The role of the MarR transcriptional regulators RrpA and RrpB in the response of *Campylobacter jejuni* to oxidative and aerobic stress

Daiani Teixeira da Silva

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Department of Pathogens Molecular Biology
Faculty of Infectious and Tropical Diseases
London School of Hygiene & Tropical Medicine

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Declaration

I, Daiani Teixeira da Silva, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. All experiments were performed at London School of Hygiene and Tropical Medicine.

Abstract

Campylobacter jejuni is a microaerobic bacterium that possesses complex mechanisms to counter oxidative stress to be able to survive in the presence of reactive oxygen species (ROS). Re-annotation of the *C. jejuni* NCTC 11168 genome sequence identified two putative MarR-type transcriptional regulators Cj1546 and Cj1556, originally annotated as hypothetical proteins, now designated as RrpA and RrpB (regulator of response to peroxide). Both *rrpA* and *rrpB* mutants exhibit increased sensitivity to hydrogen peroxide stress compared to the wild-type strain and both mutants exhibit reduced levels of catalase (KatA) activity. However, neither mutant exhibited any significant difference in sensitivity to either cumene hydroperoxide or menadione oxidative stresses, indicating that RrpA and RrpB do not regulate expression of either alkylhydroperoxide (AhpC) or superoxide dismutase (SodB). *rrpA* and *rrpB* mutants exhibit increased biofilm formation, probably due to accumulation of ROS within the cells. Preliminary RNA-seq analysis indicated reduced *katA* expression in the *rrpA* mutant, but no differences in *katA* expression was observed in the *rrpB* mutant or *rrpAB* double mutant compared to the wild-type strain. *C. jejuni* strains normally contain *rrpA*, whilst only a subset contained *rrpB*. *C. jejuni* strains containing both genes were more associated with livestock-associated MLST clonal complexes. The presence of *rrpB* is linked to a hypervariable region containing the IF subtype of the type I Restriction-Modification (*hsd*) system, whereas strains containing only *rrpA* contain the IAB subtype *hsd* system. Analysis of 43 Brazilian strains identified that most chicken meat isolates contained both genes, whilst most human isolates contained only *rrpA*. The predominant presence of *rrpB* in livestock-associated *C. jejuni* MLST-types suggests an important role for fine-tuning oxidative stress responses through the concerted actions of multiple regulatory proteins in this microaerophilic pathogen. It also highlights the potential of genetic variation in the natural population in the adaptation to different environmental niches.

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1 Introduction

1.1 *Campylobacter jejuni*

Campylobacter jejuni is a Gram-negative bacteria, non-sporulating, spiral shaped and microaerophilic (Young *et al.*, 2007). Microaerophilic bacteria require low oxygen concentrations for growth (Krieg and Hoffman, 1986). *C. jejuni* requires between 2% to 5% oxygen, 10% carbon dioxide and approximately 85% nitrogen as optimum concentrations for growth (Atack and Kelly, 2009). Due to this singular growth condition, *C. jejuni* is susceptible to environment levels of oxygen concentration (Atack and Kelly, 2009). *C. jejuni* cannot grow below 30ºC, and the optimum growth temperature is 42ºC (Park, 2002). *C. jejuni* cells are slim and S-shaped, measuring on average 1.5 to 6.0 μm in length by 0.2 to 0.5 μm in width (Ketley, 1997). *C. jejuni* possesses flagella at one or both ends of the cell (Pead, 1979). Flagella are important for motility, which is essential for cell colonisation (Young *et al.*, 2007). *C. jejuni* has a small genome compared to other enteric bacteria. The complete sequence has 1,641,481 base pair (bp) in length (Parkhill *et al.*, 2000, GSGSC, 2016). The genome contains many hypervariable sequences in genes encoding surface structures, such as lipooligosaccharide (LOS), capsular polysaccharide (CPS) and flagella modification (Parkhill *et al.*, 2000).

C. jejuni morphology can change depending upon the growth stage or depending on the environment conditions (Thomas *et al.*, 1999). *C. jejuni* cell morphology from early log phase displays typical spiral and curved shapes (Kelly, 2001). Nutrient limiting conditions (such as in late stationary phase) can change the cell morphology and cell integrity promoting the transition to coccoid forms (Thomas *et al.*, 1999, Kelly, 2001). When *C. jejuni* changes morphology, the bacteria lose culturability and this state is called viable but non-culturable (VBNC) (Kelly, 2001). Oxidative stress has been shown to decrease *C. jejuni* culturability and increase the number of coccoid cells (Harvey and Leach, 1998). Fresh cultures have uniform spiral cells, whilst older cultures tend to have more coccoid forms and reduced capacity to adhere to epithelial cells (Konkel *et al.*, 1992).

1.2 Metabolism

Unlike other enteropathogenic bacteria, *C. jejuni* is not capable of metabolising carbohydrates because *C. jejuni* lacks several enzymes from the glycolytic pathway (Parkhill *et al.*, 2000, Stahl *et al.*, 2012), such as 6-phosphofructokinase and glucokinase orthologues (Parkhill *et al.*, 2000, Velayudhan and Kelly, 2002). However, some *C. jejuni* strains have a genomic island that allows L-fucose utilization as nutrient source (Stahl *et al.*, 2011). *C. jejuni* utilises amino acids as primary source of nutrients for growth (Stahl *et al.*, 2012). Amino acids are broken down through a deamination process. Compounds generated from this process, such as ammonia, can be used as a carbon source for bacterial metabolism (Velayudhan and Kelly, 2002, Velayudhan *et al.*, 2004). Ammonia can also be utilised by *C. jejuni* as nitrogen source for amino acid metabolism (Velayudhan *et al.*, 2004).

The amino acids commonly utilised by *C. jejuni* are aspartic acid, glutamic acid, proline, asparagine and serine (Hofreuter *et al.*, 2008, Wright *et al.*, 2009). Aspartic acid, serine, asparagine and glutamic acid are the preferred amino acids. Only after the depletion of these preferred amino acids, will proline start to be consumed (Leach *et al.*, 1997, Wright *et al.*, 2009). However, there is some metabolic diversity amongst different *C. jejuni* species (Hofreuter *et al.*, 2008). Serine, aspartic acid, asparagine, glutamic acid and proline are all metabolised by *C. jejuni* and also make up some of the most common amino acids found in chick excreta (Parsons *et al.*, 1982). This fact might explain why chickens are the main reservoir of *C. jejuni* (Parsons *et al.*, 1982).

C. jejuni has the capacity to survive under different environments and hostile conditions, which could lead to a selective pressure and differences in amino acid metabolism identified in different *Campylobacter* species (Hofreuter *et al.*, 2008). Furthermore, some strains might acquire genes resulting in an enhanced ability to utilise different amino acids. This capability can enhance virulence and cell colonisation (Hofreuter *et al.*, 2008). Guccione *et al.* (2008) investigated amino acid depletion by *C. jejuni* from Mueller Hinton broth after 48 h growth. They demonstrated that glutamic acid, aspartic acid, serine and proline were depleted by more than 50% from the initial concentrations. They also demonstrated that when amino acids, such as glutamic acid, aspartic acid, serine, proline or glutamic acid, were added to MEMα media, the *C. jejuni* growth rate was increased (Guccione *et al.*,

2008).

Metabolism of amino acids generates different compounds such as acetate, lactate, pyruvate and succinate (Mendz *et al.*, 1997). Pyruvate is the first metabolite generated and is efficiently used in energy metabolism (Mendz *et al.*, 1997). *C. jejuni* metabolises serine to generate pyruvate, which is essential for synthesis of different amino acids (Berg *et al.*, 2002). Pyruvate can also be converted to acetyl-CoA to enter the Krebs cycle to generate energy for *C. jejuni* cells (Mendz *et al.*, 1997).

1.3 Epidemiology

C. jejuni is the leading cause of bacterial gastroenteritis in the world (Friedman *et al.*, 2004). Though this bacterium can be linked to outbreaks, most of the cases reported are sporadic (Pires *et al.*, 2010). *C. jejuni* infections are a burden to developed economies due to hospitalisations and treatment (Pires *et al.*, 2010). *C. jejuni* is the leading cause of food poisoning in the UK, believed to be responsible for over 280,000 cases, hospitalisations costs around £900 million, and more than 100 deaths per year (FSA, 2016). 76% of retail chickens were contaminated in UK retail market in 2015 (FSA, 2015).

