barrel porin-type structure; similar to OmpG of <i>E. coli</i>	May be involved in nutrient uptake (possibly larger oligosaccharides) as this function was suggested for OmpG by Subbarao and van der Berg (2006)

**Table 4.1. Continued.**

Functional group	Antigen name	Predicted subcellular localisation	Function	Role of gene in chicken colonisation	Other aspects to support as vaccine candidate	Other comments
Outer membrane proteins	CjaA	Periplasm and inner membrane (Wyszynska <i>et al</i> , 2008)	The binding component of an ABC-type cysteine transporter system (Mullet <i>et al</i> , 2005)	Unknown	Shown to reduce colonisation of chickens by <i>C. jejuni</i> by 6log <sub>10</sub> when vectored in an attenuated <i>S. Typhimurium</i> vector (Wyszynska <i>et al</i> , 2004)	Later shown to reduce colonisation of chickens by <i>C. jejuni</i> by 2log <sub>10</sub> CFU/g caecal contents when vectored in an attenuated <i>S. Typhimurium</i> vector or given as recombinant subunit vaccine (Buckley <i>et al</i> , 2010) and by 1log <sub>10</sub> when vectored in <i>Eimeria tenella</i> (Clark <i>et al</i> , 2012).
	CjaC	Anchored to inner membrane (Wyszynska <i>et al</i> , 2007)	Histidine binding protein, part of ABC-type transport system (Wyszynska <i>et al</i> , 2007)	Unknown	Conserved in isolates of three serotypes of <i>C. jejuni</i> common in human cases (Pawelec <i>et al</i> , 1988)	Recognised by maternal antibodies transferred in egg (Shoaf-Sweeney <i>et al</i> , 2008); Also described as OmpH1 (Pawelec <i>et al</i> , 2000)
	CjaD	Outer membrane (as reviewed in Godlewska <i>et al</i> , 2009)	Interacts with Tol proteins (as reviewed in Godlewska <i>et al</i> , 2009)	Failed attempts to obtain a mutant (as reviewed in Godlewska <i>et al</i> , 2009)	Shown to reduce colonisation of chickens by <i>C. jejuni</i> by 6log <sub>10</sub> when vectored in an attenuated <i>S. Typhimurium</i> vector (Layton <i>et al</i> , 2011)	No protection observed in another attenuated <i>S. Typhimurium</i> vectored vaccine (Laniewski <i>et al</i> , 2012)
	PorA	Outer membrane (Amako <i>et al</i> , 1996)	Forms a porin when multimerised; role in adhesion to intestinal cells (Mahdavi <i>et al</i> , 2014)	Could not be deleted but a mutant that is not glycosylated has reduced chicken colonisation potential (Mahdavi <i>et al</i> , 2014)	GST-PorA protective against heterologous colonisation in a mouse model (Islam <i>et al</i> , 2010)	Shown to be O-glycosylated (Mahdavi <i>et al</i> , 2014)
Multidrug efflux system	CmeA	Periplasmic (Lin <i>et al</i> , 2002)	Part of CmeABC multidrug resistance efflux pump (Lin <i>et al</i> , 2002)	Increased expression of CmeABC in presence of bile salts (Lin <i>et al</i> , 2005) but no direct evidence of role in chicken colonisation	None at time of writing	N/A

**Table 4.1. Continued.**

Functional group	Antigen name	Predicted subcellular localisation	Function	Role of gene in chicken colonisation	Other aspects to support as vaccine candidate	Other comments
Multidrug efflux system	CmeC	Outer membrane (Zeng <i>et al</i> , 2010)	Part of CmeABC multidrug resistance efflux pump (Lin <i>et al</i> , 2002)	$\Delta cmeC$ mutant fails to colonise chickens when given at $10^7$ CFU (Lin <i>et al</i> , 2003)	Study in chickens showed that it induces strong IgA and IgY response when given subcutaneously but was not protective against colonisation (Zeng <i>et al</i> , 2010)	Failure of protection replicated in a later study (Neal-McKinney <i>et al</i> , 2014)
Flagellar proteins	FlaA	On cellular surface (Nuijten <i>et al</i> , 1990)	The major flagellar unit (Nuijten <i>et al</i> , 1990)	FlaA rather than motility is required for chicken colonisation (Wasenaar <i>et al</i> , 1993)	Multiple studies showed protection using FlaA-based vaccines (see Section 1.5)	Other studies showed no protection using FlaA-based vaccines (see Section 1.5)
	FlgE2	On cellular surface (Power <i>et al</i> , 1992)	Flagellar hook protein (Power <i>et al</i> , 1992)	Unknown	FlgE2 mutant is non-motile and does not secrete Cia proteins (Konkel <i>et al</i> , 2004)	Recognised by maternal antibodies transferred in egg (Shoaf-Sweeney <i>et al</i> , 2008)
	FlgK	On cellular surface	Flagellar junction protein	FlgK is required for motility and chicken colonisation (Fernando <i>et al</i> , 2007)	FlgK mutant unable to secrete the Cia proteins (Neal-McKinney and Konkel, 2012)	N/A
	FliD	At end of flagellum (shown in <i>Salmonella</i> by Yokoseki <i>et al</i> , 1995)	Flagellar cap protein; acts as polymerisation template for the filament (shown in <i>Salmonella</i> by Yokoseki <i>et al</i> , 1995)	Unknown	Immunogenic in natural <i>Campylobacter</i> infection, with high prevalence of FliD specific serum IgY in broiler birds (Yeh <i>et al</i> , 2014b)	Mutant has reduced motility in vitro (Neal-McKinney and Konkel, 2012)
	FspA1	Extracellular, secreted through the flagellum (Poly <i>et al</i> , 2007)	Unknown	Unknown	Immunogenic and protected mice against illness after <i>C. jejuni</i> challenge (Baqar <i>et al</i> , 2008)	N/A
Cellular metabolism proteins	GGT	Periplasmic in <i>E. coli</i> (Suzuki <i>et al</i> , 1986)	Metabolism of glutathione and glutamine - catalyses first step in pathway that releases cysteine residues from glutathione (Tate and Meister, 1985)	Role in persistent colonisation of avian gut by <i>C. jejuni</i> (Barnes <i>et al</i> , 2007)	GGT from <i>C. jejuni</i> inhibits epithelial cell and lymphocyte proliferation but has no proapoptotic activity (Floch <i>et al</i> , 2014)	Essential role in colonisation of gastric mucosa of mice by related pathogen <i>H. pylori</i> (Chevalier <i>et al</i> , 1999)

**Table 4.1. Continued**

Functional group	Antigen name	Predicted subcellular localisation	Gene function	Role of gene in chicken colonisation	Other aspects to support as vaccine candidate	Other comments
Cellular metabolism proteins	SodB	Outer membrane (Watson <i>et al</i> , 2014)	Basal expression level is essential for resistance to oxidative stress (modelled by paraquat stress) (Garenaux <i>et al</i> , 2008)	Reduced colonisation of chickens by a $\Delta$ sodB <i>C. coli</i> mutant (Purdy <i>et al</i> , 1999)	Recombinant SodB linked to <i>Vibrio cholerae</i> toxin is protective against <i>H. pylori</i> challenge in a murine model (US Pat. No. US20010019834)	Crystal structure of <i>H. pylori</i> SodB shows an N-terminal tail thought to be involved in interaction with outer membrane; similar sequence present in <i>C. jejuni</i> SodB (Esposito <i>et al</i> , 2008)
	Tpx	Periplasmic in <i>E. coli</i> (Cha <i>et al</i> , 1995)	Antioxidant enzyme that removes peroxides and H <sub>2</sub> O <sub>2</sub> (Cha <i>et al</i> , 1995)	Unknown	None at time of writing	Found to be upregulated in a gut loop model of <i>C. jejuni</i> infection in rabbit (Stintzi <i>et al</i> , 2005)
	AccD	Cytoplasmic ( <a href="http://www.uniprot.org/uniprot/A710T4">http://www.uniprot.org/uniprot/A710T4</a> )	Component of the acetyl coenzyme A carboxylase (ACC) complex ( <a href="http://www.uniprot.org/uniprot/A710T4">http://www.uniprot.org/uniprot/A710T4</a> )	Unknown	None at time of writing	N/A
Other proteins	Cj0424	Outer membrane	Resistance to innate antimicrobial peptides *	Unknown	Mutant is more susceptible <i>in vitro</i> to antimicrobial peptides from chickens and humans *	N/A

\* At the time of selection, evidence was presented by Halah al Haideri (Prof. David Kelly's group, University of Sheffield, to indicate a role in defence against cationic antimicrobial peptides, including polymixin B, human  $\beta$ -defensin 1, human cathelicidin IL-37 and chicken fowlicidins 1 and 2.



**Table 4.2. Characteristics of the genes and encoded proteins for each of the candidate vaccine antigens identified in Table 4.1.**

Functional group	Protein name (and abbreviation)	Gene number in <i>C. jejuni</i> M1 (old locus tag)	Size of gene (bp)	Molecular weight (kD)	Gene conservation (min %)
Adhesin	Outer membrane fibronectin-binding protein (CadF)	(CJM1_1423)	960	36.023	99
	Fibronectin-like protein A (FlpA)	(CJM1_1260)	1236	46.072	97
Hypothetical proteins	CJ0088 (CJ0124*)	CJM1_RS00440 (CJM1_0088)	1362	51.237	98
	CJ0089 (CJ0125*)	CJM1_RS00445 (CJM1_0089)	369	14.043	98
	CJ0090 (CJ0126*)	CJM1_RS00450 (CJM1_0090)	624	22.446	99
	Cj0536	(CJM1_0536)	930	35.039	98
Outer membrane protein	<i>Campylobacter jejuni</i> antigen A (CjaA)	(CJM1_0957)	840	30.993	97
	<i>Campylobacter jejuni</i> antigen C (CjaC)	(CJM1_0717)	756	27.799	99
	<i>Campylobacter jejuni</i> antigen D (CjaD); Peptidoglycan-associated lipoprotein (Pal); Omp18	CJM1_RS00560 (CJM1_0112)	497	17.828	99
	Major outer membrane protein (PorA; MOMP)	(CJM1_1240)	1275	45.6	83
Multidrug efflux system	<i>Campylobacter</i> multidrug efflux protein A (CmeA)	CJM1_0345	1104	40.103	97
	<i>Campylobacter</i> multidrug efflux protein C (CmeC)	(CJM1_0343)	1479	55.437	97
Flagellar-related proteins	Flagellin A (FlaA)	CJM1_RS06450 (CJM1_1296)	1731	59.5290	79
	Flagellar hook protein (FlgE2)	(CJM1_1679)	2073	73.699	84
	Flagellar hook-associated protein; junction (FlgK)	(CJM1_1412)	1827	67.086	98
	Flagellar cap protein (FlhD)	CJM1_RS02610 (CJM1_0523)	1932	69.921	96
	Flagellar secreted protein 1 (FspA1)	(CJM1_0381)	429	16.528	98
Cellular metabolism proteins	Gamma-glutamyl transpeptidase (GGT)	CJM1_RS00190 (CJM1_0038)	1671	60.257	99
	Superoxide dismutase (SodB)	CJM1_RS00850 (CJM1_0171)	663	24.812	98
	Thiol peroxidase (Tpx)	CJM1_RS03760 (CJM1_0755)	528	18.428	99
	acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (AccD)	(CJM1_0126)	843	30.951	98
Antimicrobial defence	Cj0424 (RidL)	CJ11168_0424	633	24.198	99

\* Gene numbers in *C. jejuni* 81-176, where they were identified by Watson *et al* (2014).

the majority of the chosen genes are central metabolic genes or flagellar genes, suggesting universal presence. Cj0424 was shown to only be present in under 50% of sequenced strains available (data obtained from Halah Al-Haideri).

This chapter described the cloning and assessment of solubility for all antigens described above and large-scale recombinant protein production of a subset of 10 antigens chosen due to their higher solubility relative to the other antigens tested.

## **4.2. Materials and methods**

### **4.2.1 Construction of the *E. coli* strains for expression of recombinant *Campylobacter* antigens**

#### **4.2.1.1 Cloning of antigens encoding the glutathione-S-transferase fusions**

Each of the selected genes encoding the candidate antigens was separately cloned into the pGEX-4T1 plasmid (GE Lifesciences; details given in Section 2.1), under the control of an IPTG-inducible promoter, in order to purify the proteins as GST fusions to be tested in a vaccination and challenge model in chickens. The genes were amplified from *C. jejuni* M1 genomic DNA using the forward and reverse primers detailed in Table 4.3 using the proofreading Phusion DNA polymerase by PCR and were cloned as an in-frame C-terminal fusion to GST through ligation-dependent cloning, using the *Bam*HI (5' end) and *Not*I (3' end) restriction sites. Ligation reactions were transformed into *E. coli* XL1 Blue and individual transformed colonies validated by PCR (using the pGEX<sub>fwd</sub> and pGEX<sub>rev</sub> primers given in Table 4.3) and double strand sequencing for the presence of the expected construct as described in Section 3.2 for the construction of the pTECH2-*cjaA* plasmid. Sequencing of these constructs was contracted to Source Bioscience (UK). Verified plasmids were transformed into electrocompetent *E. coli* Rosetta (genotype given in Section 2.1) using the fast electroporation protocol. The empty pGEX-4T1 plasmid was also transformed into the Rosetta strain to purify GST that would be used as a negative control during vaccination experiments in chickens. Transformed *E. coli* Rosetta were screened for the presence of a correct plasmid by PCR as described for the construction of the pTECH2-*cjaA* plasmid in Section 3.2.

#### 4.2.1.2 Cloning of antigens cloned as maltose binding protein fusions

Nine of the GST-tagged recombinant antigens tested *in vivo* were also cloned into pMal-p2X (NEB; details given in Section 2.1) as in-frame fusions to the C-terminus of MBP in order to be utilised in ELISAs for the measurement of humoral immune responses induced by vaccination. This approach was chosen to avoid detection of antibodies against the GST fraction of each recombinant antigen. Genes were re-amplified by PCR with the proof-reading Phusion DNA polymerase using the same forward primers described above, but with different reverse primers described in Table 4.3 and denoted by pMal. These PCR fragments were cloned into the *Bam*HI (3' end) and *Sal*I (5' end) restriction sites of the pMal-p2X plasmid as described above. The same construct validation workflow as utilised for the pGEX-4T1 constructs was used, but the pMal<sub>fwd</sub> and pMal<sub>rev</sub> primers were used for PCR validation. Only pMal-p2X-*sodB* and pMal-p2X-*fliD* were transformed in *E. coli* Rosetta and validated as described above. Otherwise, expression of the MBP recombinant proteins for purification was performed using *E. coli* XL1 cells.

#### 4.2.1.3 Cloning of *cjaA* as a 6xHis fusion

The cloning of *cjaA* as a 6xHis-tagged fusion in the pET28b (Novagen, UK) plasmid was undertaken for a more direct comparison with previous studies within our group (Buckley *et al*, 2010). This plasmid has an origin of replication from pBR322 giving approximately 50 copies/cell and it also contains an *f1* origin of replication. The *T7* promoter allows chemical induction with IPTG and the *lacI<sup>q</sup>* gene to control basal level of expression. A kanamycin resistance gene is used for selection. The plasmid contains 6xHis tags at both the 5' and 3' end of the MCS. For this cloning, *C. jejuni* M1 *cjaA* gene was digested from the pGEX-4T1-*cjaA* plasmid with the *Bam*HI and *Not*I restriction enzymes and ligated into the pET28b vector digested with the same restriction enzymes to fuse 6xHis residues at the C terminus. Plasmid constructs were transformed into *E. coli* XL1 and a validation workflow similar to the one described above for pGEX-4T1 constructs was used. Sequencing-validated plasmids were transformed into *E. coli* Rosetta and the strains validated as described for the pGEX-4T1 constructs above.

#### 4.2.2 Assessment of ease of purification of GST-tagged recombinant proteins

Following construction of the *E. coli* Rosetta strain expressing each of the candidate antigens described above, small scale inductions and purifications were run to assess the

**Table 4.3. Primers used for the construction of recombinant *Campylobacter* antigen expression plasmids.** Underlined sequences represent sites for restriction enzymes utilised in cloning. All sequences are given as 5' to 3'.

Primer	Sequence (5'-3')
Primers for cloning of <i>C. jejuni</i> M1 antigens into pGEX-4T1	
pGEX-4T1- <i>cme</i> Cfwd	CGCGCGGGATCCATGAATAAAATAATTTCAAT
pGEX-4T1- <i>cme</i> Crev	CGCGCGGGCGCCGCTATTCTCTAAAAGACATAT
pGEX-4T1- <i>por</i> Afwd	CGCGCGGGATCCATGAAACTAGTTAACTTAG
pGEX-4T1- <i>por</i> Arev	CGCGCGGGCGCCGCTTAGAATTTGTAAAGAGCTT
pGEX-4T1- <i>acc</i> Dfwd	CGCGCGGGATCCATGAATTTTGCAGATATTTT
pGEX-4T1- <i>acc</i> Drev	CGCGCGGGCGCCGCTTATTTTCCCTAAAAAATTT
pGEX-4T1- <i>cad</i> Ffwd	CGCGCGGGATCCATGAAAAAATATTCTTATG
pGEX-4T1- <i>cad</i> Frev	CGCGCGGGCGCCGCTTATCTTAAAATAAATTTAG
pGEX-4T1- <i>flp</i> Afwd	CGCGCGGGATCCATGACAACCCACATCTATGA
pGEX-4T1- <i>flp</i> Arev	CGCGCGGGCGCCGCTCAAATTTTTTCATTTAAAA
pGEX-4T1- <i>cj0586</i> fwd	CGCGCGGGATCCATGCTAGTAAATAAATTTAA
pGEX-4T1- <i>cj0586</i> rev	CGCGCGGGCGCCGCTTAATAATCGATGATATTTT
pGEX-4T1- <i>cja</i> Cfwd	CGCGCGGGATCCATGAAAAAATATTAAGCAT
pGEX-4T1- <i>cja</i> Crev	CGCGCGGGCGCCGCTTATTCTAATTCATATTTT
pGEX-4T1- <i>por18</i> fwd	CGCGCGGGATCCATGAAAAAATCTTTTTAC
pGEX-4T1- <i>por18</i> rev	CGCGCGGGCGCCGCTTATCTTGATAATTTAAAT
pGEX-4T1- <i>flgE2</i> fwd	CGCGCGGGATCCATGATGAGATCACTTTGGTC
pGEX-4T1- <i>flgE2</i> rev	CGCGCGGGCGCCGCTTAAGATCCATCATTATTA
pGEX-4T1- <i>fsp</i> Afwd	CGCGCGGGATCCATGCAAATTAACAATTCCTT
pGEX-4T1- <i>fsp</i> Arev	CGCGCGGGCGCCGCTCAAGCTTGTGGCTTTGGA
pGEX-4T1-L7- <i>por</i> Afwd	CGCGCGGGATCCATGGGTAAAAAAGATAAACTAC
pGEX-4T1-L7- <i>por</i> Arev	CGCGCGGGCGCCGCTTAAATATCGCCATTAGGTTAG
pGEX-4T1- <i>flgK</i> fwd	CGCGCGGGATCCATGGGTATTTTTGGAACATT
pGEX-4T1- <i>flgK</i> rev	CGCGCGGGCGCCGCTTAAAGATTTAAGCCCTAGTA
pGEX-4T1- <i>fliD</i> fwd	CGCGCGGGATCCATGGCATTGGTAGTCTATC
pGEX-4T1- <i>fliD</i> rev	CGCGCGGGCGCCGCTTAATTATTAGAATTGTTTG
pGEX-4T1- <i>flgL</i> fwd	CGCGCGGGATCCATGAGAATTAACAATAAACT
pGEX-4T1- <i>flgL</i> rev	GCGCGCGCTCGAGTTACATATAATTTAATAAGC
pGEX-4T1- <i>cj0088</i> fwd	CGCGCGGGATCCATGAAGATAAAAGTTGGGTT
pGEX-4T1- <i>cj0088</i> rev	CGCGCGGGCGCCGCTTATTTTTCCATGATAGCAA
pGEX-4T1- <i>cj0089</i> fwd	CGCGCGGGATCCATGAAAAAATATTTTTATT
pGEX-4T1- <i>cj0089</i> rev	CGCGCGGGCGCCGCTTAAATTTTTGCTTTAATTT
pGEX-4T1- <i>cj0090</i> fwd	CGCGCGGGATCCATGAAAAAACAATAATTTT
pGEX-4T1- <i>cj0090</i> rev	CGCGCGGGCGCCGCTTACCAAGTAACTGATTTAC
pGEX-4T1- <i>tpx</i> fwd	CGCGCGGGATCCATGAGTACAGTAAATTTTAA
pGEX-4T1- <i>tpx</i> rev	CGCGCGGGCGCCGCTTAATGGCAACCACAACCAC
pGEX-4T1- <i>sodB</i> fwd	CGCGCGGGATCCATGTTTGAATTAAGAAAATT
pGEX-4T1- <i>sodB</i> rev	CGCGCGGGCGCCGCTTATTTTACAGGGTGAAGTT
pGEX-4T1- <i>ggt</i> fwd	CGCGCGGGATCCATGCGTTATTTAGCTATATT
pGEX-4T1- <i>ggt</i> rev	CGCGCGGGCGCCGCTATACAAGAAAAGAATTTCTAA
Primers for cloning of <i>C. jejuni</i> antigens in pMAL-p2X	
pMAL-p2X- <i>tpx</i> rev	CGCGCGCGGGTTCGACTTAATGGCAACCACAACCAC
pMAL-p2X- <i>sodB</i> rev	CGCGCGCGGGTTCGACTTATTTTACAGGGTGAAGTT
pMAL-p2X- <i>cj0089</i> rev	CGCGCGCGGGTTCGACTTAAATTTTTGCTTTAATTT
pMAL-p2X- <i>flgE2</i> rev	CGCGCGCGGGTTCGACTTAAGATCCATCATTATTA
pMAL-p2X- <i>porA-L7</i> rev	CGCGCGCGGGTTCGACTTAAATATCGCCATTTAGGTTAG
pMAL-p2X- <i>cja</i> Arev	CGCGCGCGGGTTCGACTTAGATCTTGCCGCCCTCAATA
pMAL-p2X- <i>fsp</i> Arev	CGCGCGCGGGTTCGACTCAAGCTTGTGGCTTTGGA
pMAL-p2X- <i>fliD</i> rev	CGCGCGCGGGTTCGACTTAAATTTAGAAATTTGTT TG
Diagnostic primers	
pGEXfwd	GGGCTGGCAAGCCACGTTTGGTG
pGEXrev	CCGGGAGCTGCATGTGTGAGAGG
pMALfwd	GGTCGTCAGACTGTGATGAAGCC
M13fwd (for pMALrev)	TGTAAACGACGGCCAGT

solubility of each GST-tagged recombinant antigen. These were run as described in Section 2.11.1 in a total volume of 50 ml LB broth, grown at 37 °C and induced with 1mM IPTG. The antigens that showed poor solubility under these conditions were re-assessed for improved yield under growth at 27 °C and induction with 0.1mM IPTG. To establish whether antigens had adequate solubility to ensure that they could be tested *in vivo* the amount of total soluble protein present within the pooled eluates of each of the recombinant proteins was measured through a Bradford assay. Only proteins that were estimated to be obtainable in sufficient quantity from 6 litres of culture to vaccinate all birds in a group once (between 264 and 926 µg, depending on molecular weight of the antigen) were tested *in vivo*.