The gastrointestinal tract of avians is the main habitat of *C. jejuni* (Stanley et al., 2014). *C. jejuni* can also be found in different animal species, such as cattle, swine, dogs, cats and a variety of wild animals (Stanley *et al.*, 2014). Most of the strains that infect humans come from poultry (Golz *et al.*, 2014, FSA, 2016). However, pigs, ruminants and wild birds are also important sources of infection (Golz *et al.*, 2014). Healthy animals can be infected without showing any symptoms (Young *et al.*, 2007). Flies, wild birds, rodents, environmental contamination and staff are some of the important sources of infection for poultry flocks (Golz *et al.*, 2014, Stanley *et al.*, 2014). *C. jejuni* is considered to be commensal bacteria present in the chicken gut (Corry and Atabay, 2001). However, Humphrey *et al.* (2014) demonstrated that infected birds developed inflammatory response against *C. jejuni*, and in certain poultry breeds the *C. jejuni* infection lead to diarrhoea. Other breeds have a more tightly regulated gut immune response to prevent excessive inflammation, leading to little or no symptoms in these birds (Humphrey *et al.*, 2014). Variation in symptoms is suggested to be due to

differences in innate immunity amongst the different poultry breeds (Humphrey *et al.*, 2014). *C. jejuni* optimum growth temperature ranges from 37°C to 42°C. Avians have body temperature of 42°C, which is ideal for *C. jejuni* growth and may be another reason why poultry are the main reservoir of this microorganism (Corry and Atabay, 2001).

The consumption of undercooked poultry meat is considered to be the main source of Campylobacteriosis for humans (van Vliet and Ketley, 2001, Coker *et al.*, 2002). Inappropriate handling of contaminated poultry meat can lead to cross contamination to other food sources (Luber *et al.*, 2006). Unpasteurised milk and untreated water are also important sources of infection for humans (Levesque *et al.*, 2008). *C. jejuni* and *Campylobacter coli* are the main species present in poultry flocks and in poultry meat (Powell *et al.*, 2012). *C. jejuni* is responsible for around 85% and *C. coli* for around 15% of the infectious cases in humans (Moore *et al.*, 2005). However, infection by other *Campylobacter* species can also occur, such as *Campylobacter lari*, *Campylobacter upsaliensis*, *Campylobacter fetus*, *Campylobacter concisus* and *Campylobacter ureolyticus* (Moore *et al.*, 2005, Mukhopadhya *et al.*, 2011). Studies indicate a low infectious dose with 400 to 500 bacteria enough to cause a disease in humans (Robinson, 1981, FDA, 2013).

Chickens are usually contaminated during the first days of life. *C. jejuni* spreads rapidly through flocks, colonising close to 100% of birds (Berndtson *et al.*, 1996, Golz *et al.*, 2014). Birds are colonised at very high levels, between 10^6 to 10^9 CFU/g in the caecal contents (Corry and Atabay, 2001). Infected flocks cause cross contamination to carcasses due to faecal contamination during the slaughter process (Berndtson *et al.*, 1996, Golz *et al.*, 2014). Flocks free from *C. jejuni* can also become contaminated during the slaughter process through contact with contaminated equipment (Perko-Makela *et al.*, 2011).

1.4 Campylobacteriosis in developed and developing countries

C. jejuni is the predominant species in poultry meat in many countries around the world, including both developed and developing countries (Suzuki and Yamamoto, 2009). In addition, chicken meat exhibits higher contamination than other poultry

meat, such as duck and turkey, becoming the most important source of contamination for humans (Little *et al.*, 2008).

In developed countries, the disease is most commonly seen in children under 4 years of age and young adults (Coker *et al.*, 2002, Padungton and Kaneene, 2003a), while in developing countries, the disease is more relevant in children under 2 years of age and is characterised as a milder form of gastroenteritis (Coker *et al.*, 2002, Padungton and Kaneene, 2003a). In such countries, *C. jejuni* is also commonly isolated from asymptomatic older children (Coker *et al.*, 2002, Quetz *et al.*, 2010). In Brazil, for example, *C. jejuni* is a common gastrointestinal infection amongst young children, and cases of infected children without symptoms have also been reported (Fernandez *et al.*, 1985, Quetz *et al.*, 2010, Quetz *et al.*, 2012). In contrast, in developed countries, it is not common to isolate *C. jejuni* from asymptomatic humans (Coker *et al.*, 2002).

C. jejuni infection usually presents in developed countries as inflammatory, bloody diarrhoea, whilst in developing countries infection presents as watery, non-inflammatory diarrhoea (Coker *et al.*, 2002). It is believed that this occurs due to the early and frequent contact of children in developing countries with *C. jejuni*, which allows the development of immunity against *Campylobacter* (Blaser, 1997, Coker *et al.*, 2002). Most likely, the first infection of a child in a developing country will result in inflammatory, bloody diarrhoea, although no published studies have investigated this (Taylor *et al.*, 1993). However, studies have demonstrated that children in developing countries have higher levels of antibodies against *C. jejuni* than children in developed countries (Taylor *et al.*, 1993). Therefore, *C. jejuni* infection becomes less common in older children and is not common in adults (Taylor *et al.*, 1993, Coker *et al.*, 2002).

Developed countries commonly have surveillance programs to monitor the incidence of cases of *C. jejuni* infection (Padungton and Kaneene, 2003a). This is not the case in developing countries that normally do not have surveillance programs for this pathogen and neither are clinical laboratories able to detect this microorganism (Blaser, 1997, Padungton and Kaneene, 2003a). For this reason, the number of cases in developing countries is not accurately understood (Padungton and Kaneene, 2003a). In fact, the number of studies and published data is much higher in developed countries than in developing ones (Coker *et al.*, 2002). Furthermore, the lack of information

about *C. jejuni* infections contributes to the non-existence of national programs to control poultry contamination (Padungton and Kaneene, 2003a). Some developing countries have low biosecurity measures to control pathogens and infectious diseases (Conan *et al.*, 2012). Although enhanced biosecurity measures are used in farms in developed countries, contamination of poultry flocks still occurs (Ridley *et al.*, 2011). Biosecurity measures help to reduce flock contamination. However, such measures still fail to prevent *C. jejuni* colonisation (Ridley *et al.*, 2011).

In Brazil, there are no surveillance programs to report *Campylobacter* infections. Therefore, the real dimension of Campylobacteriosis in Brazil is not known. Furthermore, clinical laboratories cannot isolate *Campylobacter* from faecal samples from patients suffering from diarrhoea because the methodology to isolate *Campylobacter* is different from other traditional enteropathogens (CCamp, 2016). *Campylobacter* has a fastidious growth and requires special incubation conditions and can easily lose culturability (Park, 2002).

The prevalence of *Campylobacter* in chicken in Brazil varies. Some studies found the prevalence of *Campylobacter* in poultry in slaugtherhouses as high as 70% (Franchin *et al.*, 2005, Franchin *et al.*, 2007, Kuana *et al.*, 2008, Chaves *et al.*, 2010). Different studies also detected presence of *Campylobacter* in poultry products sold in Brazilian markets (Carvalho and Cortez, 2003, Freitas and Noronha, 2007, Silva *et al.*, 2014).

Fernandez *et al.* (1985) and Mendes *et al.* (1987) isolated *Campylobacter* from children with acute diarrhoea, but also from healthy children. A more recent study showed that 10% of children with diarrhoea had *Campylobacter* (Quetz et al., 2010). However, *C. jejuni* and *C. coli* were also isolated from asymptomatic children (Quetz *et al.*, 2010, Quetz *et al.*, 2012), which is a common occurrence in developing countries.

1.5 Disease symptoms

C. jejuni infection causes gastroenteritis in humans (Friedman *et al.*, 2004). The symptoms can vary from watery, non-inflammatory diarrhoea to inflammatory, bloody diarrhoea (Coker *et al.*, 2002). The disease is normally acute and self-limiting, with a 2 to 5-day incubation period and symptoms that persist for 7 to 10 days (Coker

et al., 2002, FDA, 2013). Other symptoms such as fever, vomiting and headaches can also occur (Allos, 2001). The infection can be severe if the infected individual has a compromised immune system (Coker *et al.*, 2002, FDA, 2013). Irritable bowel syndrome can also be an outcome following *Campylobacter* infection (Spiller, 2007).