#### **4.2.3 Production of GST-tagged recombinant subunit vaccines**

The antigens that were deemed to have adequate solubility from the preliminary small scale screen were purified in large quantities. For each purification, volumes between 500 ml and 6 l were grown in LB broth at either 37 °C or 28 °C and induced with either 1mM or 0.1 mM IPTG. Purification of each protein was undertaken as described in Section 2.11. To allow constitution of vaccines in a total volume of 50µl per dose, multiple eluates of each protein were passed through Amicon® Ultra (Milipore, UK) centrifugal concentrator columns with a molecular weight cut-off of either 30kD or 50kD as appropriate. Final protein preparations were quantified through a Bradford assay before use *in vivo*.

Each large scale protein preparation was resolved through SDS-PAGE on two separate 10% MiniProtean® TGX gels (BioRad). One of the gels was silver stained and the other transferred to PVDF membrane and assessed for the purity of the protein preparations via a Western blot using an anti-GST mouse IgG1 monoclonal antibody (Santa-Cruz Biotechnology, USA) at a 1:10,000 dilution. Bound primary antibody was detected with a rabbit anti-mouse HRP-conjugated secondary antibody (Sigma, UK) used at a 1:10,000 dilution. Western blots were developed as described in section 2.14.

#### **4.2.4 Production of 6xHis-tagged recombinant subunit vaccines**

Production of 6xHis-CjaA was undertaken from *E. coli* Rosetta. The *E. coli* BL21 strain for expression of 6xHis-Cj0424 from a pET plasmid was constructed by Halah Al Haideri (Prof. David Kelly's group, University of Sheffield). Expression and purification was undertaken using Ni-NTA resin affinity chromatography as described in Section 2.11.3. Final protein preparations were verified through a Western blot with a mouse IgG anti-6xHis antibody (Invitrogen, UK) used at a 1:10,000 dilution and detected with an HRP-conjugated

goat-anti mouse IgY antibody (AbD Serotec, UK) used at a 1:10,000 dilution. Final protein preparations were quantified through a Bradford assay before being used *in vivo*.

#### 4.2.5 Production of MBP-tagged recombinant antigens for use in ELISAs

##### 4.2.5.1 MBP-tagged protein production

Production of the MBP-tagged *Campylobacter* antigens was undertaken in *E. coli* XL1 due to lower amounts of antigen being required for ELISAs compared to the vaccination trials. Volumes of LB broth cultures between 500ml and 2 litres were induced with either 0.5 mM IPTG (as per manufacturer's recommendations) or 0.1 mM IPTG at either 37 °C or 28 °C. Purification was undertaken as described in section 2.11.2 and multiple eluates were passed through the same concentrator column.

##### 4.2.5.2 Validation MBP-tagged protein preparations

Validation of the MBP-fusion antigens was as described for the GST-fusion antigens, however the Western blot utilised an anti-MBP mouse IgG2a monoclonal primary antibody (NEB, UK) at a 1:10,000 dilution, followed by detection with the same secondary antibody as above. Before use in ELISAs, final protein preparations were quantified on the Direct Detect<sup>®</sup> machine (Milipore, UK), following manufacturer's instructions.

#### 4.2.6 Assessment of the role of Cj0424 in virulence and colonisation

Strains of *C. jejuni* 11168H lacking *cj0424* or having a double deletion of *cj0423-cj0424* and their complemented strains were constructed by Halah Al Haideri (Prof. David Kelly's group, University of Sheffield). The entire *cj0424* gene or *cj0423-cj0424* doublet were replaced by homologous recombination with a kanamycin resistance gene. The complemented strains had pCmetK::*cj0424* or pCmetK::*cj0424-0423* (pCmetK was described by Gaskin *et al*, 2007) integrated at the *cj0046* pseudogene locus, allowing selection through chloramphenicol resistance.

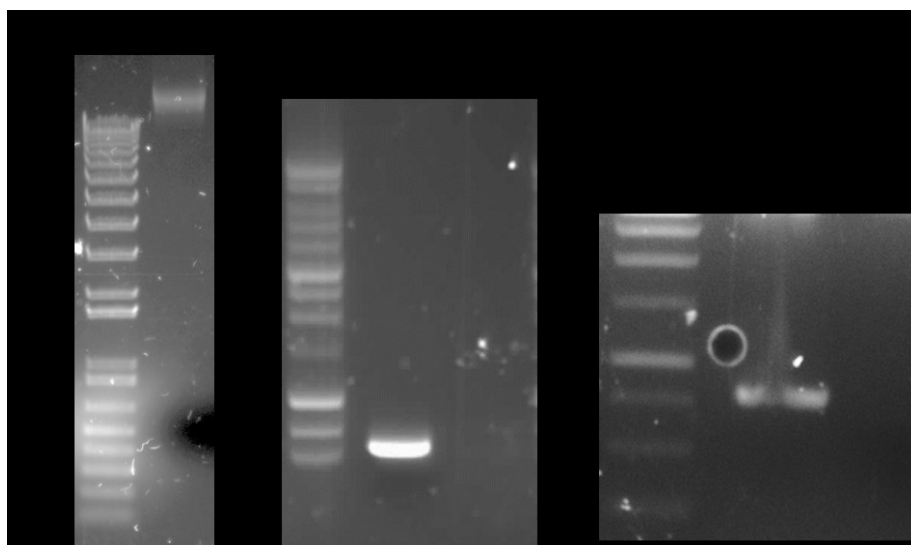
Initially, the single mutant and its complemented strain were tested for attenuation in a model of virulence using wax moth (*Galleria mellonella*) larvae as described in Section 2.18. Subsequently, the single mutant was tested *in vivo* in a chicken model of colonisation, as described in Section 2.15, using challenge doses of 10<sup>6</sup> and 10<sup>8</sup> CFU/bird. Lastly, the double mutant was tested in the chicken model of colonisation using challenge doses of 10<sup>4</sup> and 10<sup>6</sup> CFU/bird. In each experiment, the mutant strains were compared against wild-type and complemented control strains. Before each experiment the motility of the three strains

used was assessed through a soft agar diffusion test. For this, early stationary phase cultures were inoculated in the centre of a Petri dish containing 0.3% (w/v) MH agar using a 10 µl plastic loop. Plates were incubated for 24 hours and the diameter of the diffusion zone measured.

### 4.3. Results

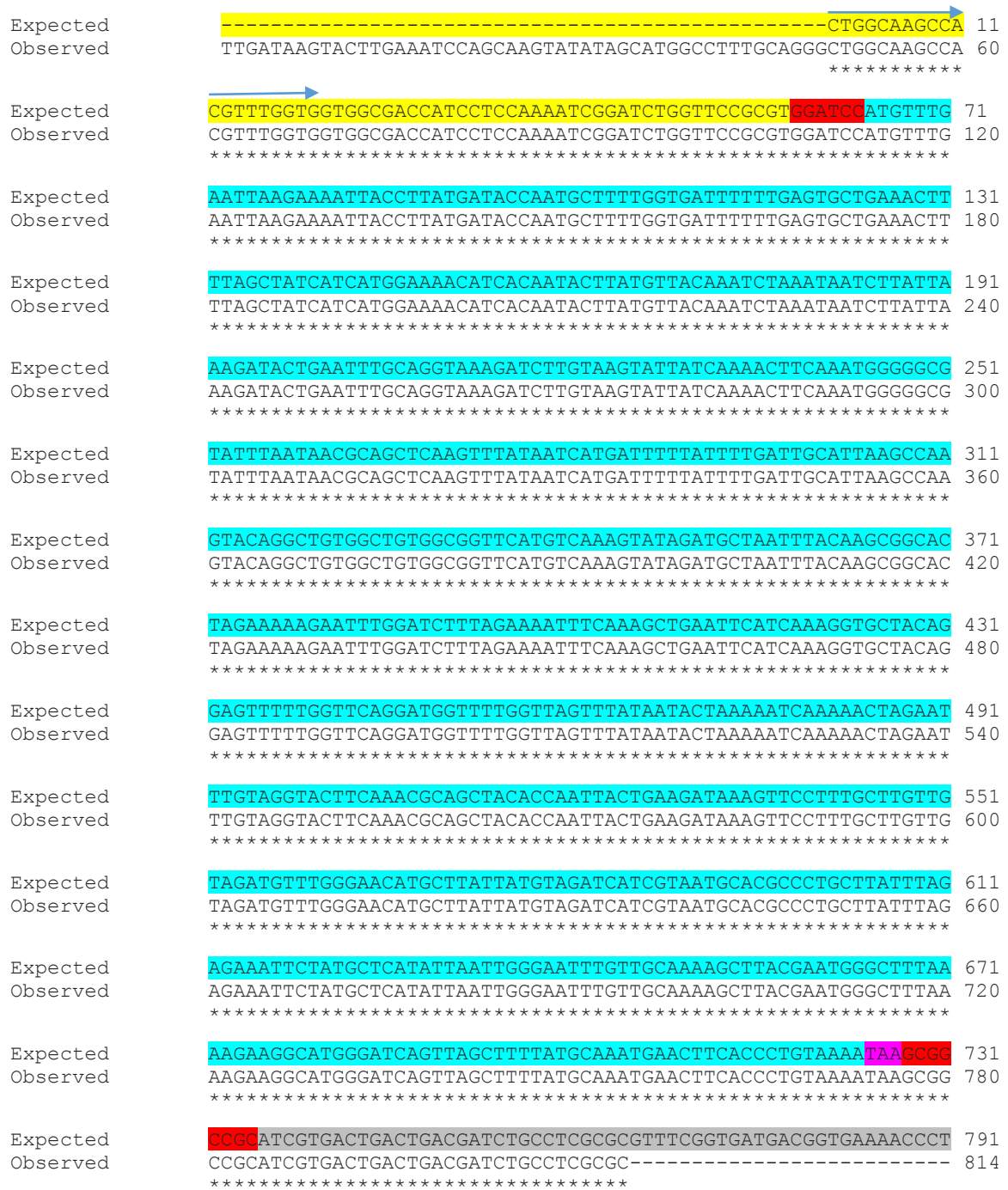
#### 4.3.1 Construction of strains for expression of GST-tagged *Campylobacter* antigens

The preparation of gDNA used for antigen cloning is shown in Figure 4.1.A and demonstrates the presence of high molecular weight DNA only. All antigens were successfully amplified by PCR from gDNA (data not shown), cloned into pGEX-4T1 and transformed into *E. coli* XL1 (data not shown). The *cj0089* gene only amplified when the PCR reactions contained double the amount of MgCl<sub>2</sub> present in the HF Buffer of the Phusion polymerase. The *Bam*HI-*Not*I double digest of the PCR amplicon for *sodB* and the PCR validation of the *sodB* gene ligated in pGEX-4T1 and transformed in *E. coli* XL1 are given as illustrative examples in Figure 4.1.B and C respectively. Using dideoxy chain termination sequencing, all of the plasmids were shown to have the expected sequence (data



**Figure 4.1 Construction of the pGEX-4T1-*sodB* plasmid.** A. gDNA extracted from *C. jejuni* M1. B. *Bam*HI and *Not*I double-digested *sodB* PCR amplicon (using primers pGEX-4T1-*sodB*fwd and pGEX-4T1-*sodB*rev) from the gDNA preparation shown in panel A. The size of the expected product is 669bp. The negative control reaction contains no template DNA. C. PCR validation of an *E. coli* XL1 single colony containing the pGEX-4T1-*sodB* plasmid, using primers pGEXfwd and pGEXrev. The size of the expected product is 867bp. The negative control reaction contains *E. coli* XL1 without the pGEX-4T1 plasmid.

not shown). The alignment between the expected and obtained sequence for pGEX-4T1-*sodB* only is given in Figure 4.2 as an illustrative example.

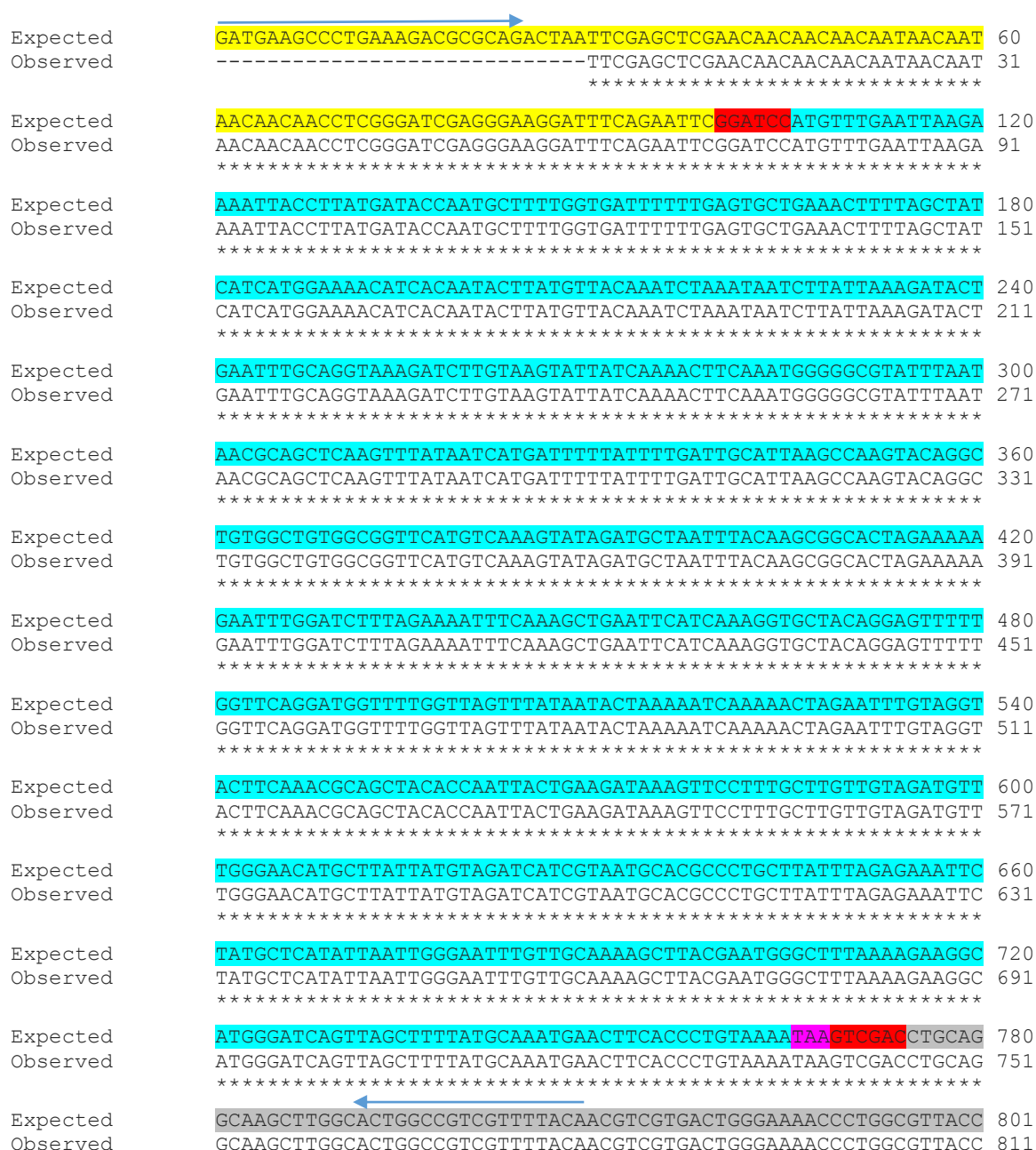


**Figure 4.2** Alignment of the expected and constructed sequence of the insert in pGEX-4T1-*sodB*. The colours and symbols in the figure represent: pGEX 5' sequencing primer (arrows), *gst* (yellow), restriction enzyme recognition sites: *Bam*HI at 5' end and *Not*I at 3' end (red), *sodB* sequence (blue), stop codon (purple), plasmid backbone (grey).



### 4.3.2 Construction of strains for expression of MBP-tagged *Campylobacter* antigens

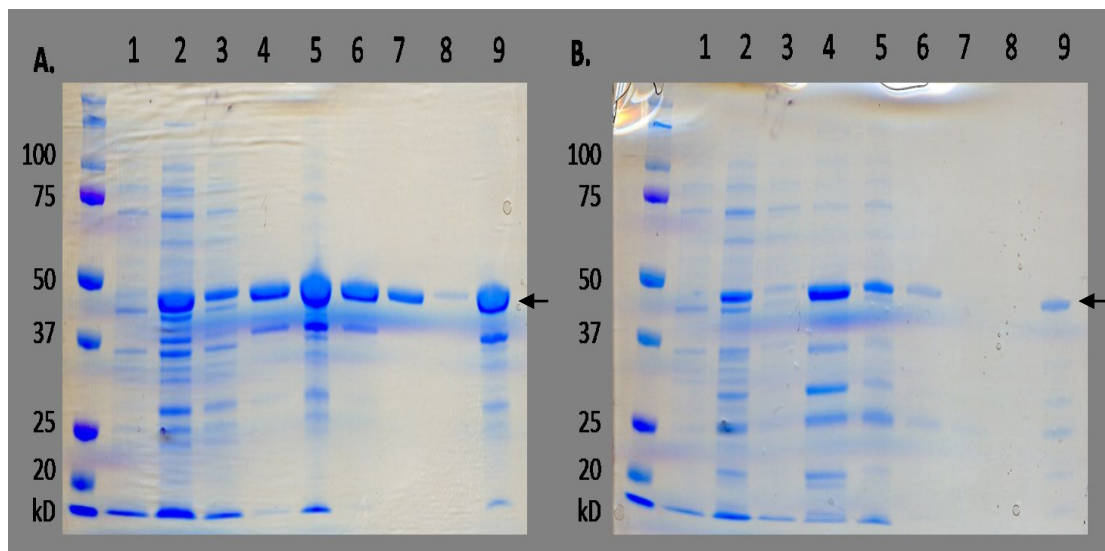
Apart from *cj0089*, which failed to re-amplify by PCR, each of the nine genes intended for purification of the cognate proteins through the MBP system were successfully amplified from the pGEX-4T1 plasmids, ligated into the pMal-p2x plasmids and transformed in *E. coli* XL1 Blue. Sequencing of plasmid DNA from these strains indicated that none of the constructs contained mutations (data not shown). Alignment of the expected and obtained sequences for pMal-p2x-*sodB* only is given in Figure 4.3 as an example.



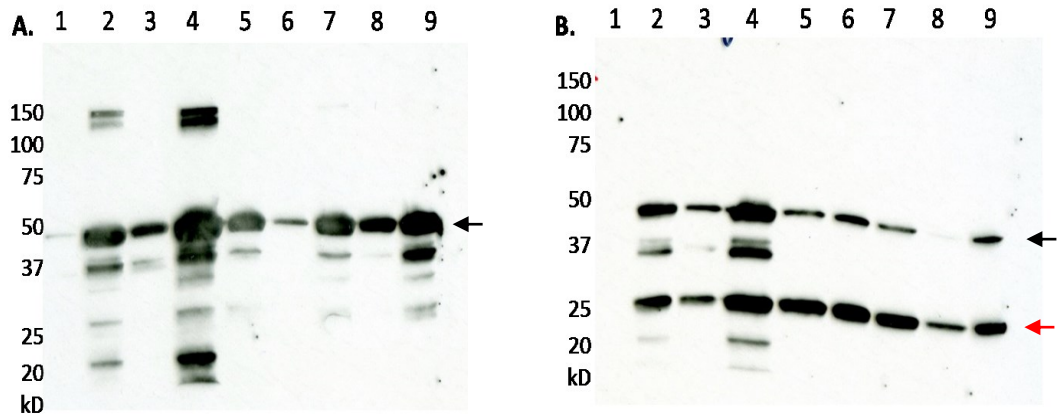
**Figure 4.3. Alignment of the expected and observed insert sequence of pMal-p2x-*sodB*.** Colour coding is as for Figure 4.2. Restriction enzymes sites are for *Bam*HI at the 5' and *Sal*I at the 3' end.

#### 4.3.3 Assessment of ease of purification of the GST-tagged recombinant antigens

Small scale induction and purification experiments for each of the cloned *Campylobacter* antigens revealed that the following proteins had sufficient yield and solubility to allow their testing *in vivo*: GST-Cj0089, GST-FspA, GST-Tpx, GST-SodB, GST-CjaA, GST-FliD, GST-FlgK, GST-FlgE2 and GST fused to the 7<sup>th</sup> extracellular loop of PorA (GST-PorA<sub>L7</sub>). An example of a relatively soluble recombinant antigen (GST-SodB) in contrast to a poorly soluble one (GST-Cj0090) is given in Figure 4.4. To further demonstrate the poor solubility of GST-Cj0090, a Western blot with an anti-GST antibody was run (Figure 4.5). This revealed the presence of a GST-only truncation in the GST-Cj0090 preparation, which accounted for the majority of the protein observed within this preparation. No such truncation was observed for GST-SodB.



**Figure 4.4 Preliminary small scale purifications for assessment of ease of purification of recombinant proteins.** **A.** SDS-PAGE followed by Coomassie staining of samples collected during purification of SodB, given as an example of a recombinant protein that could be purified in sufficient quantity. **B.** SDS-PAGE followed by Coomassie staining of samples collected during purification of Cj0090, given as an example of a protein that could not be purified in adequate quantities. The final yield is an interplay between factors such as protein solubility, yield during induction, affinity for the purification resin and ease of elution. In each figure lanes are as follows: (1) whole cell lysate of cells pre-induction; (2) whole cell lysate post-induction; (3) supernatant after cell lysis; (4) pellet after cell lysis; (5) glutathione sepharose beads after sample binding; (6) glutathione sepharose beads after three washes; (7) the first eluate; (8) the second eluate; (9) glutathione sepharose beads after elution. The expected size of both GST-SodB and GST-Cj0090 is 50kD and is indicated by the black arrows.



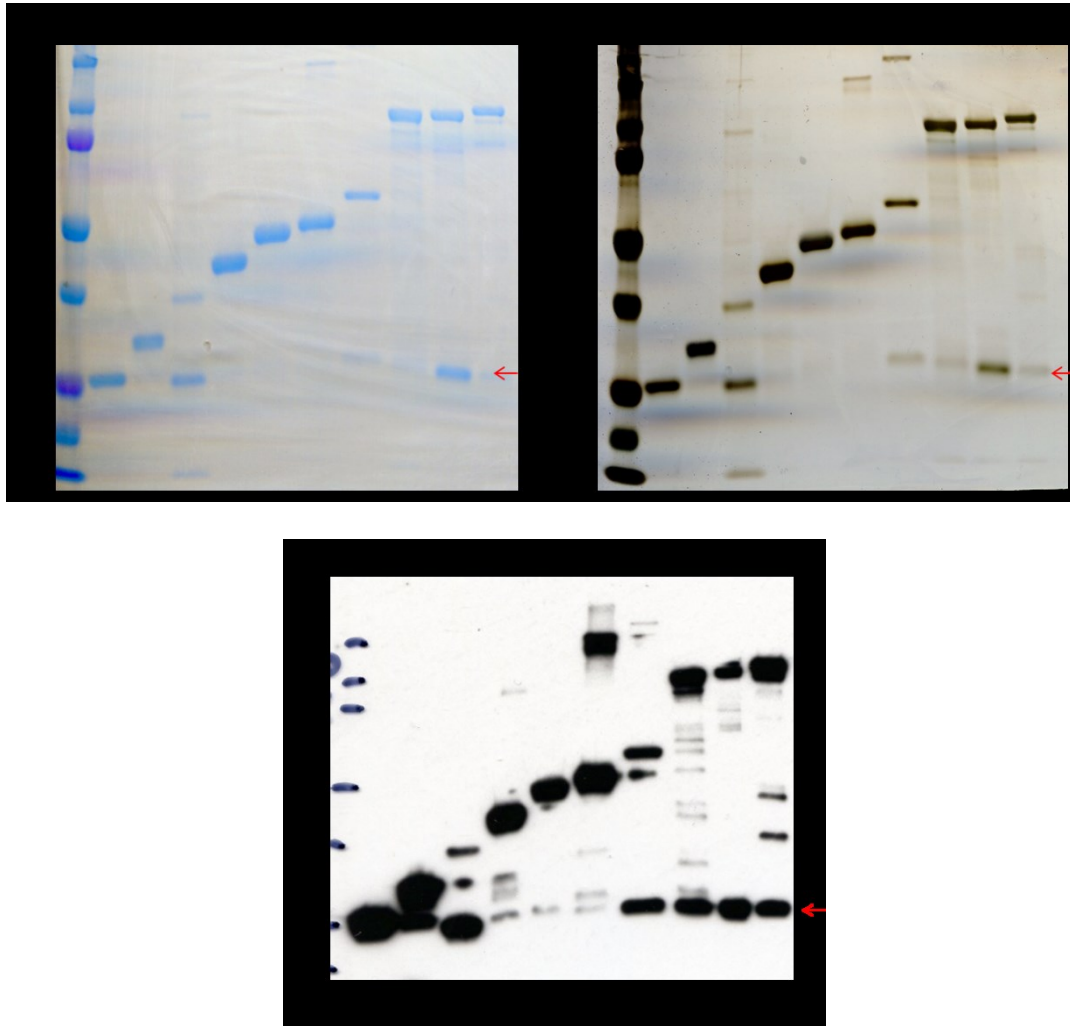
**Figure 4.5. Assessment of purity of the GST-SodB and GST-Cj0090 protein preparations used for assessment of ease of purification.** This was done through Western blotting with an anti-GST antibody. Panels A. and B. and lanes in each panel are the same as in Figure 4.4. In both figures, for both antigens, the insoluble pellet obtained following lysis of bacterial cells in the 50ml cultures was re-suspended in 1ml of PBS, resulting in a 50x concentration of the sample compared to the supernatant. Within the eluates, no obvious bands are seen on the Coomassie gel that do not react with the anti-GST antibody, suggesting the protein preparations contain low levels of contamination with proteins other than those of interest. The black arrows indicate the expected band corresponding to GST-SodB and GST-Cj0090 and the red arrow indicates a likely GST-only truncation (the band is of the size of GST alone and reacts strongly with the anti-GST antibody).

#### 4.3.4 Production of the GST-tagged recombinant antigens tested in vivo

Cultures of *E. coli* Rosetta strains expressing GST-CjaA, GST-FlgE2, GST-FlgK, GST-FlhD and GST-Cj0089 were grown at 28 °C and induced with 0.1 mM IPTG. Cultures of the strains expressing GST, GST-PorA<sub>L7</sub>, GST-FspA, GST-SodB and GST-Tpx were grown at 37 °C and induced with 1 mM IPTG. For the purification of the latter antigens, 500ml of culture were used each time. For the purification of GST-CjaA, GST-FlhD and GST-Cj0089 6 litres of culture were used each time. For the purification of the remainder of the antigens 2 litres of culture were used each time. In total, two protein preparations were made for GST-SodB and different protein preparations for each vaccination in the case of GST-CjaA and GST-FlhD. As far as could be discerned from SDS-PAGE and Western blot analysis, independent preparations of the same antigen were similar. The remainder of the recombinant antigens were obtained in sufficient quantity from a single purification.

Typical preparations of each antigen are shown in Figure 4.6. Silver staining indicated the presence of a band of the size of GST alone in the GST-Cj0089, GST-CjaA and the flagellar component antigens (Fig 4.6B). Assessment of the identity of this band was made through a Western blot with an anti-GST antibody (Fig 4.6C) and strong reactivity

indicated that the 25kD band was most likely GST. Furthermore, this Western blot indicates the presence of a number of other protein species that are possible truncations within the flagellar component antigens, however the bands with the strongest reactivity were of the expected size for fully translated proteins. Truncations may reflect premature termination or post-translational processing. Yield during purification is given for each protein in Table 4.4.



**Figure 4.6. Typical protein preparations of each of the *Campylobacter* antigens used as vaccines in vaccination and challenge experiments in chickens.** Coomassie staining (A) and silver staining (B) of typical protein preparations. The identity and expected molecular weight of the proteins are as follows: (1) GST, 25 kD; (2) GST-PorA<sub>L7</sub>, 29 kD; (3) GST-Cj0089, 35 kD; (4) GST-FspA, 40.5 kD; (5) GST-Tpx, 43kD; (6) GST-SodB, 50kD; (7) GST-CjaA, 55kD; (8) GST-FlgK 92kD; (9) GST-FliD, 95kD; (10) GST-FlgE2, 99kD. C. Assessment of purity of typical protein preparations of each of the *Campylobacter* antigens used as vaccines in *in vivo* trials in chickens via a Western blot with an anti-GST antibody. In all panels the red arrows indicate truncations of the proteins of a size equal to GST only. The high molecular weight band in lane six may indicate a multimer of this protein species.

**Table 4.4 Approximate yield of recombinant GST-tagged *Campylobacter* antigens from single protein purification experiments.** Yield is given as mg per litre of LB broth culture.

Recombinant protein	Approximate yield (in mg/l)
GST	13.4
GST-Por <sub>AL7</sub>	17.4
GST-Cj0089	0.25
GST-FspA	14.6
GST-Tpx	15.4
GST-SodB	19.8
GST-CjaA	0.3
GST-FlgK	0.9
GST-FliD	0.4
GST-FlgE2	2.8

#### 4.3.5 Production of MBP-tagged antigens used in ELISAs

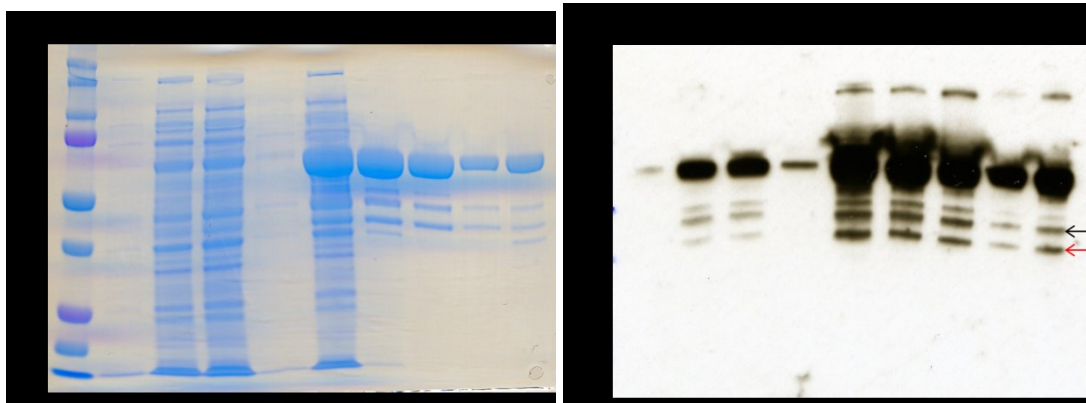
The *E. coli* XL1 strains expressing the MBP-CjaA and MBP-FliD recombinant antigens were grown at 27 °C and induced with 0.1 mM IPTG. Strains expressing the remainder of the MBP-tagged antigens were grown at 37 °C and induced with 0.5 mM IPTG as per manufacturer's instructions. The entire purification process is exemplified through samples taken during purification of MBP-SodB and shown through a Coomassie stained SDS-PAGE gel in Figure 4.7A. Identification of the MBP-SodB recombinant protein was undertaken through a Western blot with an anti-MBP antibody (Figure 4.7B). This revealed the presence of a protein species of the size expected for a MBP-only truncation expressed from the pMal-p2X plasmid (c. 46kD) and of a protein species of the size expected for the endogenous MBP expressed from the chromosome of *E. coli* XL1 (43.4 kD). All purified proteins were validated in the same way.

#### 4.3.6 Role of Cj0424 in virulence and colonisation

##### 4.3.6.1 Role of Cj0424 in virulence in a wax moth (*Galleria mellonella*) model

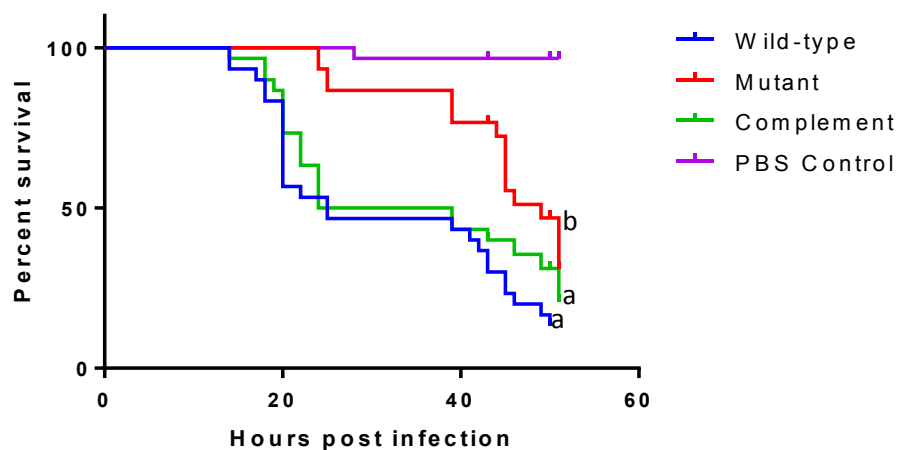
In order to strengthen the rationale for testing Cj0424 as a candidate antigen and to apply 3R principles for reduction of animal use, the role of Cj0424 *in vivo* was initially assessed through the use of Greater wax moth (*Galleria mellonella*) larvae. This model has been reported to detect the attenuating effect of mutations in factors known to influence chicken colonisation (Champion *et al*, 2010).

When tested in the wax moth larvae model of virulence, a statistically significant increase in the median time to death was observed in the group inoculated with the *cj0424* mutant (67.17h) compared to the wild-type group (median time to death: 27.84h;  $p = 0.0013$ ; Figure 4.8). Survival of larvae inoculated with the complemented strain was similar to the



**Figure 4.7. Purification of MBP-SodB as an example of MBP-tagged recombinant *Campylobacter* antigen purification.** **A.** Coomassie staining of an SDS-PAGE gel of samples collected during purification of MBP-SodB. **B.** Assessment of purity of the samples shown in panel A via a Western blot with an anti-MBP antibody. In each figure lanes are as follows: (1) whole cell lysate of cells pre-induction; (2) whole cell lysate post-induction; (3) supernatant after cell lysis; (4) pellet after cell lysis; (5) amylose beads after sample binding; (6) amylose beads after three washes; (7) the first eluate; (8) the second eluate; (9) amylose beads after elution. The expected size of MBP-SodB is 60kD. The black arrow indicates a truncation of the protein of a size equal to the size of MBP alone as expressed from the pMal-p2x plasmid (c. 46kD). The red arrow indicates a protein of size equal to the size of the endogenous MBP in *E. coli* (43.4 kD).

levels observed with the wild-type and the median time to death (26.29h) was significantly lower than for the mutant group ( $p = 0.0002$ ). The proportion of larvae alive at 24 hours (as used to score virulence of *C. jejuni* 11168H mutants by Champion *et al*, 2010) was on average 55% in the wild-type group, 50 % for the complemented group and 13% in the  $\Delta cj0424$  mutant group.



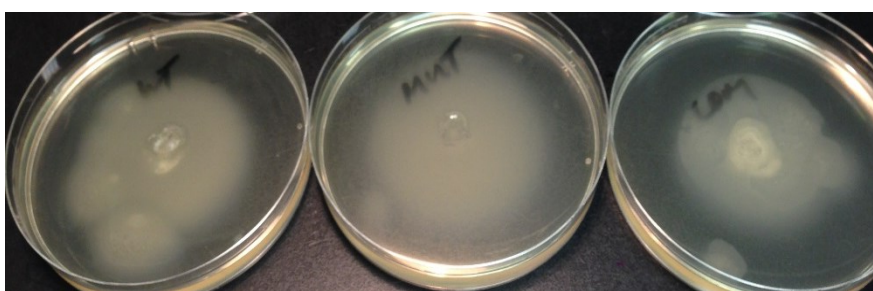
**Figure 4.8. Survival of *Galleria mellonella* larvae inoculated with *C. jejuni* 11168H wild-type,  $\Delta cj0424$  mutant and  $\Delta cj0424::cj0424$  complemented strains.** Data is presented as the mean of three independent replicates each using ten larvae per treatment. Plots marked with different letters were significantly different ( $p < 0.001$ ).

#### 4.3.6.2 Role of Cj0424 and Cj0423 in chicken colonisation

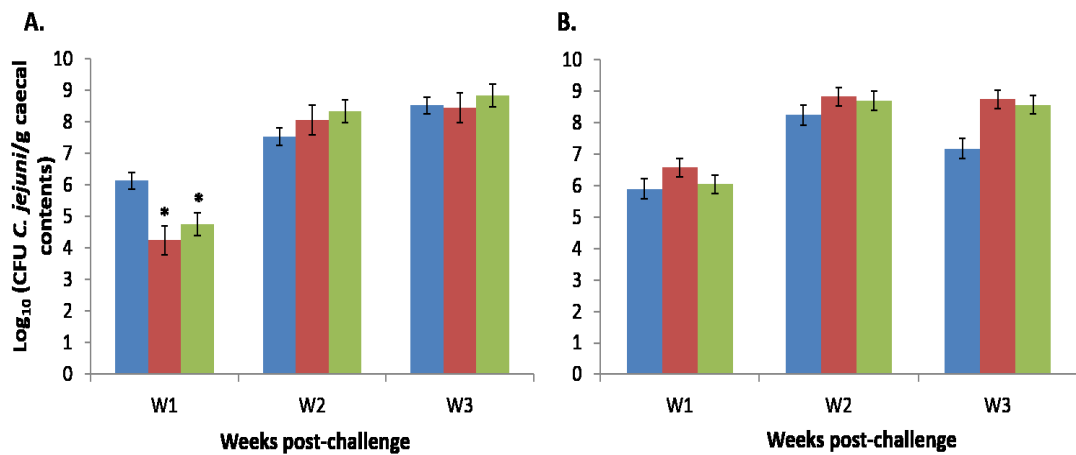
When testing the wild type,  $\Delta cj0424$  mutant and the  $\Delta cj0424::cj0424$  complemented strains in chickens, no bacteria were recovered from any of the birds during the first experiment. The failure of the three *C. jejuni* strains to colonise during the first experiment was most likely due to their lack of motility as observed visually under the microscope and through soft agar diffusion test (data not shown). While the strains were re-selected for motility through clonal expansion of single colonies for the second experiment, this only partially improved motility. During a second experiment low bacterial numbers were recovered from only 30% of the birds (data not shown).

In order to firmly establish whether Cj0424 plays a role in chicken colonisation, each of above three *C. jejuni* 11168H strains were re-made in a parent stock originating from a new vial ordered from the National Collection of Type Cultures (NCTC). The newly made strains exhibited high and similar levels of motility as observed visually under the microscope and in the soft agar diffusion test (Figure 4.9). The newly made strains were then tested *in vivo* in chickens at two different challenge doses and all birds were colonised to high levels. During this experiment, the only statistically significant reductions were observed at 1 week post-challenge in the mutant and complemented groups compared to the wild-type strain (Figure 4.10). However, complementation did not restore the phenotype and no reductions were observed at the two subsequent sampling time-points.

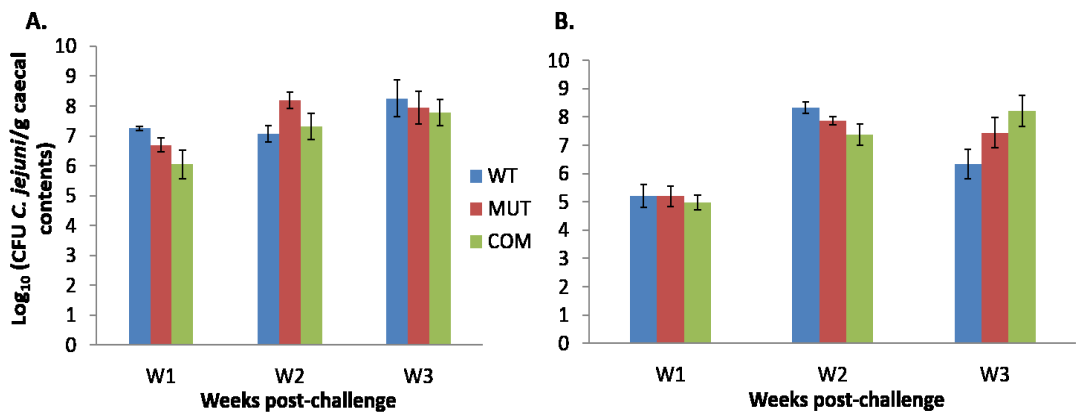
As both Cj0424 and Cj0423 were claimed by the Kelly laboratory to mediate resistance to cationic antimicrobial peptides at the time of the study, the possibility of redundancy within the system was assessed through the testing of a double mutant that does not produce either protein. When compared to the wild-type and complemented strain in chickens no reductions in colonisation were observed at any time-points, at neither of the  $10^4$  or  $10^6$  CFU/bird challenge dose (Figure 4.10).



**Figure 4.9. Soft agar diffusion test for the wild-type,  $\Delta cj0424$  mutant and  $\Delta cj0424::cj0424$  complemented strains.** Plates in the image are in the order given in the figure title from left to right.