Guillain-Barré Syndrome (GBS) is a complication arising from *C. jejuni* infection, with about 1.3 cases per 1,000 *C. jejuni* infections (Hughes and Rees, 1997). GBS is an autoimmune disease that affects the nervous system and leads to muscular paralysis (Hughes and Rees, 1997). GBS occurs due to the molecular mimicry by carbohydrates present in the bacterial LOS of human gangliosides (Yuki *et al.*, 2004). This similarity can be due to the presence of sialic acid moieties in the LOS structure (Godschalk *et al.*, 2007). Hence, once infected with *C. jejuni*, the human host generates antibodies that target initially the bacterial LOS structures and, once infection is cleared, the antibodies then attack the human peripheral nerve gangliosides, causing acute inflammatory demyelination and axonal degeneration of the peripheral nerves (Yuki *et al.*, 2004, Nyati and Nyati, 2013). IgG antibodies are produced against the GM1 ganglioside that are present in human peripheral nerves (Yuki *et al.*, 2004). The damage to the peripheral nervous system leads to acute flaccid paralysis (Hughes and Rees, 1997). The individual presents with progressing weakness of the limbs and respiratory muscles. Most of the isolates obtained from patients with GBS have been reported as *C. jejuni* (Nachamkin *et al.*, 1998, Nyati and Nyati, 2013). The risk of developing GBS is increased after infection with certain *C. jejuni* serotypes, such as the serotype O:19 based on Penner serotyping scheme (Yuki *et al.*, 1997). Other serotypes, such as O:1, O:2, O:4, O:4 complex, O:5, O:10, O:16, O:23, O:37, O:44, and O:64 have also been linked to GBS (Nachamkin *et al.*, 1998). However, host susceptibility factors may also affect the antiganglioside antibody production (Nachamkin *et al.*, 1998).

Miller Fisher Syndrome (MFS) is a variant of GBS causing ataxia, areflexia, and ophthalmoplegia (Willison and O'Hanlon, 1999). MFS is also an immune mediated neuropathy arising from the *C. jejuni* LOS structure that mimics human GQ1b and GT1a gangliosides (Willison and O'Hanlon, 1999, Dingle *et al.*, 2001). 40% of people presenting with GBS or MFS can be associated with a prior *C. jejuni* infection (Dingle *et al.*, 2001).

1.6 Bacterial surface structures

The genome sequence of *C. jejuni* NTCT 11168 demonstrated the presence of hypervariable sequences that are responsible for expressing proteins involved in the biosynthesis or modification of carbohydrates structures, such as capsule, LOS and flagella (Parkhill *et al.*, 2000). These hypervariable sequences arise from slip-strand mispairing during replication, which, in turn, lead to a phase variation altering surface proteins and antigenicity (Parkhill *et al.*, 2000). Phase variation is a change in the translational reading frame leading to variation in protein expression or changes in the level of expression of one or more proteins in a bacterial population (Henderson *et al.*, 1999, van der Woude and Baumler, 2004). This change in gene expression is reversible (van der Woude and Baumler, 2004). Phase variation can be modulated by environmental conditions and can lead to antigenic variation (Deitsch *et al.*, 2009). Phase variation is an important mechanism to evade host immune system. Through the alteration of surface antigens, the bacteria can avoid cross-immunity and enhance bacterial survival (Henderson *et al.*, 1999, van der Woude and Baumler, 2004, Deitsch *et al.*, 2009). Most phase variation systems require DNA replication (van der Woude and Baumler, 2004). Therefore, the reason for such extensive *C. jejuni* phase variation could be because of the lack of DNA repair enzymes that are commonly present in other bacteria (Parkhill *et al.*, 2000).

1.6.1 Capsule

C. jejuni has a capsular polysaccharide (CPS) with a highly variable polysaccharide composition (Karlyshev *et al.*, 2005a). CPS structures are the main components for serotyping determined by the Penner serotyping scheme of *C. jejuni*. Penner serotyping scheme identified 42 serotypes based on the diversity of antigens of the polysaccharide capsular structures of *C. jejuni* (Penner *et al.*, 1983).

Karlyshev and Wren (2001) were the first to demonstrate the presence of capsule in *C. jejuni*. They used the Alcian blue dye to stain the capsular polysaccharide, which was visualised by electron microscopy. This dye was shown to be better than traditional stains, such as silver stain (Karlyshev and Wren, 2001).

C. jejuni kpsM gene is involved in CPS biosynthesis and capsule formation (Karlyshev

and Wren, 2001). *kpsM* gene is also a member of ABC transporter family involved in polysaccharide export (Karlyshev *et al.*, 2000). A *C. jejuni* 11168H *kpsM* mutant failed to produce polysaccharide capsule and resulted in serotype changes based on Penner serotyping method (Karlyshev *et al.*, 2000, Karlyshev and Wren, 2001). A *C. jejuni* 81-176 *kpsM* mutant also failed to express capsule, and exhibited reduced adhesion to and invasion of epithelial cells (Bacon *et al.*, 2001). *kpsE* gene is also involved in capsule transport mechanism through the bacterial cell surface (Bachtiar *et al.*, 2007). A *C. jejuni* 81-176 *kpsE* mutant failed to express capsule and had reduced capacity to adhere and invade human epithelial cells (Bachtiar *et al.*, 2007).

The capsule possesses highly variable polysaccharides due to slip-strand mispairing (Bacon *et al.*, 2001). The host innate immune response is an important defense mechanism against *C. jejuni* infection, so phase variation of the CPS can modulate the host immune response (Maue *et al.*, 2013). The genetic variation of CPS can change the surface structure, therefore, favoring evasion of the host immune response, which can also be important for adaptation to hostile environments (Karlyshev *et al.*, 2005b). Keo *et al.* (2011) demonstrated that *C. jejuni* strains were resistant to human serum and that the capsule was essential for the serum resistance. CPS is important for *C. jejuni* survival in the environment. However, the role of CPS in bacterium–host relationships is not clarified yet (Karlyshev *et al.*, 2005b).

1.6.2 Lipooligosaccharide

Lipooligosaccharide (LOS) plays a role in adhesion to and invasion of intestinal epithelial cells (Young *et al.*, 2007). Strains with different LOS structures have shown variation in the ability to invade and colonise epithelial cells (Muller *et al.*, 2007). LOS is highly variable in *C. jejuni* with 19 distinct classes of LOS identified in the biosynthesis loci (Parker *et al.*, 2006). LOS can undergo phase variation altering the expression of LOS surface structures, which plays an important role in avoiding the host immune response (Guerry *et al.*, 2002, Prendergast *et al.*, 2004). Besides phase variation, insertion and deletion events can also generate variation in LOS structure (Parker *et al.*, 2006). Furthermore, some strains can synthesise and incorporate sialic acid within the LOS structure, which affects immunogenicity and increases the bacteria resistance to human sera (Guerry *et al.*, 2000). Sialylated LOS is also

important for invasion to host cells, as strains with sialylated LOS have been shown to invade epithelial cells at higher levels than strains with unsialylated LOS (Louwen *et al.*, 2008). Sialylated LOS also reduces immunogenicity compared to unsialylated LOS, which has been shown to have a much stronger signal in a Western blot (Karlyshev *et al.*, 2000).

LOS is an important structure that contributes to antimicrobial resistance as this structure forms a diffusion barrier for macrolides across the outer membrane (Jeon *et al.*, 2008, Keo *et al.*, 2011). LOS can also protect against cationic antimicrobial peptides (Jeon *et al.*, 2008, Keo *et al.*, 2011). Conversely, CPS does not appear to have an important role in *C. jejuni* antimicrobial resistance (Jeon *et al.*, 2008).

Phase variation can hinder vaccine development because of the variability in LOS structures (Prendergast *et al.*, 2004). Vaccines with LOS structures could lead to the induction of antiganglioside antibodies and to the development of autoimmune disease (Prendergast *et al.*, 2004).

1.6.3 Flagella

C. jejuni is a highly motile bacterium which is mediated by polar flagella (Hendrixson and DiRita, 2004). The flagellum is located at one or both bacteria poles and has three main parts: the basal body, the hook, and the filament (Young *et al.*, 2007). The filament is composed of two flagellins: FlaA (the major flagellin) and FlaB (the minor flagellin) (Nuijten *et al.*, 1990). Both flagellins are around 59kDa and are 95% identical (Nuijten *et al.*, 1990). The flagellins have different promoters, σ^{28} for *flaA* and σ^{54} for *flaB* (Hendrixson and DiRita, 2003). Both flagellins are required for a fully functional flagella (Joslin and Hendrixson, 2009). A *C. jejuni* 81116 *flaB* mutant exhibits reduced motility whilst a *C. jejuni* 81116 *flaA* mutant produces a truncated flagella and exhibits no motility (Wassenaar *et al.*, 1991, Guerry, 2007). *rpoN* and *fliA* encode sigma factors which are important for flagellar expression in *Helicobacter* species (Sterzenbach *et al.*, 2008). *C. jejuni* also demonstrated to have both regulators, *fliA* encodes σ^{28} and *rpoN* encodes σ^{54} (Jagannathan *et al.*, 2001). A *C. jejuni* 11168 *fliA* mutant produced a truncated flagellum, whereas a *C. jejuni* 11168 *rpoN* mutant showed complete absence of flagella (Jagannathan *et al.*, 2001).