**Figure 4.10. Effect of  $\Delta cj0424$  mutation on the ability of *C. jejuni* 11168H to colonise chicken caeca.** The three strains were tested at a dose of  $10^6$  CFU/bird (A) or  $10^8$  CFU/bird (B). Each graph shows the mean level of colonisation in each of the groups tested. Six birds were sampled in each group at each of the time-points. The groups tested included a wild-type strain (blue bars), a  $\Delta cj0424$  strain (red bars) and a  $\Delta cj0424::cj0424$  strain (green bars). Error bars represent the standard error of the means (SEM). Significant differences are denoted by \* for  $p \leq 0.05$ .



**Figure 4.11. Effect of  $\Delta cj0424$ -*cj0423* double mutation on the ability of *C. jejuni* 11168H to colonise chicken caeca.** The three strains were tested at a dose of  $10^4$  CFU/bird (A) or  $10^6$  CFU/bird (B). Each graph shows the mean level of colonisation in each of the groups tested. Six birds were sampled in each group at each of the time-points. The groups tested included a wild-type strain (blue bars), a  $\Delta cj0424$ -*cj0423* strain (red bars) and a strain trans-complemented with both genes (green bars). The error bars represent the SEM.



#### 4.4. Discussion

Affinity purification of *Campylobacter* antigens via the GST system was chosen due to previous vaccination studies reporting the successful induction of protection against colonisation in a murine model using GST-PorA (Islam *et al*, 2010). In specialised laboratories using highly optimised conditions and pipetting robots, the 6xHis-tag has been found to be able to give similar purity but higher yield compared to the GST tag (Scheich *et al*, 2003). However, studies in other laboratories using manual purification found the 6xHis tag to result in protein preparations of lower purity even though it may give better yield compared to the GST-system (Lichty *et al*, 2005; reviewed by Vaugh, 2005). Additionally, GST-tagged protein purification has been advocated as having a good balance between ease of use, yield and cost (Lichty *et al*, 2005).

Cloning of all the 21 selected antigens into the pGEX-4T1 plasmid, for expression as GST-tagged recombinant proteins, was successful at the first attempt and sequencing showed no mutations in any of the clones obtained. Purification of the GST-tagged proteins was met with various levels of success and proteins that have been reported to be free in the cytosol (Tpx, SodB) or normally secreted (FspA1) were easily purifiable. The flagellar proteins, while they were purified in sufficient quantities for *in vivo* testing, yielded lower amounts of recombinant protein per unit of culture volume. Lastly, while some of the membrane-bound proteins were purified (CjaA, Cj0125) they required large volumes of culture or the use of truncation to known extracellular domains that normally exist in aqueous phase (PorA<sub>L7</sub>).

A study into the purification of a large spectrum of recombinant mammalian proteins in *E. coli* through affinity tags found that molecular weight, number of contiguous hydrophobic residues and the presence of low complexity regions correlate with soluble protein expression, however, the grand average of hydropathicity (GRAVY) did not correlate with successful soluble expression (Dyson *et al*, 2004). Furthermore, the TM Finder programme (Deber *et al*, 2001) has been shown to be able to predict transmembrane domains in proteins based on thresholds of segment hydrophobicity and non-polar phase helicity. Work leading to the development of this programme showed that 97% of transmembrane domains available in SWISS-PROT and TMbase have an index of hydrophobicity approximately equivalent or higher to that of a string of 20 alanine residues and as such this threshold can be used for prediction of transmembrane domains (Liu and Debber, 1998). Work from the same group also showed that an index of helicity can be also used for improving transmembrane domain predictions when helicity was determined in non-polar media but not in polar media (Wang *et al*, 1999). As the majority of the proteins that were difficult to purify in this study have numerous transmembrane domains, it is possible that the

presence of long sections of numerous consecutive hydrophobic residues within these proteins may have resulted in them being insoluble, rather than having high GRAVY.

Additionally, large tags used in purification, such as GST, can interfere with folding of recombinant proteins into their native conformation (as reviewed by Smith *et al*, 2003). This may have been another factor that accounted for the difficulty of purification of some of the antigens selected in this study. Protein folding may have also accounted for the increased yield observed with induction of some of the other proteins at lower incubation temperatures as it has been proposed that this observation could be due to improved protein folding, allowing for either the GST tag or the recombinant antigen to fold correctly under these conditions. Studies showed improved expression of individual proteins at lower temperatures in bacteria (e.g. Kataeva *et al*, 2005) and yeast (e.g. Li *et al*, 2001) but this is protein dependent and may only apply to proteins of larger sizes as a study of 20 human protein with molecular weights of under 20kD did not find enhanced solubility or yield at lower temperatures (Hammarstrom *et al*, 2002).

The same aspects discussed above apply to purification of the 9 *Campylobacter* antigens using the MBP-system. Esposito and Chatterjee (2006) review methods of enhancing solubility of recombinant proteins and describe numerous studies that report the successful use of MBP as a fusion partner for enhanced solubility. However, the effect may be dependent on particular proteins and is likely to need empirical resolution. In this study, although not formally assessed, the change to the MBP tag did not appear to enhance solubility as the purification of the less soluble GST-tagged proteins required large volumes of cultures (2 litres) even when MBP-tagged, compared to the purification of the highly soluble proteins (500ml in the case of each tag).

As with expression of GST-CjaA and TetC-CjaA, truncations of the size of GST alone were observed in some of the protein preparations, particularly in those that had poorer solubility. The presence of rare codons in the DNA sequence at the start of heterologous genes has been discussed in Section 3.4 as a possible reason for premature termination. Additionally, it is possible that secondary structures at the 3' end of the *gst* gene are involved in termination of translation. Signals beyond the stop codon, such as modification of the DNA strand and features encoded within the secondary structure of the mRNA at the 3' end of genes have been shown to contribute to accurate termination of transcription in eukaryotes (Birnstiel *et al*, 1985).

With regard to the assessment of the role of Cj0424 in colonisation, the failure of the *C. jejuni* 11168H strains to establish infection in the first two experiments was most likely due to them being non-motile. The importance of motility and the presence of flagella for

chicken colonisation have been described in Chapter 1. The passage history of the 11168H isolate from the Kelly laboratory was unclear. Even though initial evidence suggested that Cj0424 mediates resistance to cationic antimicrobial peptides *in vitro* and primed our studies in *Galleria* and chickens, more recent studies undertaken by Jonathan Butler (Prof. David Kelly's group, University of Sheffield) have shown no significant differences in resistance to cationic peptides between the wild-type,  $\Delta cj0424$  and  $\Delta cj0424::cj0424$  strains *in vitro*. As such, the functions of Cj0424 in *C. jejuni* 11168H and other strains in which it is present remains unknown. The lack of a colonisation defect in the  $\Delta cj0424$  or the double mutant is perhaps unsurprising given that Cj0424 is only present in less than 50% of the *Campylobacter* strains sequenced and there appears to be no association of these strains with poultry. A nucleotide BLAST of the *Cj11168\_0424* sequence reveals 14 *C. jejuni* strains that have a gene with over 99% sequence identity. Out of these, the host of origin of 4 strains is not given but 8 of the remaining strains were isolated in human clinical cases and two originate from ruminants.

The nine GST-tagged *Campylobacter* antigens that were described in this chapter as having been purified in adequate quantities were tested in chickens as recombinant subunit vaccines for their protective effect against *Campylobacter* colonisation. Furthermore, the magnitude and nature of the immune response induced by vaccination was measured and the data related to these aspects are given in the next chapter.

## **5. Evaluation of recombinant subunit vaccines against *Campylobacter* colonisation in chickens**

### **5.1 Introduction**

Nine recombinant subunit vaccines were produced in sufficient quantities for their protective efficacy against *Campylobacter* colonisation to be tested in chickens. Published literature reporting the use of subunit recombinant anti-*Campylobacter* vaccines in broilers was summarised in Section 1.5.4. Rational selection of antigens was discussed in Section 4.2.

Previous experiments using *Salmonella*-vectored CjaA undertaken in our group (Buckley *et al*, 2010) and independent unpublished reports using both live vectored and subunit recombinant vaccines, suggest high variability of the protective effect observed when CjaA is used as an antigen, with some experiments demonstrating protection while others showed no differences compared to mock-vaccinated controls. This effect appears to be independent of the experimental and vaccine design, suggesting this may be an intrinsic property of CjaA or due to variability in the challenge strain. The underlying mechanisms for this variability are unknown. CjaA is processed as a lipoprotein and is also glycosylated in *Campylobacter* (Wyszynska *et al*, 2008) and expression of recombinant CjaA in *E. coli* may lead to different processing to that of the native protein in *Campylobacter*. Furthermore, variation of glycosylation may be used as an immune evasion mechanism by *Campylobacter* in vaccinated birds. Due to the variability in CjaA's protective effect, recombinant subunit vaccines were tested with the aim of identifying an antigen or combination of antigens that confers improved protection compared to CjaA or that would provide more replicable protection across independent experiments. Such an antigen would allow future testing of optimised vaccines across a reduced number of independent replicates.

### **5.2 Materials and methods**

#### **5.2.1 Validation of immunogenicity of CjaA, FliD and Cj0089**

Immunogenicity of CjaA, FliD and Cj0089 following *Campylobacter* infection was described previously (Shoaf-Sweeney *et al*, 2008). At the time of their use in vaccination experiments, their antigenicity in *Campylobacter*-infected but non-vaccinated birds was assessed through a Western blot using sera from PBS-vaccinated birds (Section 3)

challenged at 4 weeks of age with *C. jejuni* M1. Pooled sera were used at a 1:100 dilution and bound serum IgY detected using an HRP-conjugated rabbit anti-chicken Ig (Sigma Aldrich) at a 1:3000 dilution.

### 5.2.2 Vaccination experiments

Nine GST-tagged recombinant *Campylobacter* antigens were tested for their protective efficacy in chickens. For parity with previous experiments in our group, an experimental design similar to that described by Buckley *et al* (2010) was used. This was described in detail in Section 2.15.2. However, there were a number of modifications to the study design. Firstly, the GST tag was used for purification of recombinant antigens instead of the 6xHis tag as it was found that this tag resulted in protein preparations with higher purity (data not shown). Secondly, due to local availability White Leghorn birds were used instead of Light Sussex birds. Furthermore, the TiterMax<sup>®</sup> Gold adjuvant (Sigma, UK) was used instead of TiterMax<sup>®</sup>. According to the manufacturer, this adjuvant has improved efficacy compared to the standard version and induces higher levels of antigen-specific antibodies. Lastly, the amount of each antigen was standardised according to molecular mass, in order to deliver  $4.3 \times 10^{-10}$  moles, the number of moles present in 14 µg of 6xHis-CjaA, as used by Buckley *et al* (2010). For this calculation, the molecular weight of each entire GST-tagged *Campylobacter* antigen was taken into account. The amount of each antigen delivered to each bird at each of the vaccinations is given in Table 5.1.

**Table 5.1. Amount of GST-tagged recombinant antigens used in vaccination trials.**

Antigen	Molecular mass (in kD)	Mass delivered per bird per vaccination (in µg)
GST	25	13.2
GST-PorA <sub>L7</sub>	29	14.9
GST-Cj0089	35	17.7
GST-FspA	41	20.4
GST-Tpx	43	19.5
GST-SodB	50	22.4
GST-CjaA	55	27.2
GST-FlgK	92	41.7
GST-FliD	95	44.5
GST-FlgE2	99	46.3

During each vaccination experiment, GST and GST-CjaA were used as negative and positive controls respectively. As in the study by Buckley *et al* (2010) vaccines were delivered in a 1:1 mix with TiterMax<sup>®</sup> Gold, with 50 µl being delivered on each side of the thorax subcutaneously, using a calibrated Hamilton's repeat dispenser and syringe. In total, 5

vaccination experiments were run using recombinant antigens. Excluding the aforementioned control groups, the groups tested in each vaccination experiment and their purpose are given in Table 5.2. Where multivalent vaccines were administered, each vaccine was delivered to a dose equal to that administered for univalent vaccines. As such, the total dose of vaccine was 2 or 3 fold higher than when administered individually.

### 5.2.3. Assessment of humoral immune responses

For each vaccination experiment, the induction of antigen-specific serum IgY and bile IgA was assessed in individual birds in each of the groups. This was done by ELISA, as described in Section 2.15.4. In order to avoid detection of antibodies against the GST portion of each recombinant antigen, the MBP-tagged recombinant proteins described in Section 4.3.5 were used. Appropriate coating concentrations and serum dilutions for each antigen were determined by checkerboard analysis and chosen to give readings within the linear range of representative serial dilutions.

The MBP-tagged antigens were used in ELISAs for measurement of antigen-specific serum IgY at the following coating concentrations: 2 µg/ml for measurement of responses against SodB and FlgK, 1 µg/ml for measurement of responses against FspA, FliD and FlgE2 and 0.5 µg/ml for measurement of responses against CjaA, PorA<sub>L7</sub> and Tpx. Sera were diluted 1:500 for measurement of responses against CjaA, FliD and PorA<sub>L7</sub>, 1:250 for measurement of responses against SodB, FspA and TpX and 1:100 for measurement of responses against FlgK and FlgE2. Responses against Cj0089 were not measured as attempts to re-clone *cj0089* into pMal-p2X failed.

Antigen-specific bile sIgA responses were measured for CjaA and SodB vaccinated birds only and both antigens were used at a 1 µg/ml concentration. Sera were diluted 1:250 for measurement of sIgA against both antigens.

Additionally, for the FliD, SodB and CjaA antigens, fold-changes in serum IgY levels were determined in individual birds at each time-point by dividing the OD<sub>450nm</sub> reading in vaccinated birds by the mean OD<sub>450nm</sub> reading of GST-vaccinated control birds averaged across the respective time-point. A linear regression of these measurements on caecal *Campylobacter* counts in each bird was used to assess whether the level of antigen-specific serum IgY induction correlated with caecal *Campylobacter* counts.

**Table 5.2. Groups tested in each of the vaccination experiments using recombinant subunit vaccines.** In each experiment GST and GST-CjaA groups were included as negative and positive controls respectively.

Vaccination experiment	Aim of the experiment	Groups included	Aim of individual group
1	Testing individual antigens	GST-PorA	Identifying an antigen with improved protection compared to CjaA
		GST-FspA	Identifying an antigen with improved protection compared to CjaA
		GST-FliD	Identifying an antigen with improved protection compared to CjaA
		GST-FlgE2	Identifying an antigen with improved protection compared to CjaA
2	Testing individual antigens	GST-SodB	Identifying an antigen with improved protection compared to CjaA
		GST-FlgK	Identifying an antigen with improved protection compared to CjaA
		GST-Tpx	Identifying an antigen with improved protection compared to CjaA
3	Repeat testing of antigens conferring reduction in caecal <i>Campylobacter</i> colonisation in the previous experiments	GST-FspA	Re-testing an antigen that conferred a reduction of minimum 1log <sub>10</sub> in the preliminary screen, even if it was not statistically significant
		GST-SodB	Re-testing an antigen that conferred a reduction of minimum 1log <sub>10</sub> in the preliminary screen, even if it was not statistically significant
		GST-FliD	Re-testing an antigen that conferred a reduction of minimum 1log <sub>10</sub> in the preliminary screen, even if it was not statistically significant
		GST-Cj0089	Identifying an antigen with improved protection compared to CjaA; could not be tested in experiment 2 due to low number of birds hatched
4	Testing of protective antigens singly and in combination	GST-SodB	Control group for the groups testing a multivalent vaccine
		GST-FliD	Control group for the groups testing a multivalent vaccine
		GST-SodB + GST-FliD	Assessing if multivalent vaccines confer improved protection
		GST-SodB + GST-FliD + GST-CjaA	Assessing if multivalent vaccines confer improved protection
5	Assessing heterologous protection and testing Cj0424 as a vaccine candidate	GST-SodB	Assessment of heterologous protection
		GST-FliD	Assessment of heterologous protection
		GST-Cj0424	Assessment of potential as a vaccine candidate of a novel protein involved in resistance to cationic antimicrobial peptides*
		GST-6xHis-CjaA	Assessing whether tag used for purification may affect variability in protection observed with CjaA.

\*As described by Halah Al-Haideri at the time of testing.

#### **5.2.4 Determination of the subcellular localisation of SodB**

The subcellular localisation of SodB was assessed through immunofluorescent (IF) staining of *C. jejuni* 11168H cells, as described in Section 2.16. *C. jejuni* 11168H were permeabilised for 30 minutes in 10% (v/v) Triton-X100 (where indicated) and fixed for 30 minutes in 4% PFA (w/v), both diluted in PBS. Pooled sera from GST-SodB vaccinated birds, collected at 24 dph, before challenge, were used as primary antibody at a 1:500 dilution. Bound serum IgY was detected with an AlexaFluor-488-conjugated goat anti-chicken IgY (AbCam), used at a 1:500 dilution. Images were taken on a DLMB fluorescent microscope (Leica), with the fluorescence intensity set for the image of interest and kept constant throughout the experiment.

The results of the IF staining were confirmed through Western blotting of outer membrane, periplasmic and inner membrane subcellular fractions of *C. jejuni* 11168H with the same sera from GST-SodB vaccinated birds used for immunofluorescent staining. The subcellular fractions were made by Halah Al-Haideri (University of Sheffield) using the method described in Section 2.17. Sera were used at a 1:100 dilution and bound IgY was detected with an HRP-conjugated rabbit anti-chicken IgY antibody (Simga Aldrich) used at a 1:3000 dilution.

#### **5.2.5. Ability of anti-FliD serum IgY to neutralise its target**

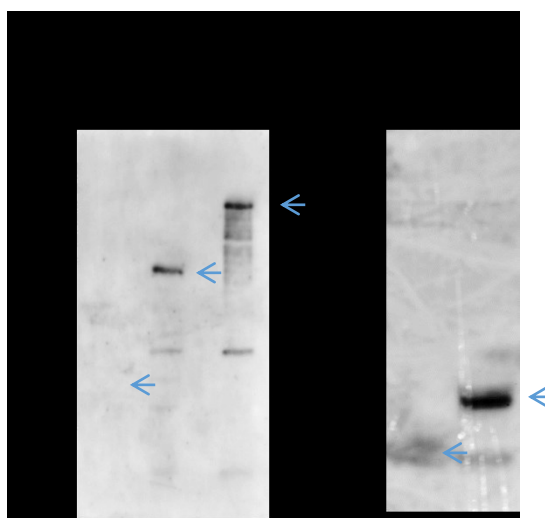
The ability of anti-FliD serum IgY to recognise and neutralise its target was assessed through a soft agar diffusion test. For this, a 10 µl sterile plastic loop was used to inoculate an overnight culture of *C. jejuni* M1 into the centre of 0.3% (w/v) MH agar plates by stabbing. Plain MH agar plates were used as negative controls and plates containing 1:100, 1:1000 or 1:10,000 (v/v) serum from GST-FliD vaccinated birds collected at 24dhp (before challenge) were used to assess the ability of anti-FliD antibodies to neutralise their target. Furthermore, this was also assessed by mixing an overnight culture of *C. jejuni* M1 with an equal volume of undiluted serum from GST-FliD vaccinated birds, followed by a 1 minute incubation with gentle agitation. Slides were then examined for evidence of agglutination and motility both with the naked eye and under the microscope, using a 40x magnification.



## 5.3 Results

### 5.3.1 Validation of natural immunogenicity of CjaA, FliD and Cj0089

Western blotting with pooled sera collected at three weeks post-challenge from PBS-vaccinated birds used as negative controls during the first vaccination experiments which were challenged with *C. jejuni* M1 at 4 weeks old, confirmed previous reports that CjaA, FliD and Cj0089 are antigenic in *Campylobacter* infections in chickens (Figure 5.1). However, the protein samples were prepared through denaturing SDS-PAGE and as such only linear epitopes were detected by Western blotting. While the other antigens were also assessed for antigenicity, they did not react with sera from *Campylobacter* infected chickens suggesting that linear epitopes of these antigens are not recognised by the chicken's immune system during infection with this bacterium (data not shown) or that these epitopes are not expressed during *in vitro* culture.



**Figure 5.1. Confirmation of antigenicity of CjaA, FliD and Cj0089 in primary *C. jejuni* infection in chickens.** This was tested through Western blotting with sera collected from birds injected with PBS and infected with *C. jejuni* M1 during the first vaccination trial testing attenuated live-vectored vaccines. Sera were obtained at three weeks after challenge with *C. jejuni* M1. Antigens are shown in two panels (A and B) as they were each tested at the time of their use in vaccination experiments. Arrows to the right of each lane indicate the expected molecular weight of each antigen tested.