The expression of many flagella biosynthetic genes are controlled by σ^{54}. However, the activation of the FlgSR two-component system is required for full expression of σ^{54}-dependent flagellar genes (Hendrixson and DiRita, 2003, Joslin and Hendrixson, 2009). FlgS is a sensor protein kinase and FlgR a response regulator (Wosten *et al.*, 2004). The sensor protein FlgS detects a signal and is activated by phosphorylation (Wosten *et al.*, 2004). When FlgS is phosphorylated, FlgS transfers the phosphate to FlgR (Hendrixson and DiRita, 2003). The activation of FlgS is important for the activation of flagellar export apparatus (Joslin and Hendrixson, 2009). Phosphorylated FlgR activates σ^{54}-dependent transcription of flagellar biosynthesis (Hendrixson and DiRita, 2003, Wosten *et al.*, 2004). Activation of σ^{54} is essential for flagellar genes expression and, therefore, for the formation of the flagella structure (Joslin and Hendrixson, 2009). σ^{54} initiates the transcription of flagellar genes encoding proteins that form the basal body, rod and hook and FlaB, whereas σ^{28} controls expression of *flaA* (Hendrixson and DiRita, 2003, Guerry, 2007).

FlgM is an anti-σ^{28} factor that represses σ^{28} activity in *Helicobacter pylori* (Colland *et al.*, 2001). However, in *C. jejuni* 81-176, FlgM has only limited σ^{28}-repressive activity. The overexpression of *flgM* did not result in any reduction in *flaA* transcription (Hendrixson and DiRita, 2003). Furthermore, mutation of *fliA*, the gene which encodes σ^{28}, demonstrated that in the *C. jejuni* 81-176 strain there is a *flaA* σ^{28}-independent promoter as the mutant expressed *flaA* at the same levels as the wild-type strain (Hendrixson and DiRita, 2003).

Many *C. jejuni* genes undergo phase variation to regulate gene expression. Phase variation also regulates the expression flagellar biosynthesis (Hendrixson, 2006). Phase variation modulates gene expression and protein expression via reversible change in the length of short DNA sequence repeats (van der Woude and Baumler, 2004). Phase variation mechanism regulates the expression of FlgSR (Hendrixson, 2006). This regulatory system alters *C. jejuni* motility and colonisation capacity by adding or removing a nucleotide in the *flgR* gene (Hendrixson, 2006). This phase variable expression of FlgR has been suggested to be the reason for the commensal behaviour of *C. jejuni* strains in chickens as opposed to the invasion of host cells in humans (Hendrixson, 2006).

Motility is important for invasion and colonisation of intestinal epithelial cells, and

the flagella is responsible for chemotaxis towards the host cell (Guerry, 2007). Motility is important for chick ceacal colonisation (Nachamkin *et al.*, 1993). However, mutants displaying less motile phenotypes are still able to colonise the chick ceacum, but not at the same level as a fully motile wild-type strain (Nachamkin *et al.*, 1993, Hendrixson and DiRita, 2004). The *in vitro* motility of *C. jejuni* increases in highly viscous solutions, probably because viscous solutions simulate the thick mucus lining of the intestinal epithelium (Guerry, 2007). Adhesion and invasion can also be increased *in vitro* in presence of viscous conditions (Guerry, 2007).

Chemotaxis is the ability of a bacteria to migrate towards or away from chemical substances, allowing the bacteria to migrate towards more favourable conditions (Adler, 1966). *C. jejuni* has a chemotactic attraction towards amino acids, organic acids, components of mucin and bile (Hugdahl *et al.*, 1988, Vegge *et al.*, 2009). The highest chemotactic activity was towards L-serine and pyruvate (Vegge *et al.*, 2009). L-serine and pyruvate are the preferred growth substrates, together with glutamine, glutamic acid, asparagine and proline (Hofreuter *et al.*, 2008). Konkel *et al.* (2004) showed that mutation of flagellar genes responsible for the flagella assembly resulted in mutants that lost both motility and the ability to secrete proteins. *C. jejuni* uses the flagellum as type III secretion system for secretion of proteins (Goon *et al.*, 2006). Flagellar mutants failed to produce FlaA and to secrete any of the *Campylobacter* invasion antigen (Cia) proteins (Konkel *et al.*, 2004).

A fully functioning flagella apparatus is required for secretion of proteins and for fully capacity to invade intestinal epithelium cells (Konkel *et al.*, 2004). *C. jejuni* invasion antigens (Cia) identified to date are: CiaB, CiaC, CiaD and CiaI (Konkel *et al.*, 2004, Christensen *et al.*, 2009, Buelow *et al.*, 2011, Samuelson *et al.*, 2013). CiaB is required by *C. jejuni* F38011 strain for fully invasion of epithelial cells (Konkel *et al.*, 2004). Mutation of *ciaB* in *C. jejuni* 81116 strain also had reduced ability to invade intestinal epithelium cells (Rivera-Amill *et al.*, 2001). Novik *et al.* (2010) mutated *ciaB* in *C. jejuni* 81-176 strain. However, the mutant did not display defect in the ability to invade epithelial cells. These findings suggest that different wild-type strains may behave differently, or that there were significant differences amongst assays performed in the different studies (Novik *et al.*, 2010). It is also possible that previous phenotypes observed were a result of phase variation (Novik *et al.*, 2010). CiaC is required for maximal invasion of host epithelial cells by *C. jejuni* F38011 (Christensen *et al.*,

2009). CiaD is required for cell invasion and for inducing secretion of IL-8 from epithelium cells (Samuelson *et al.*, 2013). CiaI is required for intracellular survival in epithelial cells by probably preventing the fusion of *Campylobacter*-containing vacuoles with the lysosome (Buelow *et al.*, 2011).

FlaC has amino acid similarities to FlaA and FlaB and is highly conserved amongst different *Campylobacter* species (Song *et al.*, 2004, Faber *et al.*, 2016). However, FlaC is not required for flagella structure nor for motility as *flaC* mutants showed no defect to the flagellum structure nor alteration of the cell shape (Song *et al.*, 2004). FlaC is secreted to the extracellular milieu but can also be found in the cytoplasm and periplasm (Song *et al.*, 2004). Secreted FlaC binds to the surface of epithelial cells of both human and chicken cells (Song *et al.*, 2004, Faber *et al.*, 2016). Further investigation has shown that a *flaC* mutant binds to HEp-2 at the same level as the wild-type strain, but exhibits reduced invasion (Song *et al.*, 2004). Recently, FlaC has been shown to bind to human toll-like receptor 5 (TLR) and to have the capacity to modulate the host immune system (Faber *et al.*, 2016). Recombinant FlaC did not induce pro-inflammatory cytokines in both human and chicken cells (Faber *et al.*, 2016). However, both *C. jejuni* 11168 *flaC* mutant and a *C. jejuni* 81-176 *flaC* mutant demonstrated to induce pro-inflammatory cytokines at higher level than the corresponding wild-type strains (Faber *et al.*, 2016). This indicates the importance of FlaC in the modulation of the innate immune response and suggests that FlaC plays an important role in chronic colonisation (Faber *et al.*, 2016).

1.7 Toxin production

Cytolethal Distending Toxin (CDT) is a toxin produced by *C. jejuni.* CDT arrests both HeLa and Caco-2 cells in the G2 phase of the cell cycle during mitosis (Whitehouse *et al.*, 1998). The cells become distended and with chromatin anomalies that lead to cell death (Whitehouse *et al.*, 1998). Cyclin-dependent kinases (CDKs) are essential for regulation of the cell cycle in yeast and bacteria (Doree and Hunt, 2002). The protein kinase activity of cdc2 is important for cell mitosis, and in order to become active, cdc2 needs to be dephosphorylated (Comayras *et al.*, 1997). CDT interferes with cdc2 phosphorylation. CDT blocks dephosphorylation, maintaining the hyperphosphorylated form of cdc2, which is the non-active form, thus not allowing

mitosis to continue (Comayras *et al.*, 1997, Whitehouse *et al.*, 1998).