### 5.3.2 Testing of individual recombinant antigens for protection against homologous *C. jejuni* colonisation

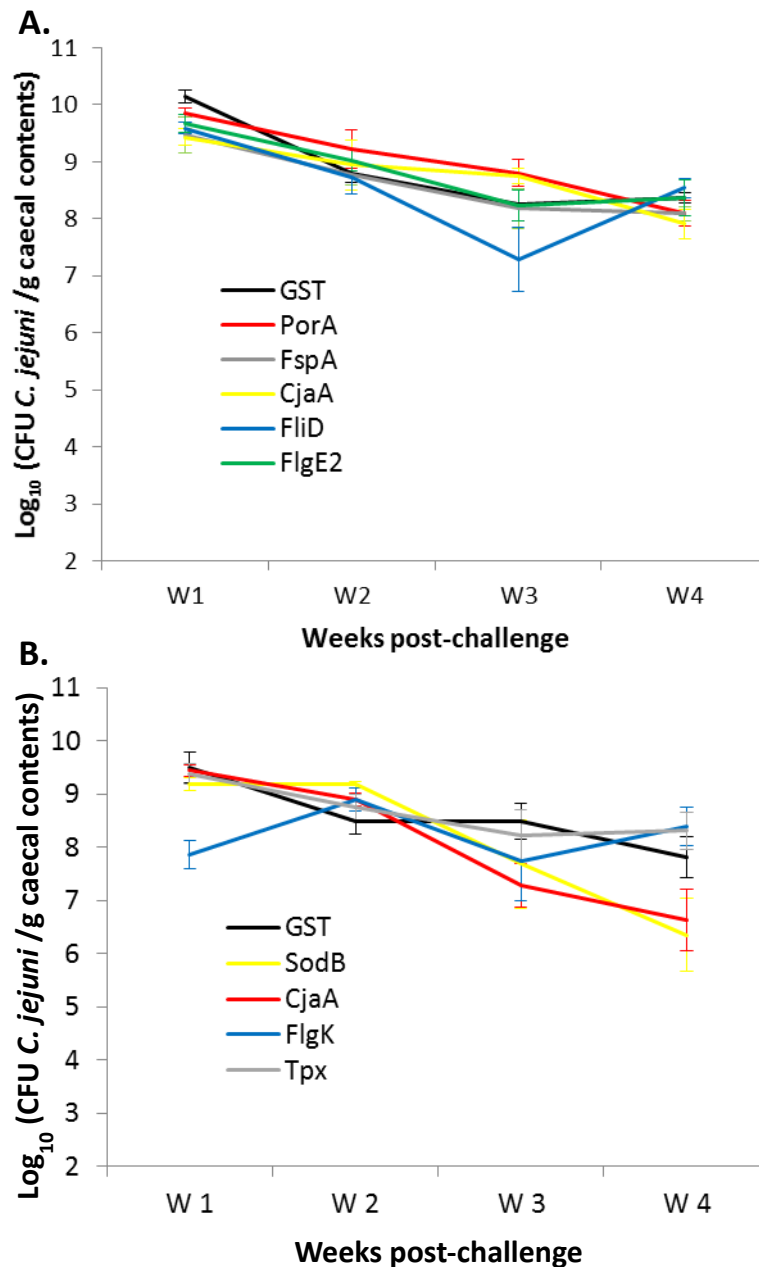
Nine novel antigens were separately tested for their ability to confer protection against *C. jejuni* colonisation, initially across two independent experiments. Two separate experiments were run due to space constraints within the containment animal facility.

### 5.3.2.1. Protection against caecal colonisation

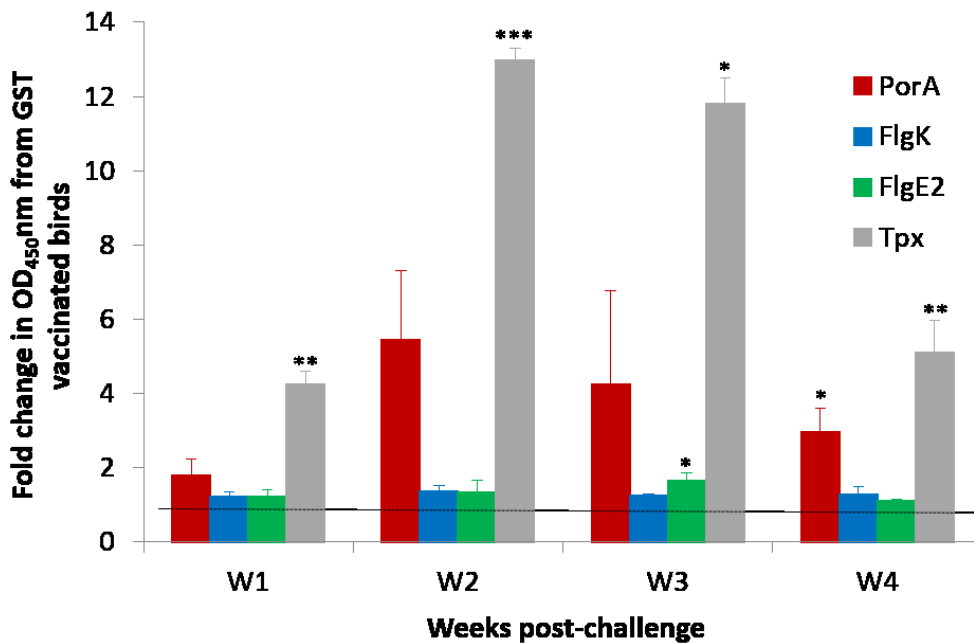
The colonisation kinetics in each of the groups vaccinated with individual GST-tagged *C. jejuni* antigens in the first two vaccination experiments are shown in Figure 5.2. Second order hierarchical general linear models (GLMs) taking into account the time of vaccination and treatment group were fitted for each individual experiment ( $R^2 = 0.5$  for each experiment). Even though reductions of  $1 \log_{10}$  CFU/g were observed with FliD at 48 dph (Figure 5.2A) and with SodB and CjaA at 56 dph (Figure 5.2B), these were not statistically significant. No statistically significant differences were detected between groups when the entire course of colonisation was considered. As no statistical significance was detected, average colonisation across the entire duration of the experiments was used as a measure of potential protective effect. The largest differences between groups vaccinated with *Campylobacter* antigens and the GST-only control group were observed in the GST-FliD (-0.24), GST-CjaA (-0.18) and GST-FspA (-0.14) groups within the first experiment and the GST-CjaA (-0.34) and GST-SodB (-0.27) groups within the second experiment. The numbers in brackets are the differences in average course of colonisation as estimated by each GLM.

### 5.3.2.2. Antigen-specific serum IgY responses

Apart from FlgK, antigen-specific serum IgY induction was observed in all vaccinated groups within the first two experiments at a minimum of a single time point. Antigen-specific serum IgY against CjaA, SodB and FliD was induced at all time-points and are shown in a later section as an average of all the experiments in which they were tested. The data for FlgK, FlgE2, Tpx and PorA<sub>L7</sub> are given in Figure 5.3. GST-Tpx induced significant increases in serum IgY at all time-points ( $p = 0.04$  at week 3 and  $p < 0.01$  at all other time-points). GST-FlgE2 only induced significant increases in serum IgY at 49 dph ( $p = 0.04$ ) and GST-PorA<sub>L7</sub> only induced significant increases at 56 dph ( $p = 0.03$ ). Even though the fold-increase in serum IgY was higher at 42 and 49 dph than at 56dph in the GST-PorA<sub>L7</sub> vaccinated group, these were not statistically significant due to a low number of birds being samples ( $n=4$ ) and high variability observed amongst responses in individual animals. These results suggest that the vaccines were successfully administered.



**Figure 5.2** Caecal colonisation by *C. jejuni* M1 of birds vaccinated with different recombinant GST-tagged *Campylobacter* antigens. Antigens were tested in two separate trials (A. and B.) due to space constraints. Each trial included a GST and a GST-CjaA vaccinated group as negative and positive controls respectively. Caecal *Campylobacter* counts were determined by serial dilution of caecal contents collected at post-mortem examinations. Between 3 and 6 birds were sampled at each of the time-points in each of the treatment groups. The error bars represent the SEM. For all treatment groups colour-coded in the legend GST-tagged recombinant antigens were used.



**Figure 5.3. Induction of antigen-specific serum IgY in birds vaccinated with the GST-tagged recombinant *Campylobacter* antigens that were tested once only.** Data is expressed as fold-change in OD<sub>450nm</sub> from the average OD<sub>450nm</sub> reading of the GST only vaccinated group, calculated at each time-point individually. Between 3 and 6 birds were sampled per time-point per treatment group. Significant differences from the GST only vaccinated group are denoted by \* for p<0.05, \*\* for p<0.01 and \*\*\* for p<0.001. For all treatment groups colour-coded in the legend GST-tagged recombinant antigens were used. The dotted line marks no change from the GST-vaccinated controls.

### 5.3.3. Repeat testing of antigens that were indicated as potentially protective during the initial experiments

Antigens that were identified as being potentially protective within the first two vaccination experiments were re-tested in a third experiment.

#### 5.3.3.1 Protection against caecal colonisation

The data obtained in the third vaccination experiment are given in Figure 5.4.A. When analysed independently, a second order hierarchical GLM taking into account the time of sampling and the treatment group ( $R^2 = 0.22$ ) revealed significant reductions in the entire course of colonisation of the GST-SodB ( $p = 0.004$ ) and GST-FliD ( $p = 0.02$ ) vaccinated groups compared to the GST-only vaccinated group. Furthermore, the GST-SodB vaccinated group also induced significantly improved protection compared to the GST-CjaA vaccinated group ( $p = 0.013$ ). *Post-hoc* Dunnett's tests were not able to distinguish individual time-points at which reduction were statistically significant. No significant reductions were

observed in the GST-Cj0086 vaccinated group, where GST-Cj0086 was tested for the first time due to insufficient birds being hatched during the previous experiment.

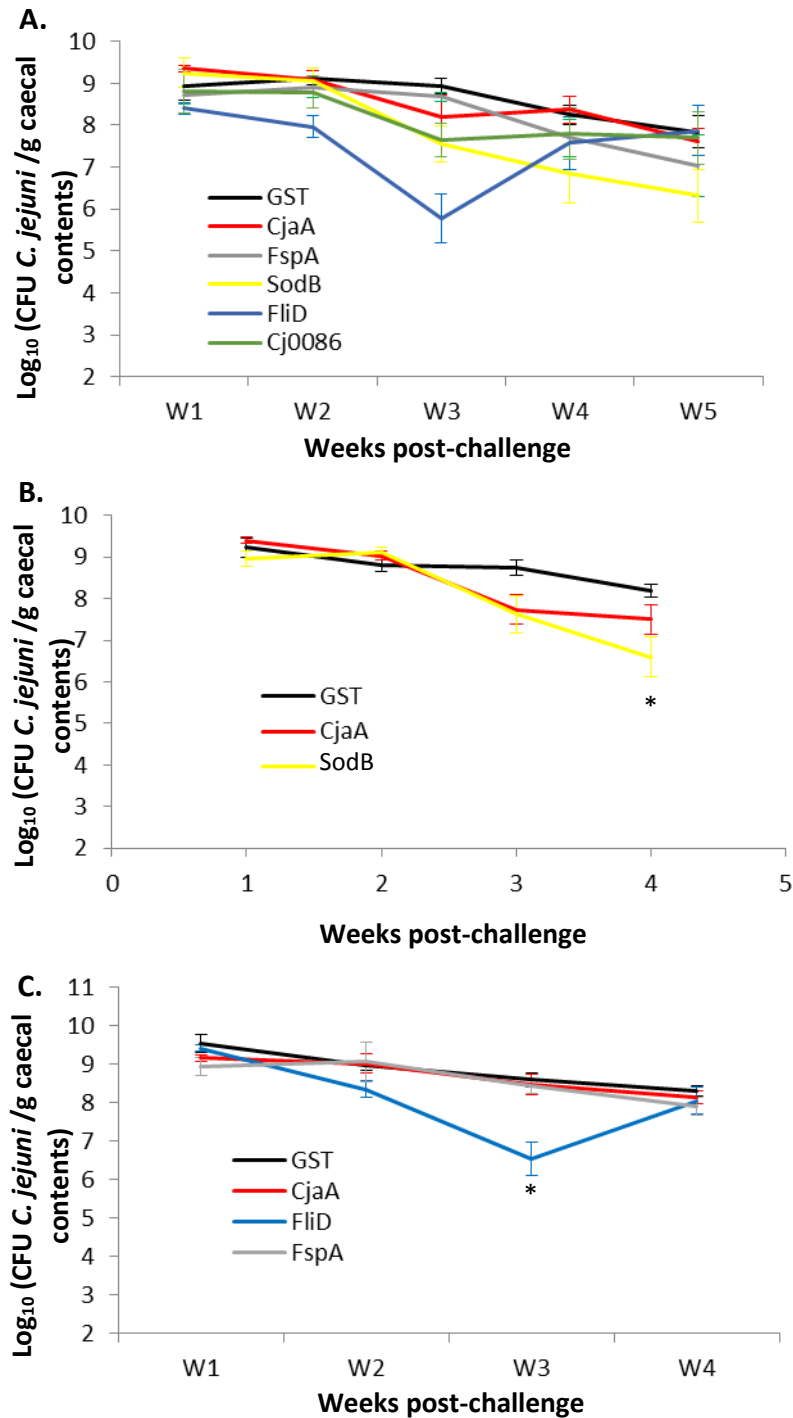
The data was also analysed averaged with the previous experiments using two separate third order hierarchical GLMs in which only control groups tested concomitantly were included. These GLMs took into account interactions between experiment number, time of sampling and treatment group. Average data of experiments testing GST-SodB relative to controls is given in Figure 5.4.B. The GLM ( $R^2 = 0.39$ ) indicated that the course of colonisation of the GST-SodB vaccinated group was significantly different from that of the GST vaccinated group ( $p < 0.023$ ). *Post-hoc* Dunnet's tests identified a significant difference at 56 dph, when the caecal colonisation in the GST-SodB vaccinated groups was reduced by  $1.6 \log_{10}$  CFU *C. jejuni*/g caecal contents compared to the GST ( $p = 0.004$ ) group. No significant differences were observed compared to the GST-CjaA vaccinated group or between the GST-CjaA vaccinated group and the GST vaccinated group. Average data of experiments testing GST-FliD or GST-FspA relative to controls is given in Figure 5.4.C. The GLM ( $R^2 = 0.47$ ) indicated that the course of colonisation of the GST-FliD vaccinated group was significantly different from that of the GST ( $p < 0.001$ ) and the GST-CjaA ( $p < 0.001$ ) vaccinated groups. *Post-hoc* Dunnet's tests identified significant differences at 49 dph, when the caecal colonisation in the GST-FliD vaccinated groups was reduced by  $2 \log_{10}$  CFU *C. jejuni*/g caecal contents compared to both the GST ( $p < 0.001$ ) and the GST-CjaA groups ( $p < 0.001$ ).

#### 5.3.3.2 Antigen-specific serum IgY responses

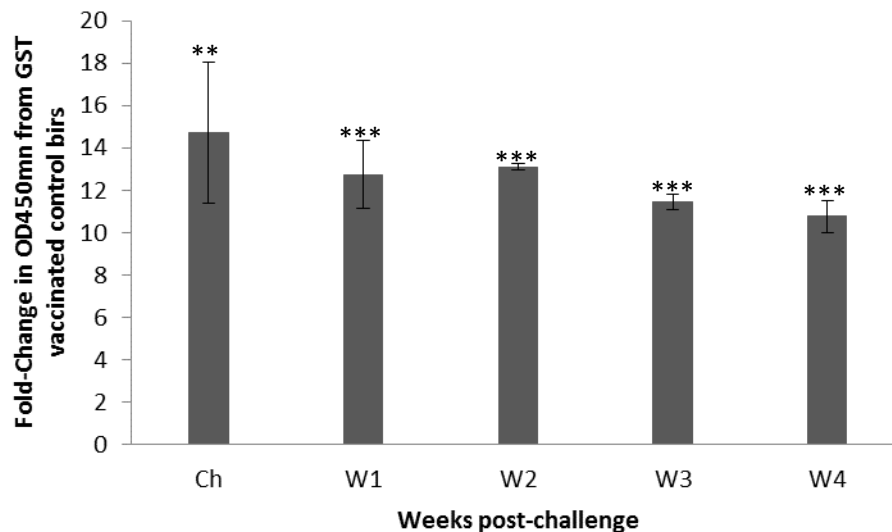
Significant induction of antigen-specific serum IgY antibodies was observed in each of the groups tested in the third vaccination experiment. The average induction of serum IgY against GST-FspA is shown in Figure 5.5. Significant induction was observed at all time-points ( $p = 0.01$  at challenge and  $p < 0.001$  at all other time-points). The induction of serum IgY against SodB and FliD is shown in a later section averaged across all experiments in which it was tested.

#### 5.3.4. Multivalent vaccines against *C. jejuni* colonisation in chickens

Following the observation that subunit recombinant vaccines based on FliD and SodB conferred protection against caecal colonisation by *C. jejuni* in chickens, an experiment was carried out to assess whether multivalent vaccines that combine SodB and FliD, with or without CjaA, can confer improved protection compared to univalent vaccines.



**Figure 5.4. Caecal colonisation by *C. jejuni* M1 of birds vaccinated with recombinant GST-tagged *Campylobacter* antigens tested in duplicate. A.** Caecal colonisation data from the third vaccination trial. Between 4 and 6 birds were sampled at each time point in each of the groups. Significant differences are described in Section 5.3.2.1. Cj0086 was tested for the first time in this study (See Table 5.2) **B.** and **C.** Average caecal colonisation across the two experiments testing GST-SodB relative to controls and GST-FliD or GST-FspA relative to controls respectively. Data is presented separately with concomitantly tested controls. Between 7 and 12 birds were sampled at each time-point in each of the groups. Error bars represent standard error of the mean. Significant differences are denoted by \* ( $p < 0.05$ ). For all treatment groups colour-coded in the legends GST-tagged recombinant antigens were used.

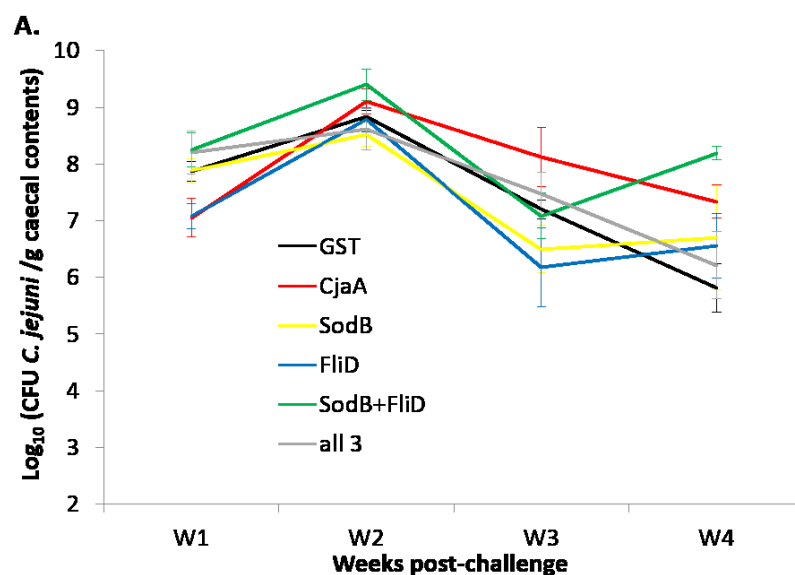


**Figure 5.5. Induction of antigen-specific serum IgY against FspA in vaccinated birds.** Data is shown as an average of both vaccination experiments in which GST-FspA was tested. ELISAs were run on MBP-FspA. Error bars represent SEM. Significant differences from the GST only vaccinated group are denoted by \*\* for  $p < 0.01$  and \*\*\* for  $p < 0.001$ .

#### 5.3.4.1 Protection against caecal colonisation

A second order hierarchical general linear model on the data collected during the fourth vaccination experiment alone ( $R^2 = 0.46$ ) found that the colonisation course of the group vaccinated with a bivalent vaccine comprising of both GST-SodB and GST-FliD was significantly different from that of the groups vaccinated with GST only ( $p = 0.012$ ), GST-SodB only ( $p = 0.01$ ) and GST-FliD only ( $p < 0.001$ ), but surprisingly owing to higher levels of caecal colonisation within this group. *Post-hoc* Dunnet's tests indicated significant difference between the bivalent SodB-FliD and the GST only ( $p < 0.001$ ) vaccinated groups at 56 dph. No individual time-points were distinguished when compared to the GST-SodB and the GST-FliD vaccinated groups. The caecal colonisation by *C. jejuni* M1 within this experiment is given in Figure 5.6 and shows that the group vaccinated with the combination of GST-SodB and GST-FliD had higher counts than the aforementioned groups to which its course of colonisation was significantly different. This suggests that combining SodB and FliD does not have a beneficial additive effect and that it may, in fact, decrease the protective effect observed with individual antigens. However, interpretation of this data should be made with caution given no significant reduction were observed in the GST-SodB and GST-FliD individually vaccinated groups compared to the GST only control. Furthermore, while a decrease in caecal *Campylobacter* colonisation was seen from weeks 1 to 4 post-challenge during the previous experiments, it was not as marked as in the current

trial. During this experiment, the caecal colonisation of *C. jejuni* M1 declined by more than  $2.5\log_{10}$  between weeks 2 and 4 post-challenge in the GST control group.



**Figure 5.6. Caecal colonisation data in birds vaccinated with univalent and multivalent recombinant *Campylobacter* vaccines.** Caecal *Campylobacter* counts were determined by serial dilution of caecal contents collected at post-mortem examinations. Between 4 and 6 birds were sampled at each of the time-point in each of the treatment groups. The error bars are the SEM. Significant differences in the course of colonisation between different groups are described in Section 5.3.4.1. For all treatment groups colour-coded in the legend GST-tagged recombinant antigens were used.

#### 5.3.4.2 Antigen-specific serum IgY responses

The induction of antigen-specific serum IgY responses in the GST-SodB group was measured in ELISAs using MBP-tagged coating antigens and this data is given in a later section as an average of all experiments. The data for GST-CjaA tested singly and in combination with GST-SodB and GST-FliD is given in Figure 5.7.A and demonstrates the induction of antigen-specific serum IgY in both groups at all time-points. MBP-CjaA was used in these ELISAs. MBP-tagged antigens were initially purified in insufficient quantities to measure the induction of specific responses against SodB and FliD in the groups vaccinated with the multivalent vaccines and in the univalent FliD vaccinate group. Subsequent attempts to purify MBP-SodB and MBP-FliD failed to produce recombinant antigens that cross-reacted with serum IgY in ELISAs (data not shown). However, these antigens reacted with an anti-MBP antibody and with antigen-specific serum IgY (using sera from vaccinated but non-challenged birds) in Western blots (data not shown), suggesting



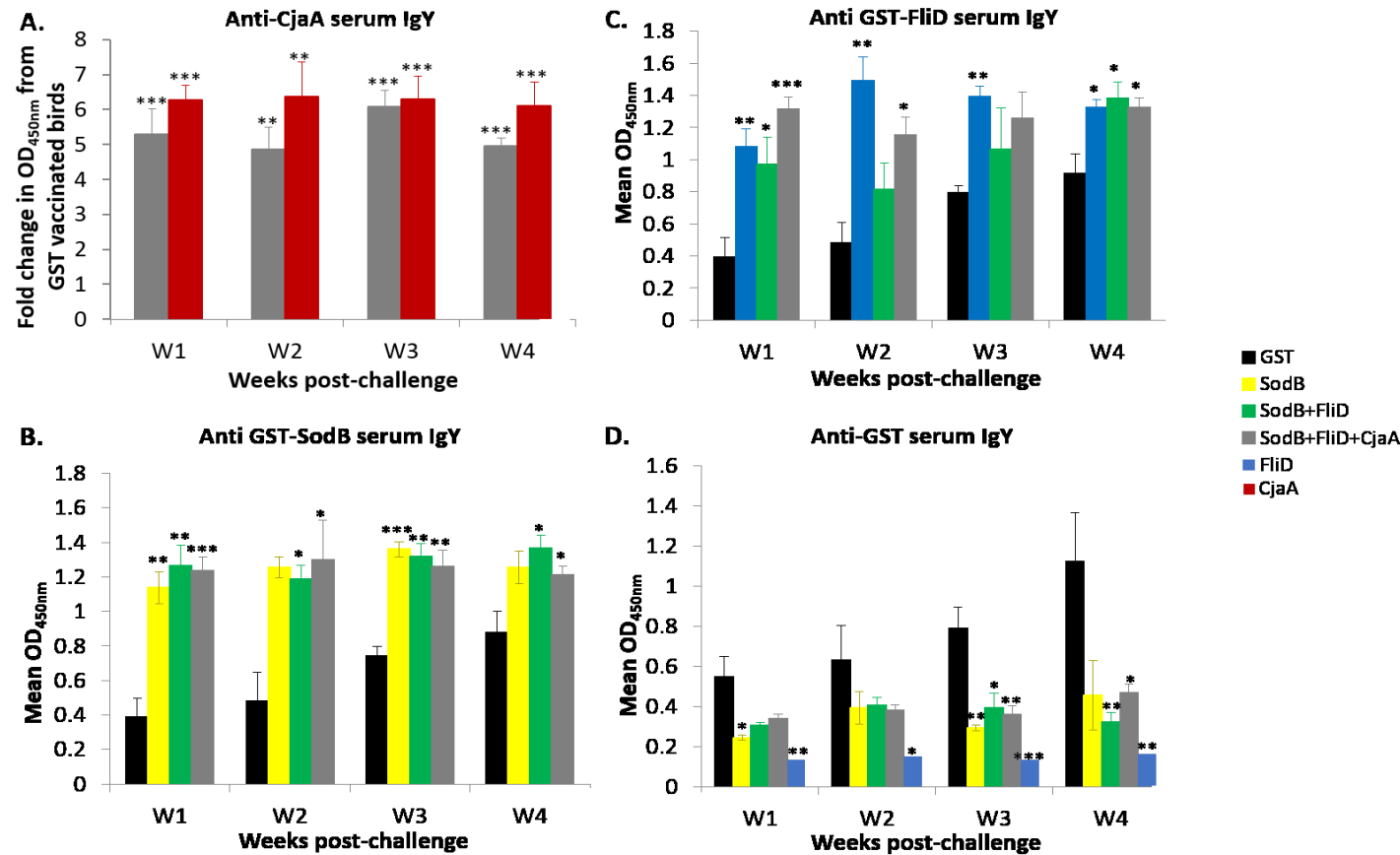
possible mis-folding of the recombinant antigens or failure of these recombinant antigens to bind to the ELISA plates. Due to this, the GST-tagged SodB and FliD recombinant antigens were used in ELISAs to measure the response against the entire recombinant protein and these were compared to the reactivity of each serum to GST alone. The data is shown in Figure 5.7.(B-D) and significant increases in serum IgY against the GST-tagged antigens compared to the GST only control group indicate an increase in antibodies due to vaccination. Furthermore, the significant decrease in reactivity to GST only in the groups vaccinated with the GST-tagged recombinant antigens may indicate that the increase in serum IgY against the GST-tagged antigens was due to induction of *Campylobacter*-antigen specific serum IgY. As the molecular weight of each GST-tagged antigen was taken into account to deliver an equal number of moles of each antigen, the molar amounts of GST were identical in the GST-only control group and the other treatment groups. This suggests that the *Campylobacter* antigens delivered in this study may have higher intrinsic immunogenicity compared to the GST tag, resulting in a significant decrease in the amount of serum IgY produced against GST in birds vaccinated with *Campylobacter* antigens.

#### **5.3.5. Average data across all trials testing protection against homologous challenge**

Given the variability in the protective effects observed across independent replicates, the ability of SodB and FliD to reduce caecal *Campylobacter* counts was assessed in comparison with the GST and GST-CjaA control groups tested in all vaccination experiments using homologous challenge, even when not tested concomitantly. This comparison was made in order to increase the statistical power and it was enabled by the design of the vaccination experiments being kept identical across all vaccination trials and the use of a challenge strain originating from the same bacterial stock.

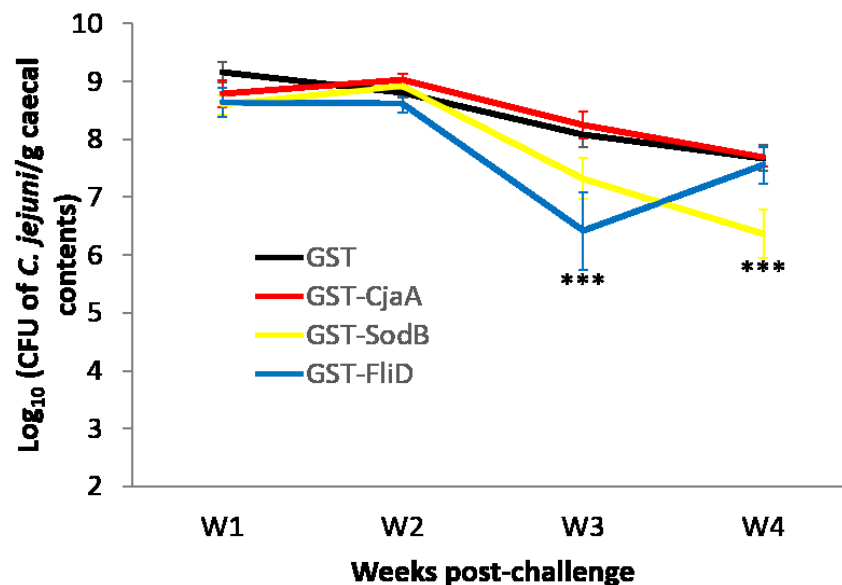
##### **5.3.5.1 Protection against caecal colonisation**

The mean caecal colonisation levels across all four experiments using homologous challenge with the *C. jejuni* M1 strain is given in Figure 5.8. A second order hierarchical GLM was used as fitting of a third order model was not possible due to the GST-SodB and GST-FliD vaccines not being tested in all four experiments. The initial model highlighted the presence of a large number of data points from individual birds with large residuals. Residuals are calculated as the difference between the observed and expected value for individual animals. Large residuals are used as an indication of data points that do not fit the model well and can be considered outliers. In order to optimise the model, the first two



**Figure 5.7. Induction of antigen-specific serum IgY in birds vaccinated with univalent and multivalent vaccines. A.** Induction of CjaA-specific serum IgY measured in ELISAs using MBP-CjaA as a coating antigen. **B-D.** Induction of serum IgY against GST-SodB, GST-Flid and GST only expressed as average OD<sub>450nm</sub> readings obtained in ELISAs using GST-tagged recombinant antigens. Between 4 and 6 birds were sampled per group per time-point. Error bars represent the SEM. Significant differences from the GST only vaccinated group are denoted by \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\* for  $p < 0.001$ . All treatment groups colour-coded in the legend were vaccinated with GST-tagged recombinant proteins.

outliers with the largest residuals in each of the GST, GST-CjaA and GST-SodB groups were removed. The residuals for these were at least  $2\log_{10}$  for the GST group and  $3\log_{10}$  for the GST-CjaA and GST-SodB groups. No outliers were identified in the GST-FliD vaccinated group. The re-fitted model ( $R^2 = 0.44$ ) indicated that the course of colonisation in the GST-SodB vaccinated birds was significantly different compared to that in the GST ( $p = 0.005$ ) and the GST-CjaA ( $p = 0.003$ ) vaccinated groups. Furthermore, the course of colonisation in the GST-FliD vaccinated group was also significantly different to that of both the GST ( $p < 0.001$ ) and the GST-CjaA ( $p < 0.001$ ) vaccinated groups. *Post-hoc* Dunnet's tests identified significant reductions of  $1.3 \log_{10}$  CFU/g in the SodB vaccinated group at 56 dph compared to both control groups and of approximately  $1.7 \log_{10}$  CFU/g in the FliD vaccinated group at 49 dph compared to both control groups (all  $p < 0.001$ ). These data suggest that both the FliD and the SodB antigens confer improved protection compared to CjaA when using the current experimental design. No significant reduction was observed with GST-FliD at week 4 post-challenge, but the reduction in colonisation at week 3 post-challenge, followed by the lack of a reduction increase in caecal colonisation at week 4 post-challenge was observed in each of three independent trials run.



**Figure 5.8. Average caecal colonisation by the homologous strain *C. jejuni* M1 in chickens vaccinated with GST, GST-CjaA, GST-SodB and GST-FliD averaged across all experiments.** The data includes four replicates for the GST and GST-CjaA controls and three replicates for each of the GST-SodB and GST-FliD controls. Error bars are the SEM. Between 12 and 22 birds were used per antigen per time-point. Significant differences to both control groups are indicated by *post-hoc* Dunnet's tests are indicated by \*\*\* for  $p < 0.001$ .

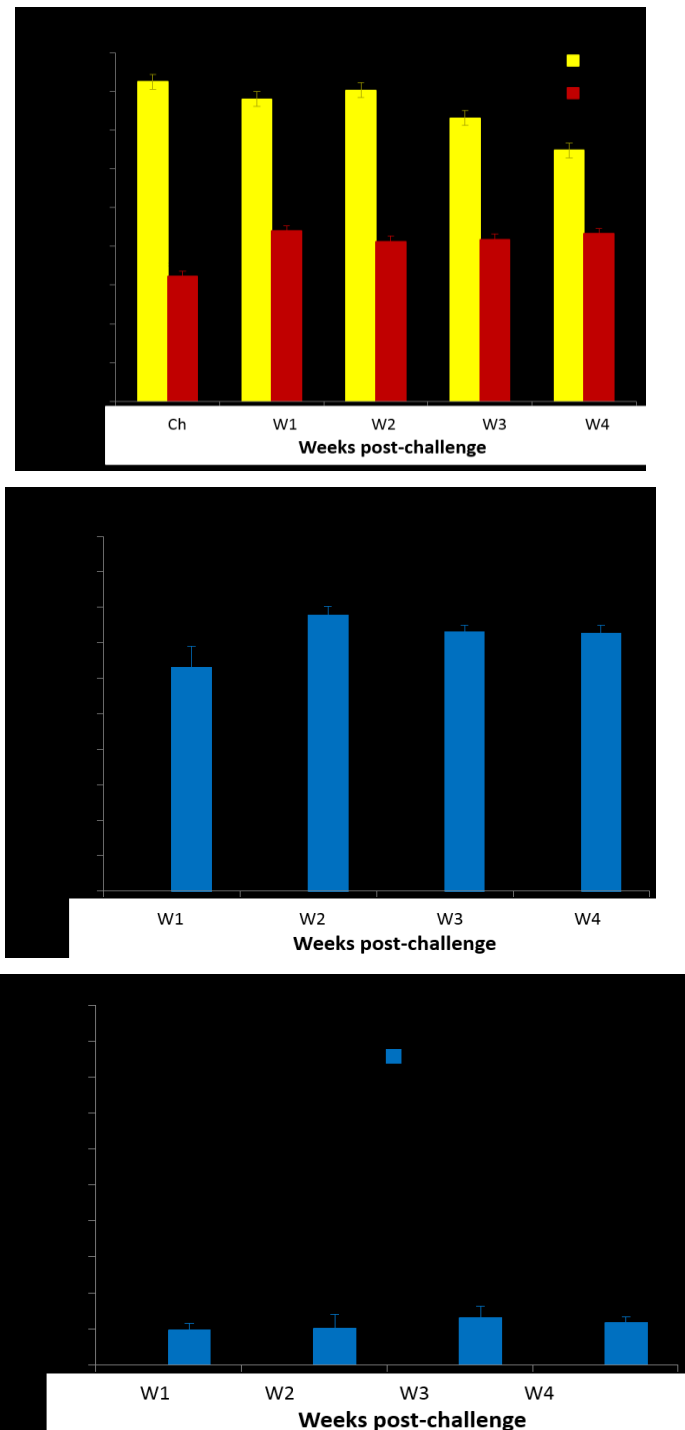
### 5.3.5.2 Antigen-specific humoral responses

The induction of SodB- and CjaA-specific serum IgY was measured through ELISAs using the MBP-tagged recombinant antigens and the data obtained is given in Figure 5.9.A. Averaged across all trials in which they were tested, both SodB (tested in 3 experiments) and CjaA (tested in 4 experiments) induced antigen-specific serum IgY at all time-points tested. The induction of antigen-specific serum IgY against FliD was measured through ELISAs using GST-FliD as the coating antigen as the amount of MBP-FliD available was insufficient to measure antibody induction in the fourth experiment. This data is shown in Figure 5.9B and 5.9C. and indicates the induction of serum IgY against FliD at all time-points measured.

The induction of antigen-specific bile sIgA was also measured for the GST-CjaA and GST-SodB vaccinated birds. These ELISAs were undertaken on MBP-tagged recombinant antigens and the average data across all experiments is given in Table 5.3. No induction of antigen-specific bile sIgA was observed at any of the time-points in any of the experiments. A lack of antigen-specific bile sIgA induction was also observed in the study of Buckley *et al* (2010). As such, measurement of bile sIgA for the other antigens used for vaccination was not performed.

### 5.3.5.3 Functional characterisation of antigen-specific serum IgY antibodies

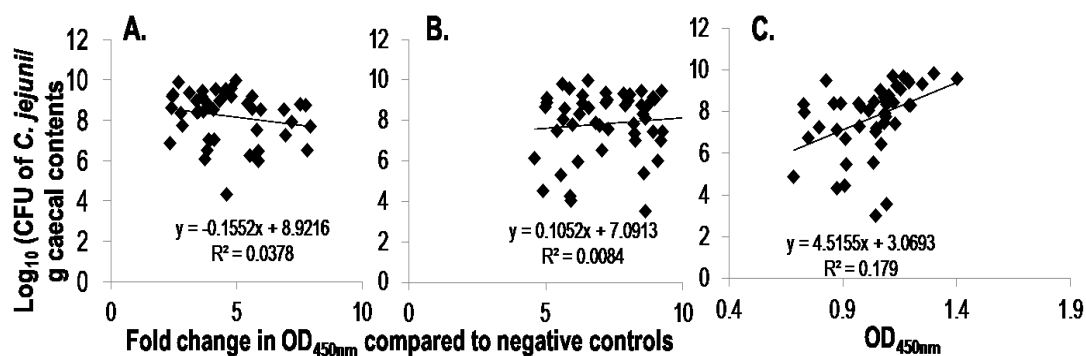
Linear regressions between caecal *Campylobacter* counts and the level of serum IgY antibodies against SodB and CjaA indicated that there was no correlation between antibody production and protection within these treatment groups (Figure 5.10.A and B). However, a linear regression between OD<sub>450nm</sub> readings (obtained in ELISAs measuring reactivity to GST-FliD) and caecal *Campylobacter* counts within the GST-FliD vaccinated group indicated a statistically significant positive correlation ( $p = 0.004$ ; Figure 5.10.C). Even though this relation was weak, it indicates that birds with higher serum IgY responses had higher levels of *C. jejuni* M1 within their caeca, suggesting that this type of immune response is not likely to be responsible for the protection observed. Furthermore, soft agar diffusion assays and slide agglutination tests suggested that serum IgY against FliD do not neutralise their target (data not shown). However, no commercial antibodies were available to use as positive control (e.g. anti-flagellin antibodies).



**Figure 5.9. Induction of antigen-specific serum IgY in birds vaccinated with GST-FliD, GST-SodB and GST-CjaA.** **A.** Induction of CjaA-specific serum IgY (averaged across four experiments) and SodB-specific serum IgY (averaged across three experiments). ELISAs were run with MBP-tagged proteins as coating antigens. **B.** Induction of serum IgY against GST-FliD in GST-FliD vaccinated birds expressed as average OD<sub>450nm</sub> reading. **C.** Induction of serum IgY against GST in GST-FliD vaccinated birds expressed as average OD<sub>450nm</sub> reading. Between 12 and 22 birds were sampled per group per time-point. Significant differences from the GST only vaccinated group are denoted by \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\* for  $p < 0.001$ .

**Table 5.3 Measurement of antigen-specific bile sIgA in GST-CjaA and GST-SodB vaccinated birds.** Average data from three experiments is shown for SodB and from four experiments for CjaA. Between 12 and 22 birds were samples per group per time point.

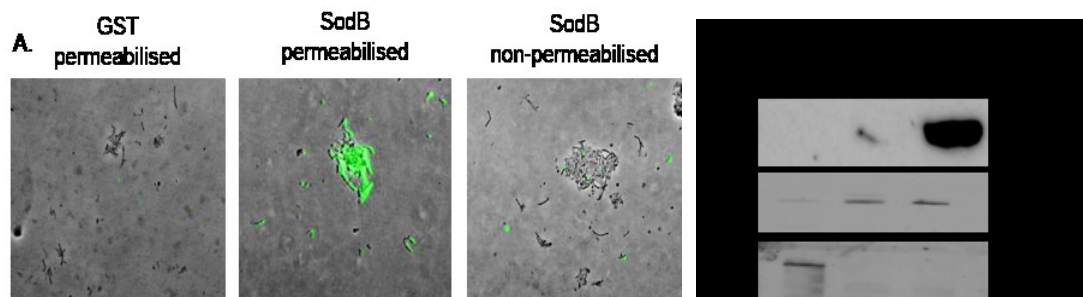
Coating antigen	Vaccine group	Mean OD <sub>450nm</sub> for antigen specific bile secretory IgA			
MBP-SodB	GST-SodB	1.01 (±0.34)	1.02 (±0.33)	1.05 (±0.33)	1.27 (±0.31)
	GST	1.04 (±0.50)	1.09 (±0.40)	1.26 (±0.31)	1.22 (±0.28)
MBP-CjaA	GST-CjaA	0.62 (±0.31)	0.93 (±0.19)	0.73 (±0.26)	0.89 (±0.21)
	GST	0.81 (±0.24)	0.83 (±0.15)	0.76 (±0.37)	0.67 (±0.37)



**Figure 5.10. Correlations between antigen-specific serum IgY induction and caecal *Campylobacter* counts for groups vaccinated with GST fusions to SodB, FliD or CjaA.** A and B. Linear regression of fold-change in antigen-specific serum IgY in individual GST-SodB and GST-CjaA vaccinated birds on their caecal *Campylobacter* counts. ELISAs were performed on MBP-tagged recombinant protein. C. Linear regression of OD<sub>450nm</sub> readings (measured in ELISAs on GST-FliD) in GST-FliD vaccinated birds on their caecal *Campylobacter* counts. ELISAs were performed on GST-FliD and as such specific reactivity against FliD could not be accurately determined in order to calculate a fold-change. For all panels, the average caecal colonisation across both caeca was used.

In addition, the ability of SodB antibodies to recognise their target on the surface of the bacterium was assessed through immunofluorescent staining of *C. jejuni* 11168H cells. Contrary to previous reports that described SodB as being present in the outer membrane proteome of *C. jejuni* (Watson *et al*, 2014), this experiment indicated that SodB is not present on the surface of *C. jejuni* (Figure 5.11.A). This finding was validated by Western blotting of *C. jejuni* 11168H subcellular fractions, which revealed the presence of SodB only within the periplasmic space (Figure 5.11.B). The purity of the fractions was demonstrated by Western blotting with  $\alpha$ CapA (an outer membrane auto-transported adhesin; Ashgar *et al*, 2007) or  $\alpha$ MfrA (a periplasmic fumarate reductase subunit associated with an inner membrane complex; Guccione *et al*, 2010). However, no assessment of cytoplasmic

contamination of the periplasmic fraction was carried out and as such the presence of SodB within the periplasm cannot be clearly dissociated from its presence within the cytoplasm. These assays also demonstrate cross-reactivity of serum IgY against *C. jejuni* M1 SodB antibodies with native *C. jejuni* 11168H SodB. Before these experiments were carried out, cross reactivity was demonstrated through a Western blot of whole cell lysate of *C. jejuni* M1 and 11168H using sera from GST-SodB vaccinated birds, which showed reactivity of SodB from both *Campylobacter* strains with the antibodies raised against *C. jejuni* M1 SodB (data not shown).



**Figure 5.11. Subcellular localisation of SodB in *C. jejuni* cells.** **A.** Immunofluorescent staining with sera from GST-SodB and GST only vaccinated birds collected on the day of challenge, before oral gavage. Bacterial cells were fixed with 4% (w/v) PFA and, where indicated, permeabilised with 10% (v/v) Triton-X100, both diluted in PBS. **B.** Immunoblotting of inner membrane, outer membrane and periplasmic subcellular fractions of *C. jejuni* 11168H with sera from the same GST-SodB vaccinated birds used in panel A.