CDT is produced by different *Campylobacter* species, such as *C. coli* and *C. fetus* (Asakura *et al.*, 2007b). CDT has also been identified in other bacteria, such as *Escherichia coli* (Comayras *et al.*, 1997) and *Actinobacillus actinomycetemcomitans* (Shenker *et al.*, 2000). CDT is encoded by three genes: *cdtA*, *cdtB* and *cdtC*, within a single operon and thus transcribed as a single mRNA (Jeon, 2005). All three genes need to be expressed for a functional toxin (Lara-Tejero and Galan, 2001). All three CDT proteins are required for the induction of IL-8 from intestinal epithelial cells (Hickey *et al.*, 2000).

CdtB has shown amino acid sequence homology to mammalian DNases (Elwell and Dreyfus, 2000). CdtB has a motif found in all DNase I (Elwell and Dreyfus, 2000). The CdtB subunit has been shown to penetrate the nucleus of the host cell and cleave DNA (Lara-Tejero and Galan, 2001). CdtB DNase activity could be responsible for the cell cycle arrest (Elwell and Dreyfus, 2000). The DNA damaged cells then recruit repair responses to the double stranded DNA breaks (Hassane *et al.*, 2003). A *C. jejuni* 11168 *cdtB* mutant showed no cytotoxicity against HeLa cells (Purdy *et al.*, 2000), whilst a *C. jejuni* 81-176 *cdtB* mutant showed a residual low level of cytotoxicity, which indicates that some species can produce more than one toxin (Purdy *et al.*, 2000). Different putative *Campylobacter* toxins have been described, such as hemolytic cytotoxins (Wassenaar, 1997). Based in microarray analysis, *Cj0183*, *Cj0588* (*tlyA*), *Cj0959* and *Cj1351* (*pldA*) encode putative haemolysins in *C. jejuni* (On *et al.*, 2006).

The roles of the CdtA and CdtC subunits are not yet fully understood. However, it is suggested that both CdtA and CdtC could adhere to cell surface receptors to translocate CdtB into the host cell since the incubation of intestinal epithelial cells with either CdtA, CdtB or CdtC alone did not result in cytotoxicity (Lara-Tejero and Galan, 2001). Cytotoxicity was only observed if all three purified proteins were incubated together with the epithelial cells (Lara-Tejero and Galan, 2001).

1.8 Adhesion and Invasion

Adherence to intestinal epithelial cells is essential for cell colonisation and disease

development (Konkel *et al.*, 2010). The ability of *C. jejuni* to cause disease relies on multiple different factors, such as motility, adhesion to the host cell, invasion, cell death, protein secretion, immune defence evasion, drug resistance, and iron acquisition (Konkel *et al.*, 2001).

Human intestinal epithelial cells are considered the best model to study *Campylobacter* pathogenicity because of the lack of a suitable animal model to represent the disease in humans (Friis *et al.*, 2005). *Campylobacter* strains that were shown to be more invasive in an *in vitro* epithelial cell model also had stronger ability to colonise the gastrointestinal tract of chicks (Hanel *et al.*, 2004). *Campylobacter* exhibits maximum adherence to epithelial cells when grown at 37ºC, and exhibits decreased adherence when grown at 42ºC, and a further decrease when grown at 30ºC (Konkel *et al.*, 1992). Different bacterial media compositions were shown not to affect the adherence capability of the bacteria (Konkel *et al.*, 1992).

LOS, flagella and other outer membrane components may also play a role in adherence, but it is not well understood how important these factors are (Konkel *et al.*, 1992). Flagella play an important role in *C. jejuni* adhesion to and invasion of human host cells (Wassenaar *et al.*, 1991). A 81116 *flaB* mutant remains motile and maintained the ability to adhere to and invade epithelial cells (Wassenaar *et al.*, 1991). However, when the *flaA* gene was mutated, this mutant lost motility and had a reduced ability to adhere to and invade epithelial cells (Wassenaar *et al.*, 1991). A fully functional flagella is important for host cell invasion as motility allows bacterial migration toward host cells to enable interaction (Wassenaar *et al.*, 1991). The authors also concluded that flagella do not have adhesive properties (Wassenaar *et al.*, 1991).

C. jejuni expresses important adhesins, such as CadF, FlpA, JlpA, PEB1 and Cj1379c (Flanagan *et al.*, 2009). *cadF*, *jlpA*, *peb1A*, *porA*, *flpA* and *Cj1349c* are highly conserved amongst *C. jejuni* strains (Pei and Blaser, 1993, Konkel *et al.*, 1997, Flanagan *et al.*, 2009).

Intestinal epithelial cells possess fibronectin, which is a glycoprotein responsible for cell-to-cell interactions and interaction with the extracellular matrix (Pankov and Yamada, 2002). The extracellular matrix is responsible for binding the cells together (Pankov and Yamada, 2002). Integrins are transmembrane receptor proteins that bind cells to fibronectin, connecting the extracellular membrane to the cell cytoskeleton

(Pankov and Yamada, 2002). *C. jejuni* was found to bind to the extracellular fibronectin (Konkel *et al.*, 1997, Moser *et al.*, 1997).

C. jejuni cells make contact with epithelial cells through surfaces adhesins, which bind to fibronectin, integrin and caveolin present on the surface of intestinal epithelial cells (Ó Croinin and Backert, 2012). This initiates the formation of pseudopods that extend around the bacteria due to cell cytoskeleton reorganisation to complete the bacterial internalisation (Konkel *et al.*, 1992). *C. jejuni* uses a combination of "zipper" and "trigger" mechanisms for cell internalisation (Ó Croinin and Backert, 2012). However, how *C. jejuni* triggers rearrangement of microfilaments and microtubules of host cells for internalisation is still unclear (Ó Croinin and Backert, 2012).

C. jejuni can also transmigrate through the epithelial cell barrier using paracellular or transcellular routes (Backert *et al.*, 2013). The paracellular route requires that the bacteria break tight junctions and E-cadherin-based adherens junctions, which are responsible for attachment of the epithelial cells together (Backert *et al.*, 2013). *C. jejuni* is able to migrate in between the intestinal epithelial cells to bind to the fibronectin located on the basolateral surface of these cells (Monteville and Konkel, 2002). With the transcellular route, the bacteria invade the host cells and exit at the basolateral membrane (Backert *et al.*, 2013). The actual transmigration mechanism in *C. jejuni* is still controversial (Backert *et al.*, 2013)

Konkel *et al.* (1997) discovered a *C. jejuni* adhesin with fibronectin binding capacity which was named CadF and later was shown to have a fibronectin binding domain (Konkel *et al.*, 2005). A *C. jejuni* 81-176 *cadF* mutant and a F38011 *cadF* mutant were shown to exhibit reduced binding to fibronectin, binding less than 10% compared to wild-type levels (Monteville *et al.*, 2003). Therefore, CadF was shown to be an important factor in host cell interactions, as the lack of CadF reduced the adherence and internalisation of *C. jejuni* to epithelial cells (Monteville *et al.*, 2003).

FlpA was also identified as an important adhesin in *C. jejuni* (Konkel *et al.*, 2010). FlpA contains fibronectin type III domains, which are extracellular exposed domains (Flanagan *et al.*, 2009, Konkel *et al.*, 2010). A *C. jejuni* F38011 *flpA* mutant exhibited reduced adhesion in *in vitro* human epithelial cell assay and also showed reduced ability to colonise chicks (Flanagan *et al.*, 2009, Konkel *et al.*, 2010). FlpA also binds to fibronectin, but a *C. jejuni* F38011 *flpA* mutant exhibited reduced fibronectin

binding affinity compared to a *C. jejuni* F38011 *cadF* mutant (Konkel *et al.*, 2010).

PEB1 is another important adhesin in *C. jejuni* and is encoded by *peb1A* (Pei *et al.*, 1998). A *peb1A* mutant exhibited reduced adhesion and invasion to *in vitro* intestinal epithelial cells compared to the *C. jejuni* 81-176 wild-type strain (Pei *et al.*, 1998). Mutation of *peb1A* also reduced the duration and the colonisation rate in mice. However, PEB1 may also play a role in nutrient transport (Pei *et al.*, 1998). PEB1 is an orthologue of the binding component in amino acid transport systems in other bacterial species (Pei and Blaser, 1993). Therefore, it has been suggested that PEB1 is the binding component of the *C. jejuni* ABC transport system as PEB1 locus has similarities with other membrane binding receptors (Pei *et al.*, 1998). Although in other bacteria the protein binding of the transport system is located in the periplasmic space, PEB1 is located on the bacteria membrane (Pei and Blaser, 1993). PEB1 is a highly antigenic protein that induces a strong immunologic response and showed to have effective immunological protection in mice (Pei *et al.*, 1998, Du *et al.*, 2008).