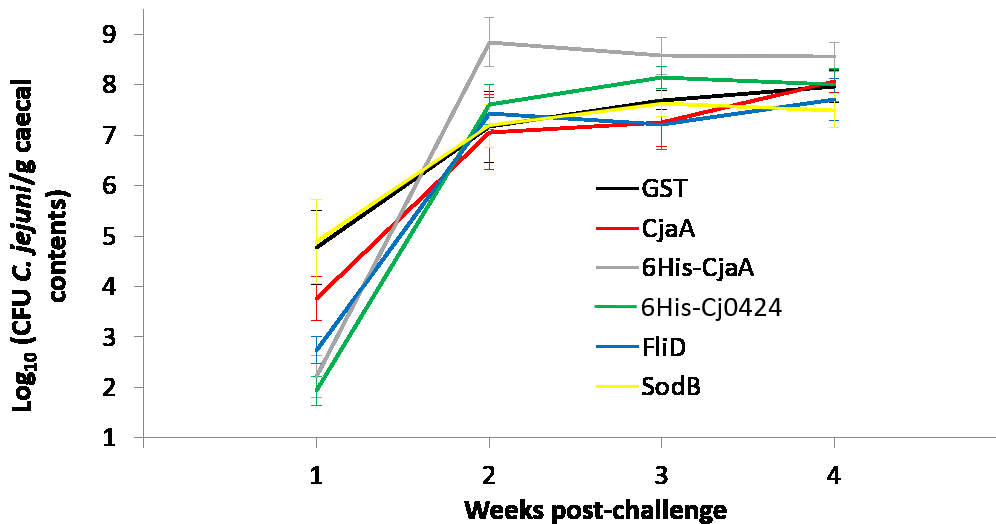
### 5.3.6 Assessment of protection against heterologous challenge

A vaccine that could be used in the field would have to induce cross-protection against different *C. jejuni* strains and following the observation above that antibodies raised against *C. jejuni* M1 SodB cross-react with *C. jejuni* 11168H SodB and the high level of primary sequence conservation of SodB in sequenced *Campylobacter* species, an experiment was carried out to assess whether SodB and FliD could confer protection against heterologous challenge. Furthermore, the same experiment aimed to assess whether the lack of the protective effect observed with GST-CjaA in the previous vaccination experiments was due to the fusion partner as previously a 6xHis-CjaA recombinant antigen was shown to be protective (Buckley *et al*, 2010). Birds were therefore vaccinated with the *C. jejuni* M1-derived antigens indicated in Table 5.2 and challenged at four weeks of age with *C. jejuni*

11168H rather than M1. The same trials also tested Cj0424 for its ability to confer homologous protection against *C. jejuni* challenge.

### 5.3.6.1 Protection against caecal colonisation

A second order hierarchical GLM ( $R^2 = 0.76$ ) did not identify significant differences between the entire course of colonisation in any of the group. The course of caecal colonisation by the *C. jejuni* 11168H heterologous challenge strain is given in Figure 5.12. In contrast with colonisation by *C. jejuni* M1, colonisation levels with *C. jejuni* 11168H were lower at 1 week post-challenge. Of particular note, 6xHis-tagged CjaA was not protective, in contrast to the observations of Buckley *et al* (2010), though several features of the experimental design differed. No protection against homologous challenge was observed with Cj0424.



**Figure 5.12. Heterologous protection against caecal colonisation by *C. jejuni* 11168H in birds vaccinated with the GST-SodB, GST-FliD, GST-CjaA and 6xHis-CjaA cloned from *C. jejuni* M1 and homologous protection against *C. jejuni* 11168H in birds vaccinated with 6xHis-Cj0424.** Between 4 and 6 birds were sampled at each of the time-point in each of the treatment groups. The error bars represent the SEM. The groups colour-coded in the legend were vaccinated with GST-tagged antigens apart from those denoted otherwise.

### 5.3.6.2. Antigen-specific serum IgY responses

Induction of serum IgY against CjaA was measured in ELISAs using MBP-CjaA and is shown in Figure 5.13.A. The same figure also shows induction of serum IgY responses against 6xHis-CjaA and 6xHis-Cj0424, measured in ELISAs using the 6xHis-



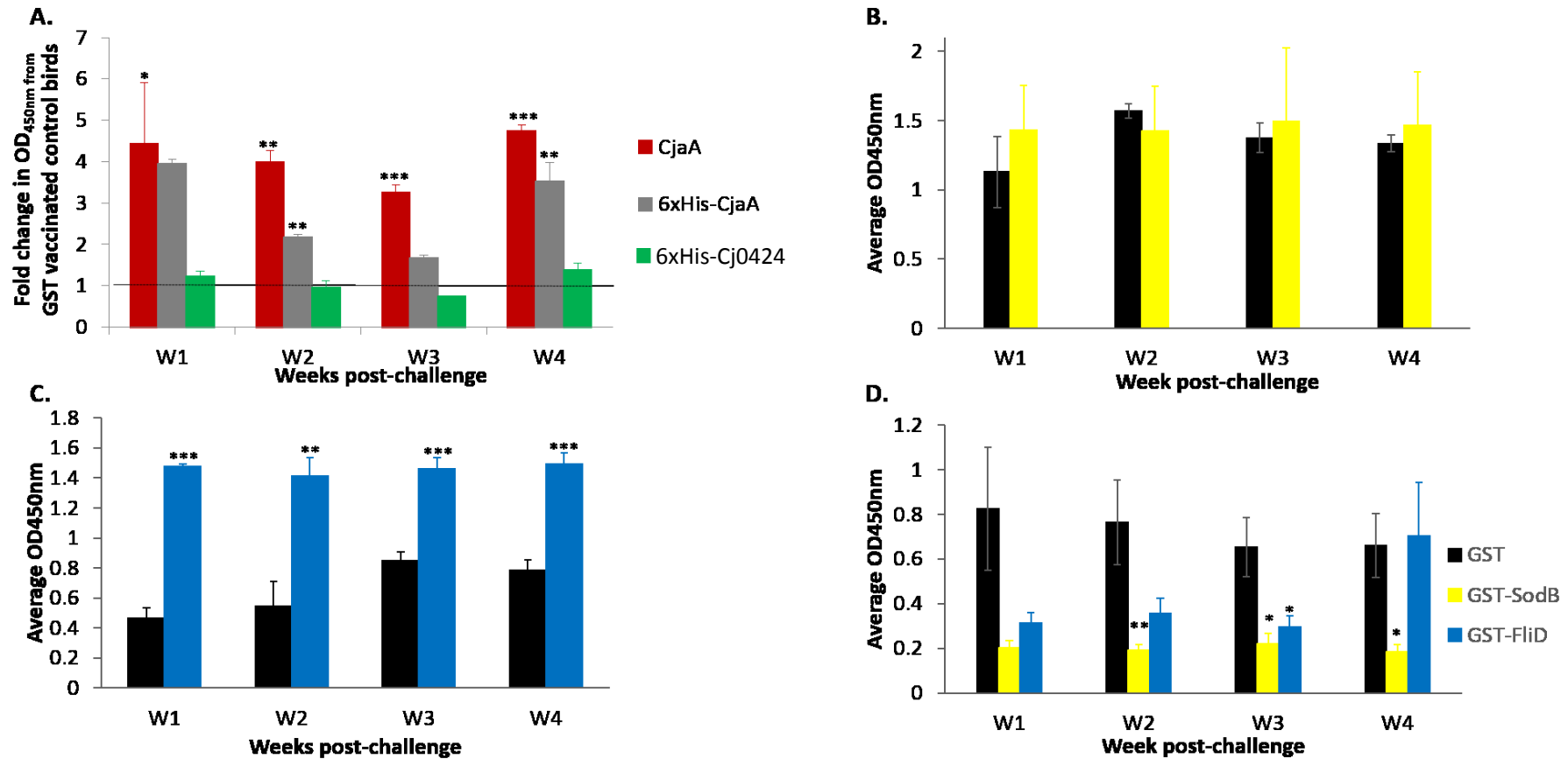
tagged recombinant proteins as coating antigens. Vaccination with GST-CjaA resulted in significantly higher anti-CjaA serum IgY levels at all time-points. Apart from at 49 dph, the 6xHis-CjaA vaccinated birds showed significant increases in serum IgY at all other time-points. Cj0424 failed to induce specific serum antibodies at any of the time-points tested, at least at the limit of detection of the assay used.

The induction of serum IgY antibodies against FliD and SodB was measured by ELISA using the GST-tagged recombinant proteins as coating antigens and the obtained data is given in Figure 5.13.B-D. Significant induction of serum IgY against FliD was observed in the GST-FliD vaccinated group at all time-points. No significant induction of serum IgY was observed against SodB in the GST-SodB vaccinated group. However, a significant reduction in the reactivity towards GST in the GST-SodB vaccinated group compared to the control group and the high reactivity of sera from GST only vaccinated birds against GST-SodB suggest possible saturation of the assay and measurements outside the linear range. More accurate determination of the serum IgY responses against GST-SodB could be determined through optimisation of this assay for this particular experiment through a separate chequerboard analysis.

## 5.4 Discussion

The experiments described above demonstrate that SodB and FliD are protective against caecal colonisation by the homologous *C. jejuni* M1 strain and add two novel protective antigens to the limited repertoire of those described in the literature. These antigens also conferred improved protection compared to CjaA using the current vaccines and delivery protocols, however CjaA failed to elicit protection. As such, whether SodB and FliD would confer improved protection using different vaccine and experimental designs, in which CjaA would be also protective, remains to be established. Furthermore, even though one aim of the study was to find antigens that may confer improved consistency of the protective effect across replicate experiments, the protective effect observed with both FliD and SodB varied within the three experiments in which they were tested. This may suggest that the variability observed may not be due to CjaA itself but it may be caused by other properties of the experimental design such as dose of antigen, adjuvant used, timing of vaccination and variability of the inoculum used for challenge. These aspects and experiments to address them are discussed in the general discussion (Section 6) of this thesis.

The lack of protection observed with CjaA is in contrast to previous observations (Buckley *et al*, 2010). As discussed in Section 3, a number of differences in experimental



**Figure 5.13. Induction of antigen-specific serum IgY in birds vaccinated with the *C. jejuni* M1 SodB, FliD and CjaA antigens or with the *C. jejuni* 11168H Cj0424 antigen and challenged with the *C. jejuni* 11168H strain. **A.** Average fold change in OD<sub>450nm</sub> in birds vaccinated with GST-CjaA, 6xHis-CjaA and 6xHis-Cj0424. **B** and **C.** Induction of serum IgY against GST-SodB (panel B) and GST-FliD (panel C) in vaccinated and control birds expressed as mean OD<sub>450nm</sub>. **D.** Induction of serum IgY against GST in GST-SodB, GST-FliD vaccinated and control birds expressed as mean OD<sub>450nm</sub>. The key for panel D applies also to panels B and C. Error bars represent the SEM. Significant changes from the GST only vaccinated group are denoted by \* for  $p < 0.05$ , \*\* for  $p < 0.01$  and \*\*\* for  $p < 0.001$ . Apart from where indicated otherwise, birds were vaccinated with GST-tagged recombinant antigens.**

design could account for the discrepancy. In addition to the use of a different breed of chickens, in the case of recombinant subunit vaccines, a different tag used for affinity purification and a different adjuvant may also account for the lack of a protective effect. While GST-PorA has been reported in the literature to induce protection against *Campylobacter* challenge in mice (Islam *et al*, 2010), the use of the GST tag in this study may have inadvertently resulted in delivery of a reduced dose. During purification of GST-CjaA, a truncation of the size of GST alone that reacted with an anti-GST antibody in Western blots was observed (Figure 4.6) This truncation accounted for approximately half the protein preparation and given that the amount of antigen delivered was standardised according to the total protein present in each antigen preparation, it is likely to have resulted in the delivery of a dose approximately half of that intended. Previous studies using various antigens report improved protection at doses over 100 µg per bird so a dose that would potentially be as low as 7 µg of CjaA per bird may be insufficient to elicit the appropriate immune responses. Whether this was the case cannot be ascertained due to lacking an immune correlate of protection, the fact that CjaA-specific serum IgY was induced in all vaccinated birds and the lack of a dose-response vaccination experiment.

With regards to the adjuvant, the TiterMax<sup>®</sup> Gold is described by its manufacturer to confer improved induction of humoral responses. However, this type of response may not be the major contributor to the protective effect observed and as such the use of this adjuvant may have contributed to decreased protection. The hypothesis that humoral responses (in particular serum IgY and bile sIgA) may not be the immune mechanism responsible for protection is supported by a number of observations in this study. Firstly, there was a lack of correlation between serum IgY induction and caecal colonisation levels in the GST-SodB vaccinated birds. Secondly, a positive correlation between these parameters within the GST-FliD vaccinated group suggests that a heightened antigen-specific serum IgY response may be detrimental to the protective effect induced by vaccination. Additionally, SodB proved protective in spite of its absence from the bacterial surface (at least at levels detectable in the assays used) suggesting that direct neutralisation of the bacteria via antibodies is unlikely to be the mechanism of protection. Also, the lack of neutralisation of FliD by serum IgY supports this latter assumption, however the results of those experiments should be interpreted with caution in light of lacking an adequate positive control. Lastly, preliminary splenocyte recall assays undertaken during the fourth vaccination experiment on birds vaccinated with GST-FliD indicated a statistically significant correlation between caecal *Campylobacter* counts and splenocyte proliferation in response to stimulation by GST-FliD (data not shown). However, no characterisation of the proliferating cellular population was

undertaken and even though a correlation was present, protection against caecal colonisation was not observed within that particular study relative to the GST control. While the findings suggest pointers towards the type of immune response induced by vaccination, the limitations of these experiments preclude firm conclusions about which arm of immunity is responsible for protection following vaccination. Future studies to quantify antigen-specific splenocyte proliferation are needed to build statistical power to the point where robust conclusions can be drawn.

Due to the variability observed within the replicate studies testing SodB-, FliD- and CjaA-based vaccines, caution should be exercised when interpreting the results obtained when testing other antigens only once, in particular those obtained within the experiments testing multivalent vaccines and heterologous protection. Ideally multiple replicates of experiments testing each parameter should have been performed. However, this would require significantly more resources than were available for this doctoral project.

Whilst this study adds two novel antigens that can be used as vaccines against *Campylobacter* to the limited repertoire described in the literature, the vaccine would require further optimisation before being suitable for field use. Furthermore, a plethora of questions with regards to the mechanism of action of these vaccines remain unanswered. Future directions of research are discussed in the final section of this thesis.

## 6. Discussion

### 6.1 Summary

The current study aimed to refine vectored vaccines for the control of *Campylobacter* in poultry, initially through the use of avian pathogenic *E. coli* (APEC) as a vector for a TetC-CjaA fusion. The use of PoulVac<sup>®</sup> *E. coli*, a  $\Delta$ aroA APEC vaccine, as a vector for CjaA resulted in induction of serum IgY against both CjaA and TetC; however, no protection was observed against *C. jejuni* with either this vaccine or with the previously described *Salmonella* Typhimurium  $\Delta$ aroA vaccine expressing TetC-CjaA (Buckley *et al*, 2010) that was used in this study as a control. Differences in experimental design between this study and those described previously, which may account for the lack of protection observed within this study, were discussed in earlier sections. They notably include the use of a different chicken line and route of administration. Additionally, codon optimisation of CjaA did not improve expression though this may reflect the culture conditions and the lack of optimisation of the N-terminal TetC fusion partner. While these experiments demonstrate the potential of inducing an immune response through vaccination with PoulVac<sup>®</sup> *E. coli*, further experiments would be required to assess its usefulness as a vaccine vector.

Due to the lack of a protective effect using live vectored vaccines and the variability in responses to vaccination observed within this and previous studies (Buckley *et al*, 2010), the remainder of this study focused on the search for an antigen or combination of antigens that may confer an improved magnitude, timing or replicability of protection compared to CjaA. Following identification and cloning of 21 candidate antigens, nine were tested in chickens for their protective efficacy against homologous challenge. Two of these antigens, SodB and FliD, were observed to confer statistically significant reductions in caecal *Campylobacter* carriage at 49 and 56 days post-hatch. However, the CjaA-based vaccine did not induce protection, in contrast with previous reports on the use of 6xHis-CjaA as a recombinant subunit vaccine (Buckley *et al*, 2010). Potential reasons for the disparity in the protective effect were discussed in Section 5.4 and include the use of a distinct bird line, fusion tag for purification and adjuvant. The FliD and SodB vaccines conferred a significant improvement over the CjaA vaccine in this study, albeit the latter was not protective. Furthermore, the timing of protection was not improved from previous studies in our group describing the use of CjaA as a vaccine (Buckley *et al*, 2010). Further experiments, using an

optimised study design, are required in order to assess whether SodB and FliD could confer improved protection compared to CjaA, in the case of the CjaA-based vaccine being itself protective.

Nevertheless, this study described two new antigens that could be used in vaccines against *Campylobacter*. Furthermore, novel insights into the nature of the protective immune response were revealed, which suggest that antibody responses, either as serum IgY or bile sIgA, may not play the major role in protection. The magnitude and timing of the antibody responses did not correlate with protection and in the case of FliD, increased serum IgY responses were observed to be detrimental to vaccine-induced protection. Furthermore, serum IgY from SodB and FliD vaccinated birds failed to agglutinate bacteria and protection was observed with SodB in spite of its cytoplasmic/periplasmic localisation. As such, it is likely that other mechanisms such as mucosal IgA or cell-mediated immunity may play a role in protection. Preliminary data obtained from assays measuring splenocyte proliferation in response to stimulation by the antigens used in vaccination identified a negative correlation between the level of splenocyte proliferation and caecal *Campylobacter* colonisation in GST-FliD vaccinated birds. However, the nature of the proliferating cells was not assessed. Future vaccination experiments should include such assays, but in addition they should also identify and characterise the cells that proliferate. Additional experiments are required to formally demonstrate the role of each of these arms of the immune response in vaccine-mediated protection and a potential study design is suggested below.

## **6.2 This study in the context of other research**

Even though live vectored vaccines were not protective against caecal *Campylobacter* colonisation, this study opens the possibility of the use of PoulVac<sup>®</sup> *E. coli* as an attenuated vector, with a demonstrated safety record (EMA, 2012), for the delivery of heterologous antigens. If optimised, the use of this vector has the potential to allow more cost-effective vaccine production, a more facile administration compared to injectable vaccines and it would have the added benefit of concomitantly controlling colibacillosis, a disease that adversely affects poultry and farmers. These needs of the poultry industry have been recognised by numerous other studies that have reported the use of attenuated bacterial vectors for delivery of recombinant subunit vaccines against *Campylobacter* and these are summarised in Section 1.5.4.

The variability observed within the vaccination experiments using live vectored vaccines but also within those using SodB, FliD and CjaA as recombinant subunit vaccines

indicate that intrinsic properties of either the study design or of *Campylobacter* are likely to account for the effect observed. Given the available literature, it may be concluded that study and vaccine design have an impact on the protective effect observed. CjaA has been described to confer a 6 log<sub>10</sub> CFU/g reduction in caecal *Campylobacter* when vectored in an attenuated *S. Typhimurium* vector (Wyszynska *et al*, 2004), a 1-2 log<sub>10</sub> CFU/g reduction when vectored within another *S. Typhimurium* vector (Buckley *et al*, 2010) and no protection when vectored in a delayed expression *S. Typhimurium* vector (Laniewki *et al*, 2014a). Furthermore, even when used in recombinant form, 6xHis-CjaA was shown to be able to confer a reduction of approximately 2.5 log<sub>10</sub> CFU/g (Buckley *et al*, 2010), but others have found no protection when a combination of GST- and 6xHis-tagged CjaA was used (Michael Konkel, personal communication). In addition, individual research groups report variation in the protective effect observed across replicates using identical experimental design, with some replicates demonstrating protection, while others do not: such variation was observed in experiments reported by Buckley *et al* (2010), within this study, within subsequent experiments using *Salmonella* vectored CjaA (Elzibeta Jagusztyn-Krynicka, personal communication) and within vaccination experiments using other antigens reported by collaborators in Zoetis and MSD Animal Health (unpublished data). Such variability in spite of a constant experimental design suggests that other factors may account for this effect.

One possible cause is the intrinsic genomic plasticity of *Campylobacter jejuni*. Detailed insights into a potential genetic mechanism for adaptation were revealed at the time of the first whole-genome sequencing of a *Campylobacter* strain (Parkhill *et al*, 2000). This revealed the presence of homopolymeric tracts that have later been demonstrated to lead to phase variation in gene expression through on/off switching of genes induced by slipped-strand mispairing during DNA replication (Gilbert *et al*, 2002; Guerry *et al*, 2002). More recently, research has identified high frequency stochastic variation within these regions that can lead to generation of as many as 50 strains from a single parent during the course of infection in a single human host (Thomas *et al*, 2014). It has been suggested that this ability to adapt may play a role in adaptation to selection pressure induced by vaccination (FSA, 2015d). Additionally, another mechanism for the remarkable adaptability of *Campylobacter* were demonstrated through studies investigating the mechanisms of adaptation to the selective pressure of phage predation during chicken colonisation (Scott *et al*, 2007). Under these conditions, *Campylobacter* have the ability to re-arrange or excise up to a third of their genome in order to give rise to strains that are resistant to phage predation. Although these strains colonise chickens poorly, they have been shown to revert to a higher colonisation

potential upon removal of phage predation, further demonstrating the plasticity of this pathogen's genome under selection or when selection is withdrawn. Furthermore, Bayliss *et al* (2012) describe high frequency mutations and on/off switching of genes involved in the variation of surface-exposed structures in *Campylobacter*, but was not able to model the drivers of these changes.

This high genomic plasticity of *Campylobacter* may be responsible for the variation in protective effect observed across different vaccination experiments. While an inoculum originating from the same single colony was administered to all birds in all vaccination experiments during this study, it is possible that during culture of the original single colony for storage as a glycerol stock a high number of *C. jejuni* variants with different combinations of on/off switches of phase variable genes and even genomic rearrangements were generated. Each subsequent culture for the purpose of preparing the challenge inoculum is likely to have resulted in further variability and, in addition, adaptation of each culture to colonisation of the avian intestinal tract is likely to have resulted in further genetic variation. It is thought this may partly explain the unstable and unpredictable dynamics of *C. jejuni* populations during mixed infection of chickens with wild-type isogenic tagged strains (Coward *et al*, 2008). It is even possible that due to the stochastic nature of strain selection, individual birds become colonised with strains of *C. jejuni* with different genetics. As such, it is likely that to improve replicability, future vaccination experiments would require the use of a higher number of birds per time-point per treatment group than were used in this study. This is supported by power calculations using the variance observed within the current vaccination experiments suggesting that approximately 10 birds should be used per group per time-point to be able to detect a 1 log<sub>10</sub> CFU/g reduction in caecal colonisation with 80% power and a type I error rate of 0.05.

This study presents preliminary data with regards to the nature of the immune response that is associated with vaccine-induced protection. While the majority of studies describing anti-*Campylobacter* vaccines in chickens published to date focus on the measurement of serum IgY as a measure of vaccine immunogenicity, the current study suggests that this type of immune response may not be associated with protection. One study attempted to describe the nature of the cellular immune responses and showed an increase in the percentage of cells in the caecal tonsils expressing CD4, CD3 and Bu-1 markers following vaccination but no significant change was detected in CD8<sup>+</sup> cells (Laniewski *et al*, 2014a). This suggests activation of T helper cells and B cells but not of cytotoxic T cells and a possible dominance of humoral immune responses. Of note is that no significant reductions



in caecal colonisation by *Campylobacter* were observed within that study. The lack of protection may be due to a lack of activation of the cell-mediated arm of immunity.

It is possible that the protective effect of vaccination is exerted through the action of cell-mediated immunity on a yet unidentified site/niche of *C. jejuni* persistence within the intestinal tissues of the chicken. In support of a role of cell-mediated immunity in vaccine-mediated protection is also the fact that, in contrast with natural infection with *Salmonella*, for which the crucial role of cell-mediated immunity in clearance at primary infection has been demonstrated (Beal *et al*, 2006), *Campylobacter* infection does not activate cell-mediated immunity efficiently in the chickens and establishes long-term colonisation. In contrast, *Campylobacter* infection activates cell-mediated immunity in humans and this has been linked to clearance of the pathogen (reviewed in Section 1.2.3). Additional experiments that could elucidate and potentiate the nature of the protective immune responses induced by vaccination are suggested below.

A small number of recent studies described the induction of high level protection against caecal colonisation using both live-attenuated vectored vaccines and subunit recombinant vaccines. Layton *et al* (2011), describe the induction of a 6log<sub>10</sub> CFU/g reduction in caecal colonisation following administration of a short peptide of CjaD surface-expressed in *S. Typhimurium*, co-delivered with the immunostimulatory molecule CD154. However, prior to field use of such a vaccine it is likely to be required to demonstrate that the vector does not remain in the birds at the point of slaughter and/or that the CD154 molecule present would not induce immunomodulatory effects in humans should the vaccine enter the food-chain. Using recombinant F1pA, Neal-McKinney *et al* (2014) demonstrate the induction of a 6log<sub>10</sub> reduction in caecal *Campylobacter* colonisation, however two doses of 250 µg of recombinant protein were used. While highly effective, the cost of producing such a vaccine would preclude its field use in broilers.

While vaccination offers the prospective of a widely-applicable and facile measure to control *Campylobacter* contamination of chicken carcasses, no effective experimental vaccine is currently close to a marketable stage. As such, it is likely that this is a long-term measure, while in the short term other measures such as improved biosecurity, carcass decontamination, consumer education, etc may have to be used to effect a reduction in human campylobacteriosis. Even in the long-term, it is likely that vaccination would be combined with other such measures in order to maximise the reduction in campylobacteriosis, as was the case with the introduction of the Red Lion Code for control of *Salmonella* in chickens.

### **6.3 Limitations of this study**

While the current study identified two novel candidate antigens that could be used in vaccines against *Campylobacter* in poultry and opened the possibility that PoulVac® *E. coli* may be used as a vector, it also has a number of limitations that are discussed herein. Firstly, in the case of the experiments testing live vectored vaccines, no protection was observed with any of the vaccines tested, including the *S. Typhimurium* vectored CjaA vaccine. This resulted in the inability to assess whether PoulVac® *E. coli* is a significant improvement compared to *Salmonella* when vectoring *Campylobacter* antigens.

Secondly, due to the high variability in protective effect observed with experimental vaccines against *Campylobacter* across individual replicates, the results of some of the experiments testing recombinant subunit vaccines should be interpreted with caution. The initial testing of the nine candidate *Campylobacter* antigens relied on a single replicate and it is possible that with further replicates other antigens may have proven protective. However, the resources available within a doctoral project precluded the repeat testing of all candidate antigens identified initially. As such, measures of statistical significance were used for initial selection, namely the modelled magnitude of reduction in caecal colonisation across the entire course of colonisation compared to the GST-only vaccinated group. Furthermore, the experiments testing multivalent vaccines and protection against heterologous challenge were also undertaken only once and as such firm conclusions cannot be drawn from these studies. Likewise, it is possible that further replicates of these studies may reveal significant reductions in caecal *Campylobacter* colonisation.

In addition, even though this study provided preliminary data on the nature of the protective immune response induced by vaccination, fine characterisation of this response was not carried out. While antibody responses did not correlate with caecal *Campylobacter* counts, only serum IgY and bile sIgA were measured. It is possible that measurement of local caecal IgA would reveal an association between *Campylobacter* colonisation and humoral immune responses. Measurement of mucosal IgA was attempted for the SodB and FliD vaccinated groups during the last vaccination experiment, however, no antigen-specific antibodies were detected even though dot-blots with an anti-IgA antibody revealed the presence of this isotype within the samples. As the birds were raised under SPF conditions, the lack of adequate controls meant that it was not possible to assess the efficacy of the extraction procedure using measurements of reactivity against an antigen known to be present and immunogenic. Furthermore, given the lack of a protective effect within this

particular experiment, it was also not possible to determine whether the lack of antigen-specific mucosal IgA was due to failure of the vaccines to induce adequate levels of immunity or whether this is genuinely a feature of experimental vaccines against *Campylobacter* in poultry. Due to all these limitations data relating to mucosal intestinal IgA was not presented within this thesis. In addition, even though a correlation between splenocyte recall and caecal *Campylobacter* colonisation was observed in the GST-FliD vaccinated birds in the experiment testing multivalent vaccines, that experiment did not demonstrate statistically significant protection in the GST-FliD vaccinated birds when analysed in isolation. This, combined with the lack of characterisation of proliferating cells, makes it impossible to distinguish even between T and B cells as the proliferating cells within this assay.

Lastly, a layer line was used in this study, reflecting the need to hatch synchronously a large number of eggs. This would be more difficult to achieve with a less prolific broiler line and if eggs were to be bought from external sources, it is likely they would originate from *Campylobacter*-positive flocks given its widespread presence in commercial flocks. The presence of maternally-derived antibodies may affect the immune responses in ways that have not yet been determined. Such antibodies may sequester antigens preventing the induction of an adequate immune response or they may opsonise vaccines, enhancing protection. Future experiments should assess the efficacy of an optimised vaccine in a broiler line given that differences between the immune response have been described between layers and broilers in certain studies. Koenen *et al* (2002) describe that broilers respond to vaccination with keyhole limpet haemocyanin (KLH) with a humoral response dominated by IgM and a low level of cellular response, whereas layer birds produce mainly IgY and show higher levels of cellular responses. However, other studies using different antigens found no differences in the magnitude of cellular responses between broilers and layers but confirmed higher levels of antibody induction in broilers (Parmentier *et al*, 2010).

Even in the face of the limitations described above, the data that demonstrated protection using SodB and FliD as GST-tagged recombinant vaccines against *Campylobacter* in chickens stand scrutiny. This data was obtained across three independent replicates for the treatment groups and four replicates for the control groups. Even though none of the individual experiments attained statistical significance due to the insufficient number of birds used, each replicate demonstrated the same pattern of reduction in caecal colonisation. However, the reduction in caecal colonisation varied from 0.5 to 2.5 log<sub>10</sub> for each of the two antigens across replicate experiments.

## 6.4 Future work

Before further optimisation of the vaccines described in this study is undertaken, future experiments should aim to determine the nature of the protective immune response associated with protection in vaccination experiments described to date. This information would then allow rational development of vaccines. One possible experiment that would allow us to formally demonstrate the role of antibody versus cell-mediated immunity would be vaccination of existing homozygous mutant chickens lacking the joining (J) segment of the Ig heavy chain gene (Schusser *et al*, 2014). Ig heavy chain production is eliminated in these birds and no antibody response is elicited on immunisation with keyhole limpet haemocyanin, while migration of B cell precursors into the bursa of Fabricius is unaffected and other immune cell types appear normal. However, subsequent maturation of B cells and their migration from the bursa are blocked. Even though such an experiment could use the vaccines described within the current study, given their limited efficacy and variability in response, a large number of transgenic birds would be required. As such, the experiment could make use of other, more protective recombinant vaccines that have recently been described in the literature, such as those based on FlpA and described by Neal-McKinney *et al* (2014). Following determination of their genotype by PCR, birds of each type would be given the FlpA-based vaccine or would be mock-vaccinated, followed by challenge with *C. jejuni* according to the experimental protocol described within the aforementioned study. The experiment could measure systemic and local humoral responses in blood and caecum respectively. Induction of specific antibodies in intact birds but not Ig<sup>-/-</sup> birds should be observed. Splenocyte proliferation should also be assessed, with additional characterisation of the type of cells that proliferate, through the use of FACS analysis. Additionally, total RNA from spleen, liver and caecal tonsil could be used for analysis of the expression of signature cytokines at each of these sites through qRT-PCR and FACS analysis could also be used to determine the cellular composition of these tissues. Immune molecules to be analysed may include the Th1 signature cytokine IFN- $\gamma$ , the Th2 signature cytokine IL-13, the proinflammatory cytokine IL-1 $\beta$  and chemokine CXCLi2, and the T regulatory cytokines IL-10 and TGF- $\beta$ 4. This experiment may also be undertaken using surgically or chemically bursectomised chickens, although additional characterisation of each bird would be required to ensure meaningful conclusions can be drawn. Use of chemical bursectomy (e.g. using cyclophosphamide or testosterone treatment) brings the risk that proliferating cells other than B cells may be targeted. Surgical bursectomy is technically difficult and concern exists regarding appropriate analgesia and/or anaesthesia during regulated procedures.

A project recently funded by the Biotechnology and Biological Sciences Research Council (BBSRC) will evaluate the use of glycosylated vaccines for the control of *Campylobacter*, vectored by *Salmonella* or APEC live-attenuated vaccines. The experiment described above for the investigation of the nature of the protective immune response induced by vaccination could also include a glycosylated form of the vaccine (e.g. engineered to express the *C. jejuni* heptasaccharide on the bacterial surface and/or fused to *Campylobacter* antigens using the pglB coupling system). Comparison of glycosylated and non-glycosylated vaccines would result not only in novel insights into the avian immune system and its response to glycosylated antigens but also in comparative immunology highlighting differences and similarities to mammalian responses to such antigens.

Following description of the nature of the protective immune response induced by vaccination, other studies could investigate the use of different adjuvants for vaccine optimisation. Such adjuvants could include not only tradition adjuvants, but also cytokines and chemokines. As experiments would have to evaluate each adjuvated vaccine compared to a group vaccinated without adjuvant and to a group receiving the adjuvant administered alone, only two adjuvants could be tested per experiment under the conditions currently available to us. One such experiment could test a mixture of IL-12 and IL-18 to augment Th1 responses or IL-6 to boost Th2 responses. A separate experiment could test a mixture of CCL19 and CCL20 (which respectively attract mature and naïve APCs) or CXCL13 (which attracts B cells and Th2 cells). In addition to testing the impact of molecules that recruit APCs, testing of molecules that drive APC proliferation and/or antigen presentation could also be undertaken. Experiments could evaluate recombinant chicken CSF-1 (which drives APC proliferation) or a combination of IL-6 and IL-21 which may drive differentiation of T follicular helper cells. All such experiments could include the characterisation of the induced immune responses in a similar manner to that in the experiment described above.

Should antibodies be shown to be vital for protection, identification of immunodominant antigens and even epitope mapping within these antigens could be carried out using antibodies induced by natural or experimental infection. Studies have previously identified antigens recognised by maternal antibodies (Shoaf-Sweeney *et al*, 2008). Such antigens could then be tested as vaccine candidates or birds could be passively immunised with antibodies directed against these antigens could be delivered through probiotic bacteria secreting fragments of these antibodies or use of hyperimmune serum/egg yolk antibodies. Should cell-mediated immunity be shown to be vital for protection, antigens and/or epitopes that induce this response during *Campylobacter* infection could also be determined, even though this is more technically challenging. Predictions of T-cell epitopes could be made *in*

*silico* and these could be tested for their ability to stimulate immune cell proliferation *in vitro*. Additionally, cultures of antigen-presenting cells could be fed whole cell *Campylobacter* lysates. MHC-epitope complexes from these cells could be extracted and the bound epitopes eluted and identified by mass spectroscopy. These epitopes or the whole antigens from which they derive may then be tested as vaccines against *Campylobacter* in chickens. Furthermore, comparative studies to identify T-cell epitopes could be done using chicken and human cell lines as these may reveal additional antigens involved in clearance of human campylobacteriosis.

Using the information gained from the experiments described above, an appropriate adjuvant could be used in future vaccination experiments that would aim to optimise the protocol of vaccination. Such optimisation could include the administration of the vaccine at different times in the life of the chicks. While previous research suggested that administration of the primary vaccination to day old, 4-day-old or 8-day-old chicks does not affect the magnitude or timing of the protective effect observed (Buckley *et al*, 2010), the data derived from a single experiment using only three birds per group per time-point. Additional experiments using higher number of birds are required to formally demonstrate the effect observed. Other studies describe highly protective vaccines when the primary vaccination is administered to 6-day-old birds (Neal-McKinney *et al*, 2014), but no comparisons are made to birds vaccinated on the day of hatch. Future experiments could also investigate the use of *in ovo* vaccination. In addition, the vaccine could be administered to layer birds and the protective effect of maternal antibody transfer to progeny may be investigated. One other aspect of the vaccination protocol that could be optimised is the dose of the vaccine. Previous studies that undertook dose-titration experiments suggest that doses over 100 µg are required for the induction of the maximal protective effect observed, with doses as high as 250 or 500 µg required in certain cases (reviewed in Section 1.5.3).

While the above experiments could be carried out using recombinant subunit vaccines, it is likely that a vaccine that would be used on a wide scale in the field would require vectoring to be cost-efficient for wide-scale use. Optimisation of the vector attributes should be undertaken and lessons learnt from the studies described above could be used. While delivery in live-attenuated bacteria is likely to stimulate a stronger immune response compared to delivery of a recombinant antigen alone, appropriate adjuvants or cytokines/chemokines could be used to further improve this response. Additionally, information from dose-titration experiments could be used to design an expression system that would deliver an appropriate amount of antigen. Furthermore, using live-attenuated vaccines, the impact of route of vaccination may also be tested. Using a single, optimised

vaccine and experimental design, trials could test in parallel vaccines delivered via the subcutaneous, intramuscular or oral routes. Delivery of a vaccine against *Campylobacter* via the oral route and/or as a spray, may be preferable in the field due to ease of application.

Lastly, a highly optimised vaccine should undertake preliminary testing under field conditions. A key requirement of an effective avian *Campylobacter* vaccine will be its ability to repel the diverse phylogenetic lineages and phase-variants of *C. jejuni* that broilers may encounter. As such, the efficacy of an optimised vaccine should be tested in broilers housed in a manner typical of commercial practice. The experimental design should use challenge with *C. jejuni* via exposure to litter contaminated with *C. jejuni* or seeder birds in contact with vaccinated birds. Litter could derive from flocks known to be highly-positive for *C. jejuni*. Vaccinated and mock-vaccinated birds could be housed in separate enclosures to prevent cross-contamination and to ensure exposure to an equal quantity of contaminated litter. Evidence from experimental and theoretical modelling studies suggests that if the level of caecal *Campylobacter* colonisation is maintained under 3 log<sub>10</sub> CFU/g transmission of the pathogen between individual birds within a flock is impaired (Rotariu *et al*, 2013). This highlights that keeping treatment and control groups separately may impact on the magnitude of the protective effect observed under field-simulating conditions. In addition, such an experiment could further evaluate the effect of reducing intestinal *Campylobacter* contamination on the contamination of the carcass following processing. Scotland's Rural College facilities at Auchincruive include an experimental slaughter line and this could be used for separately processing control flocks and vaccinated flocks, provided thorough disinfection of the plant is used between the two groups. Such an experiment would provide a direct measurement of how well a reduction in intestinal carriage would translate into reduced carcass contamination.

## **6.5 Conclusion**

The current study adds two novel antigens, namely SodB and FliD, to the limited repertoire described to date in published literature related to vaccines against *Campylobacter* in chickens. In addition, it provides preliminary evidence into the nature of the protective immune response induced by vaccination, suggesting that antibody may not be the main mediator of the observed effect. However, further experiments are needed to formally demonstrate the nature of the immune response mediating protection and future studies into development and refinement of anti-*Campylobacter* vaccines for use in chickens should be undertaken in a rational manner, informed by such evidence.

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**Appendix I**

**Conservation (% nucleotide identity) of the *C. jejuni* antigens used in these experiments as candidate vaccines across 20 *C. jejuni* strains.**

<i>C. jejuni</i> strain	Gene																					
	accD	cadF	cj0088	cj0089	cj0090	cj0536	cj11168_0424	cjaA	cjaC	cmeA	cmeC	flaA	flgE2	flgK	fliD	flpA	ggt	omp18	porA	tpx	sodB	fspA
11168	98	99	98	98	99	99	100	98	99	97	100	81	98	100	96	98	Not present	99	83	99	99	99
81176	99	99	99	99	99	99	Not present	98	99	98	99	99	84	98	98	99	99	99	83	99	99	99
81116	99	100	100	100	100	100	Not present	100	100	98	99	100	91	99	99	100	99	100	84	100	100	99
R14	99	99	99	99	99	99	99	97	99	98	99	80	86	99	96	98	Not present	99	95	99	99	99
CG8421	99	99	98	99	99	99	Not present	98	99	98	99	79	86	99	97	98	Not present	99	95	99	99	99
RM3196	99	99	99	99	99	99	99	98	100	98	97	85	84	99	96	98	99	99	83	99	98	99
YH001	98	99	98	98	99	99	99	98	99	97	100	80	98	98	100	98	Not present	99	83	99	99	99
ICDCC07001	98	99	99	99	99	99	99	98	99	98	97	85	98	99	96	98	99	99	85	99	98	99
00-0949	98	99	98	98	99	98	99	98	99	98	99	81	100	100	96	98	Not present	99	83	99	99	99
01-1512	98	99	98	98	99	98	99	98	99	98	99	80	98	100	96	98	Not present	99	83	99	99	99
IA3902	98	99	98	98	99	98	Not present	98	99	97	100	81	86	100	96	98	Not present	99	84	99	99	99
PT14	98	99	98	98	99	99	99	98	99	97	99	80	92	99	98	98	Not present	99	95	99	99	99
00-1597	98	99	98	98	99	98	Not present	98	99	98	99	81	86	98	98	98	Not present	99	94	99	99	99
S3	98	99	98	99	99	99	Not present	98	99	98	99	79	92	99	97	97	Not present	99	94	99	99	98
00-6200	98	99	98	98	99	98	99	98	99	97	100	81	86	99	96	98	Not present	99	83	99	99	99
F38011	98	99	98	99	99	99	99	98	99	98	99	82	92	98	99	98	Not present	99	83	99	99	98

<i>C. jejuni</i> strain	Gene																					
	<i>accD</i>	<i>cadF</i>	<i>cj0088</i>	<i>cj0089</i>	<i>cj0090</i>	<i>cj0536</i>	<i>cj11168_0424</i>	<i>cjaA</i>	<i>cjaC</i>	<i>cmeA</i>	<i>cmeC</i>	<i>flaA</i>	<i>flgE2</i>	<i>flgK</i>	<i>fliD</i>	<i>flpA</i>	<i>ggt</i>	<i>omp18</i>	<i>porA</i>	<i>tpx</i>	<i>sodB</i>	<i>fspA</i>
<b>00-2544</b>	98	99	98	98	99	99	99	98	99	97	100	81	87	100	98	98	Not present	99	83	99	99	99
<b>00-2426</b>	98	99	98	98	99	98	99	98	99	97	100	81	87	100	98	98	Not present	99	83	99	99	99
<b>00-2538</b>	98	99	98	98	99	99	99	98	99	97	100	81	87	100	98	98	Not present	99	83	99	99	99
<b>RM3197</b>	99	99	99	99	99	99	99	98	99	98	97	85	98	99	96	98	99	99	83	99	98	99
<b>M1</b>	100	100	100	100	100	100	Not present	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