JlpA is a surface lipoprotein with an adhesion role in *C. jejuni* (Jin *et al.*, 2001). A *jlpA* mutant decreased adhesion and invasion *in vitro* compared to the wild-type strain (Jin *et al.*, 2001). Antibodies against JlpA also reduced bacterial adherence to HEp-2 cells (Jin *et al.*, 2001). JlpA binds to HEp-2 cell receptor that activates NF-κB initiating an inflammatory response (Jin *et al.*, 2003). JlpA and Cj1349c have been shown not to be essential for *in vivo* chick colonization. However, CadF, PEB1 and FlpA exhibited an important role in chick colonisation as the respective mutants had reduced colonisation ability (Flanagan *et al.*, 2009).

porA encode a major outer-membrane protein (MOMP) in *C. jejuni* (Islam *et al.*, 2010). PorA is involved in ion transport across the outer membrane (Islam *et al.*, 2010). PorA also plays a role in adhesion to host epithelium cells and to extra-celullar matrix (Moser *et al.*, 1997). The *porA* locus has a great genetic diversity, as every distinct sequence encodes a novel peptide which provides positive immune selection (Cody *et al.*, 2009). However, PorA also has conserved regions with common antigenic epitopes amongst strains (Islam *et al.*, 2010).

1.9 Glycosylation systems

C. jejuni possesses two important glycosylation systems: *N*-linked and *O*-linked glycosylation, which add glycans to proteins as a post-translational modification (Szymanski *et al.*, 2003). This modification is important for protein folding, stability and adhesion to epithelial cells (Szymanski *et al.*, 2003). Many *C. jejuni* proteins undergo glycosylation to be fully functional (Szymanski and Wren, 2005).

1.9.1 *O*-linked glycosylation

C. jejuni O-linked glycosylation is required for flagella filament assembly (Szymanski *et al.*, 2002). Defects in the glycosylation process can cause loss of motility and deficiency in adhesion to and invasion of host cells (Szymanski *et al.*, 2002, Young *et al.*, 2007). The lack of glycosylation on key residues can hinder filament assembly, produce strains with reduced motility or produce truncated flagella filaments (Ewing *et al.*, 2009). *O*-linked glycosylation genes in the glycan biosynthetic locus are hypervariable resulting in variation between different strains (Ewing *et al.*, 2009). This region is one of the most variable between *C. jejuni* genomes (Parkhill *et al.*, 2000, Dorrell *et al.*, 2001). Therefore, flagellin glycosylation varies amongst different strains, which contributes to antigenic diversity and avoidance of the host immune response (Guerry, 2007).

O-linked glycosylation is responsible for modifying 19 serine and threonine residues in the flagellin from the *C. jejuni* 81-176 strain (Thibault *et al.*, 2001). *C. coli* VC167 had at least 16 amino acid residues glycosylated in the flagellin (Logan *et al.*, 2002). Alteration in the glycosylation process changes the serospecificity of the flagellar filament (Logan *et al.*, 2002). Most of the modified residues in *C. jejuni* 81-176 are in the central domain of the flagellin. However, one residue is exposed on the surface of the flagellin (Thibault *et al.*, 2001). The glycosylation locus in *C. jejuni* 11168 possesses 24 more genes than the 81-176 strain (Guerry *et al.*, 2006). However, only 9 common genes in both strains seem to affect the glycosylation of flagellin (Guerry *et al.*, 2006). The main sugar used in the *O*-linked glycosylation is the monosaccharide 5,7-diacetamido-3,5,7,9-tetradeoxy-L-glycero-L-manno-nonulosonic acid (pseudaminic acid, Pse, which is similar to sialic acid) and its

acetamidino derivative (PseAm) (Thibault *et al.*, 2001, Logan *et al.*, 2002). Mutation of *Cj1293* responsible for pseudaminic acid biosynthesis generated a non-motile aflagellated *C. jejuni* 81-176 strain with accumulated unglycosylated flagellin in the cytoplasm, whilst a *C. coli* VC167 *Cj1293* mutant replaced Pse with PseAm and retained motility (Goon *et al.*, 2003). Cj1324 also has a role in flagellin glycosylation, as an 11168H *Cj1324* mutant showed an absence of the glycans Leg5Am7Ac and Leg5AmNMe7Ac on the flagellin and exhibited a defect in chick colonisation (Howard *et al.*, 2009).

1.9.2 *N*-linked glycosylation

N-linked glycosylation links glycans to asparagine residues in many proteins, associated with the motif Asp/Glu-X_1-Asn-X_2-Ser/Thr, where X is any amino acid except for proline (Nothaft and Szymanski, 2010). Carbohydrates attached to proteins are important in different manners: signal transduction, protein folding, stability, cell interactions and host immune response (Nothaft and Szymanski, 2010).

Originally, a gene locus that encoded enzymes involved in the glycosylation of multiple proteins in *C. jejuni* was identified (Szymanski *et al.*, 1999). This locus was named *pgl* for protein glycosylation and is highly conserved amongst *C. jejuni* and *C. coli* strains (Szymanski *et al.*, 1999).

The *C. jejuni* glycosylation system (Figure 1.1) produces a heptasaccharide in the cytoplasm from a uridine diphosphate-activated *N*-acetylglucosamine (UDP-GlcNAc), PglF (C6 dehydratase) generates UDP-2-acetamido-2,6-dideoxy-D-xylo-4-hexulose (a UDP-4-keto-sugar), PglE (aminotransferase) transfers the amino group from L-glutamate to UDP-4-keto-sugar forming UDP-2-acetamido-4-amino-2,4,6-trideoxy-α-D-glucose (UDP-4-amino-sugar). Then PglD acetylates the compound from acetyl CoA, forming UDP-2,4-diacetamido-2,4,6-trideoxy-α-D-glucose (UDP-diNAcBac). After this, PglC forms diNAcBac-α1-PP-Und. Then PglA adds a UDP-GalNAc to form GalNAc-α1,3-diNAcBac-α1-PP-Und. PglJ adds an α1,4-GalNAc residue to the compound. PglH adds three α1,4-linked GalNAc residues to the glycan chain. Finally, PglI adds a β1,3-linked glucose branch. PglK is a flippase responsible for translocating the heptasaccharide across the inner membrane to the periplasm.

PglB is an oligosaccharyltransferase responsible for releasing the oligosaccharide into the periplasm or for transferring the oligosaccharide to a protein with the following sequence Asp/Glu-X_1-Asn-X_2-Ser/Thr, in which X_1 and X_2 are any amino acid except Proline (Nothaft and Szymanski, 2010).

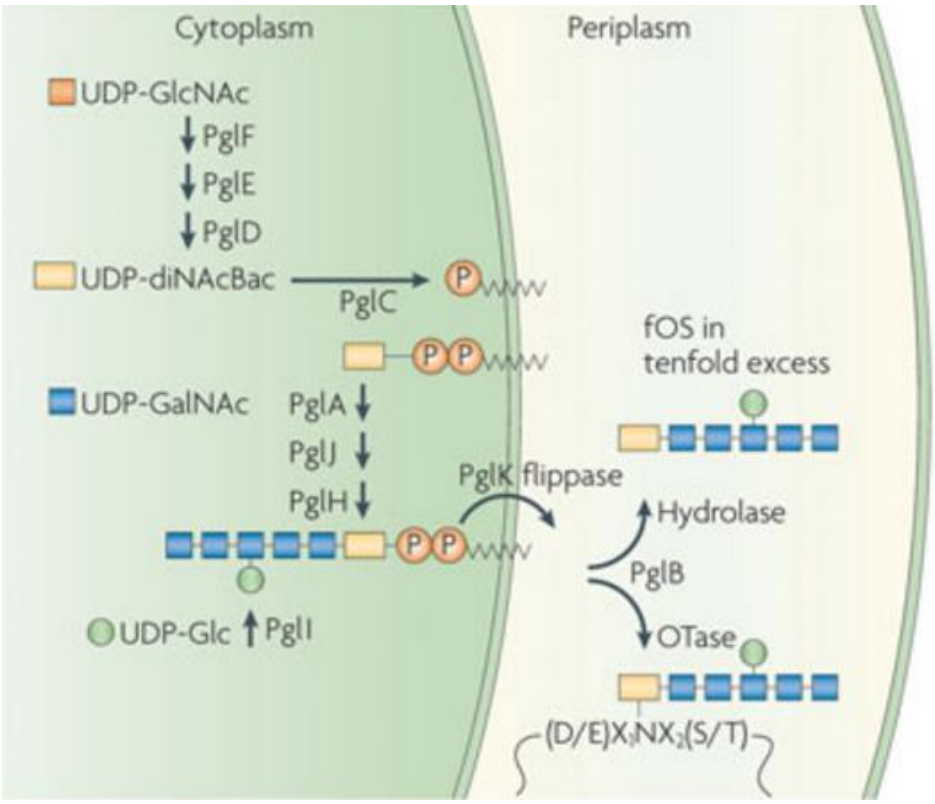

Figure 1.1 The mechanism of block transfer for *Campylobacter jejuni*, which is the prototype for the bacterial *N*-linked protein glycosylation system. The undecaprenyl pyrophosphate-linked heptasaccharide is assembled in the cytosol by the addition of the indicated sugars from nucleotide-activated donors. The complete heptasaccharide is translocated across the inner membrane into the periplasm by the protein glycosylation K (PglK) protein, an ATP-binding cassette (ABC)-type transporter. The oligosaccharide is transferred to the amino group of Asn in the protein consensus sequence (Asp/Glu-X_1-Asn-X_2-Ser/Thr, in which X_1 and X_2 are any amino acid except Proline), or released into the periplasm as free oligosaccharides (fOS) by the oligosaccharyl transferase (OTase) PglB. In *C. jejuni*, the fOS/*N*-glycan ratio is approximately 10/1 under standard growth conditions. Reproduced from Nothaft and Szymanski (2010).

Mutations in the *pgl* locus resulted in mutants with altered antigenicity (Szymanski *et al.*, 1999), as deglycosylation can result in the protein losing immunogenicity (Thibault *et al.*, 2001). A total of 130 proteins have been shown to be *N*-glycosylated with 75 glycosylation sites identified (Scott *et al.*, 2011). 81-176 *plgB* and *pglE* mutants showed a decreased capacity to adhere to and invade intestinal epithelial cells (Szymanski *et al.*, 2002).

Larsen *et al.* (2004) demonstrated that PglB and PglE are important for the natural transformation of *C. jejuni*. Larsen *et al.* (2004) also demonstrated that the type IV secretion system protein VirB10 is inactive in the non-glycosylated form. It has also been suggested that *N*-linked glycosylation of *C. jejuni* surface proteins can protect against cleavage from gut proteases (Alemka *et al.*, 2013).

The *C. jejuni pgl* locus has been demonstrated to function when cloned into *E. coli* (Wacker *et al.*, 2002) and is a better alternative for generating conjugate vaccines than conventional chemical methods (Nothaft and Szymanski, 2010). PglB attaches polysaccharides to proteins. This process enhances host immune response and can prevent colonisation and infection of pathogens (Cuccui and Wren, 2015). Therefore, polysaccharide conjugates from pathogenic bacteria can be produced in *E. coli* using the *C. jejuni N*-glycosylation system as an alternative for vaccines against bacterial pathogens (Cuccui and Wren, 2015).

1.10 Vaccines

Chickens start producing antibodies against *Campylobacter* once colonisation occurs. Chickens produce IgG, IgA and IgM *Campylobacter*-specific antibodies (Widders *et al.*, 1998, de Zoete *et al.*, 2007, Humphrey *et al.*, 2014). The increase of serum antibodies against *Campylobacter* has been demonstrated to reduce the bacterial concentration in chicken faeces (de Zoete *et al.*, 2007). No vaccine developed so far is capable of preventing *Campylobacter* infection/colonisation of poultry (Hermans *et al.*, 2011). All developed vaccines had limited success. The best vaccines could only reduce the level of *Campylobacter* colonisation in the chicken gut (de Zoete *et al.*, 2007, Hermans *et al.*, 2011). Different types of vaccines have been tested, such as whole cell lysate, surface-exposed subunit vaccines, and antigens vectored by

microrganisms (Layton *et al.*, 2011, Neal-McKinney *et al.*, 2014, Meunier *et al.*, 2016). Vaccines still need improvement and further investigation to increase the level of protection to boost a strong intestinal immune response (de Zoete *et al.*, 2007, Jagusztyn-Krynicka *et al.*, 2009). Investigation into the expression of surface proteins during human/chicken colonisation is essential to improve the vaccine development (de Zoete *et al.*, 2007, Jagusztyn-Krynicka *et al.*, 2009). A *Campylobacter* vaccine must be highly immunogenic and must provide cross protection against different *Campylobacter* strains (Hermans *et al.*, 2011). If a subunit is chosen as a good immunogenicity candidate, this protein must be conserved amongst different strains (Meunier *et al.*, 2016). *Campylobacter* vaccines for humans are also being investigated. However, tests in humans are rarely performed due to the possibility of inducing GBS (Jagusztyn-Krynicka *et al.*, 2009). One study demonstrated that humans develop anti-*Campylobacter* antibodies after being infected (Black *et al.*, 1988). High levels of antibodies in humans can reduce the risk of *Campylobacter* infection (Black *et al.*, 1988, Walz *et al.*, 2001).

The best strategy to control *Campylobacter* infections is to combine different strategies: 1 - biosecurity measures which are important to avoid flock contamination; 2 - reduce *Campylobacter* carriage in the gut (vaccination, nutritional measures, probiotics); 3 - antimicrobial alternatives, such as bacteriophages and bacteriocin treatments (Jagusztyn-Krynicka *et al.*, 2009). Farm biosecurity measures are difficult to implement, but important to prevent flock contamination effectively (Newell *et al.*, 2011). However, no clear route on how *Campylobacter* infects flocks has been established (Newell *et al.*, 2011). Vaccination can reduce the level of colonisation in poultry. However, vaccines cannot yet eliminate *Campylobacter*. To efficiently control *Campylobacter,* all these different measures need to be combined to reduce the level of contamination on flocks and, as a result, to reduce human exposure to this bacterium (Newell *et al.*, 2011). Some nutritional compounds, such as fatty acids, have an antimicrobial effect, and can reduce the level of *Campylobacter* in the chicken gut (Meunier *et al.*, 2016). Probiotics and phage treatment can also reduce the level of *Campylobacter* in the chicken gut (Messaoudi *et al.*, 2011, Ghareeb *et al.*, 2012, Meunier *et al.*, 2016). Nutritional additives and bacteriophages can be administered before slaughter to reduce the level of gut contamination since they are not efficient in the long term (Hermans *et al.*, 2011).

1.11 Secretion systems

Secretion systems are important for all bacteria to survive under environmental conditions or to interact with host cells (Wang *et al.*, 2015). Gram-negative bacteria contain numerous secretion systems to transfer bacterial proteins from the cytoplasm to the periplasm or through the outer membrane (Costa *et al.*, 2015). The secreted compounds can be released to the extracellular space. However, some secretion systems can deliver proteins directly into the cytoplasm of a target cell (Guerry, 2007). The general secretion pathway (Sec-pathway) and the twin arginine translocation (Tat-pathway) are secretion systems found in eukaryotes and prokaryotes (Mori and Ito, 2001).

Tat and Sec only span the cytoplasmic membrane and allow the passage of proteins through the cytoplasmic membrane to the periplasmic space (Natale *et al.*, 2008). The Tat system mainly transports folded proteins and cofactors, whilst the Sec system transports unfolded proteins (Mori and Ito, 2001). Tat co-factors are found in the cytoplasm and periplasm of bacteria, whilst Sec co-factors are only found in the periplasm (Natale *et al.*, 2008). Both pathways secrete proteins with diverse functions, such as Sec-pathway: substrate uptake and excretion, cell envelope structure, sensing and cell communication; Tat-pathway: respiratory energy metabolism, cell division, cell motility, iron and phosphate acquisition, quorum sensing (Natale *et al.*, 2008).

More specialised secretion systems have been described in prokaryotes. Six secretion systems have been identified in Gram-negative bacteria: type I secretion system (T1SS), type II secretion system (T2SS), type III secretion system (T3SS), type IV secretion system (T4SS), type V secretion system (T5SS) and type VI secretion system (T6SS) (Costa *et al.*, 2015).

T1SS, T2SS, T3SS, T4SS and T6SS all span both the cytoplasmic and outer membrane, whilst the T5SS spans only the outer membrane of the cell (Costa *et al.*, 2015). T2SS and T5SS use a two-step secretion mechanism, whilst all the others use only a one-step secretion mechanism, secreting proteins from the cytoplasm directly to the extracellular space or host cell (Costa *et al.*, 2015).

The T3SS is specific for the transport of factors by pathogenic bacteria (Kuehn and Kesty, 2005). However, *C. jejuni* lacks a T3SS that is important for virulence for many

other enteropathogenic bacteria (Parkhill *et al.*, 2000, Guerry, 2007).

1.11.1 Type VI Secretion System

One of the functions of the T6SS is to deliver effectors to other bacteria competing in the same environment (Salomon *et al.*, 2014). These effectors give a survival advantage in a specific niche, e.g. phospholipases (Wang et al 2015). DNases are delivered to attack neighbouring cells (Wang et al 2015). The T6SS is required for full virulence in various bacteria (Tseng *et al.*, 2009). The T6SS has a cluster of 13 conserved genes that are present with at least one copy in the genome (Basler *et al.*, 2012, Wang *et al.*, 2015).

The structure of the T6SS is very similar to a contractile tail of bacteriophages (Salomon *et al.*, 2014). VgrG (valine glycine repeat) and PAAR (repeat-containing proteins) form a structure similar to a phage spike (Pukatzki *et al.*, 2007, Salomon *et al.*, 2014). Hcp (hemolysin-coregulated protein) forms the structure homologous to a phage tube protein (Kudryashev *et al.*, 2015). Hcp and VgrG are required to form the T6SS structure, but are also secreted to the extracellular milieu (Pukatzki *et al.*, 2007). Studies demonstrated that Hcp is assembled in hexametric rings to form a tubular structure to deliver proteins or virulence factors (Ho *et al.*, 2014). It has also been shown that the Hcp tubular structure has homology to the phage lambda tube protein and shows an evolutionary relationship between the T6SS and phages (Pell *et al.*, 2009). Mutation of *hcp* or *vgrG* in *Vibrio cholerae* stopped the secretion of proteins substrates. Both mutants also showed reduced cytotoxicity towards macrophages (Pukatzki *et al.*, 2007).

The 13 conserved genes of the T6SS have been renamed *tssA* to *tssM* (Shalom et al., 2007). The 13 belonging to the T6SS are described in Table 1.1.

TssB and TssC form a tubular structure that is assembled around the TssD (Hcp) tube, similar to a sheath, and attached to the bacterial membranes (Basler *et al.*, 2012). TssB/TssC are homologues of VipA/VipB in *V. cholerae* and *E. coli*, which form a long contractile sheath structure (Basler *et al.*, 2012, Kudryashev *et al.*, 2015). It is believed that the contraction of the sheath is responsible for the process of translocating the effectors through the TssD tube across the membrane (Kudryashev

et al., 2015).

icmF homolog is a conserved gene present amongst T6SS gene cluster (Bingle *et al.*, 2008). IcmF is present in T4SS *Legionella pneumophila*, which is an accessory protein associated with ATPase (Bingle *et al.*, 2008). ATPase activity is important to power the T6SS to secrete proteins (Wang *et al.*, 2015).

TssH (ClpV) is cytoplasmic ATPase responsible for recognising the contracted T6SS sheath (Kapitein *et al.*, 2013, Kudryashev *et al.*, 2015). ClpV disassembles contracted VipA/VipB tubules in *V. cholera* allowing formation of functional elongated tubules (Kapitein *et al.*, 2013).

Table 1.1 List of core T6SS genes, homologues and putative functions.

Gene	Homologues	Putative function
tssA	*impA, vasJ*	Unknown function
tssB	*impB, vipA*	Homologous to T4 phage contractile tail sheath proteins
tssC	*impC, vipB*	Homologous to T4 phage contractile tail sheath proteins
tssD	*hcp*	Effector/Structure: Homologous to T4 phage tube
tssE	*impF, vasS*	Essential baseplate protein similar to T4 phage gp25 proteins
tssF	*impG, vasA*	Unknown function
tssG	*impH, vasB*	Unknown function
tssH	*clpV, vasG*	ATPase / effector chaperon / recycling TssB/TssC
tssI	*vgrG*	Effector/structure: forms the T6SS piercing structure
tssJ	*vasD, lip*	Anchoring T6SS to cell wall
tssK	*impJ, vasE*	Unknown function
tssL	*ompA, dotU*	Anchoring T6SS to cell wall

tssM	*vasK, icmF*	Anchoring T6SS to cell wall

Tss (type VI secretion) genes refers to the T6SS gene nomenclature proposed by (Shalom *et al.*, 2007). These genes have been shown to be essential for secretion of at least two proteins, Hcp and VgrG and are conserved in the genome sequence of over 100 different bacteria encoding a T6SS similar to the prototype described by (Pukatzki *et al.*, 2009). Table reproduced from Shyntum *et al.* (2014).

1.11.2 T6SS is important for bacterial resistance to oxidative stress

Wang *et al.* (2015) demonstrated that the T6SS has a role in oxidative stress resistance. The *Yersinia pseudotuberculosis* T6SS-4 is regulated by OxyR (a global oxidative stress regulator) and the expression of the T6SS is induced in the presence of hydrogen peroxide (H_2O_2). Furthermore, mutants lacking important structural genes of the T6SS-4 are more sensitive to oxidative stress compared to the *Y. pseudotuberculosis* wild-type strain and accumulated higher amounts of reactive oxygen species (Wang *et al.*, 2015). The T6SS has an important role not only in resistance to oxidative stress conditions, but also to high osmolality and low pH (Wang *et al.*, 2015).

Wang *et al.* (2015) also demonstrated that the T6SS is involved in uptake of ions, such as Zn^{2+} under stress conditions. Zn^{2+} is required for bacterial survival under oxidative stress conditions, specially required for attenuation of hydroxyl radicals (Wang *et al.*, 2015).

Zinc is an important co-factor for several enzymes and has an antioxidant role (Eide, 2011). However, higher concentrations of zinc can be toxic. Therefore, zinc homeostasis has to be tightly regulated in both bacterial cells and eukaryotic cells (Davis *et al.*, 2009). Host cells can release zinc at the mucosal level as an innate defence mechanism against pathogens (McDevitt *et al.*, 2011). Zinc deficiencies increase oxidative stress within the cells and cause DNA damage in rat blood cells (Song *et al.*, 2009). The mechanisms by which zinc reduces oxidative stress are not yet clear (Song *et al.*, 2009), and neither is the mechanism how zinc can provide protection against pathogens (McDevitt *et al.*, 2011).

The T6SS may be important for zinc uptake in bacteria. However, it has been suggested that *C. jejuni* has a ZnuABC system that can also uptake zinc (Davis *et al.*, 2009). The ZnuABC system is required for *C. jejuni* survival in a low zinc environment and for chick colonisation (Davis *et al.*, 2009). Zinc homeostasis is very important for *C. jejuni* during the infectious cycle, playing a major role in the ability of *C. jejuni* to survive within host cells (Davis *et al.*, 2009).

Bacillus subtilis was shown to survive H_2O_2 stress in the presence of zinc, demonstrating the important role of zinc in oxidative stress resistance (Gaballa and Helmann, 2002). The same study also showed that a *B. subtilis perR* mutant was dependent on *zosA* (P-type metal-transporting ATPase) expression, which is up-regulated by H_2O_2 and repressed by PerR, which also has an important role in protecting cells against peroxide stress (Gaballa and Helmann, 2002).

1.11.3 Outer membrane vesicles

Outer membrane vesicles (OMV) are utilized by Gram-negative bacteria to deliver virulence factors to the extracellular environment (Kuehn and Kesty, 2005). *C. jejuni* lacks the virulence-associated secretion systems present in most enteric pathogens, such as a T3SS or T4SS (Guerry, 2007). The flagella apparatus is similar to a T3SS that could deliver virulence factors outside *C. jejuni* cells (Konkel *et al.*, 2004). Therefore, the delivery of pathogenicity factors through OMVs is an especially important feature for *C. jejuni* (Elmi et al., 2012).

OMVs are secreted by Gram-negative bacteria, both pathogenic and non-pathogenic species (Beveridge, 1999). OMVs consist of a spherical bilayer membrane with a diameter ranging from 50 to 250 nm (Beveridge, 1999, Kuehn and Kesty, 2005). The external membrane of the OMV is composed of LOS/LPS and the internal membrane is composed of phospholipids and lipoproteins (Logan and Trust, 1982, Kuehn and Kesty, 2005). Surface proteins and carbohydrates are antigens for the host immune response (Logan and Trust, 1982). OMVs also have surface-exposed adhesins and receptors (Kulp and Kuehn, 2010). OMVs are formed from small portions of the outer membrane that bleb away and are released from the cell (Figure 1.2) (Kulp and Kuehn, 2010).

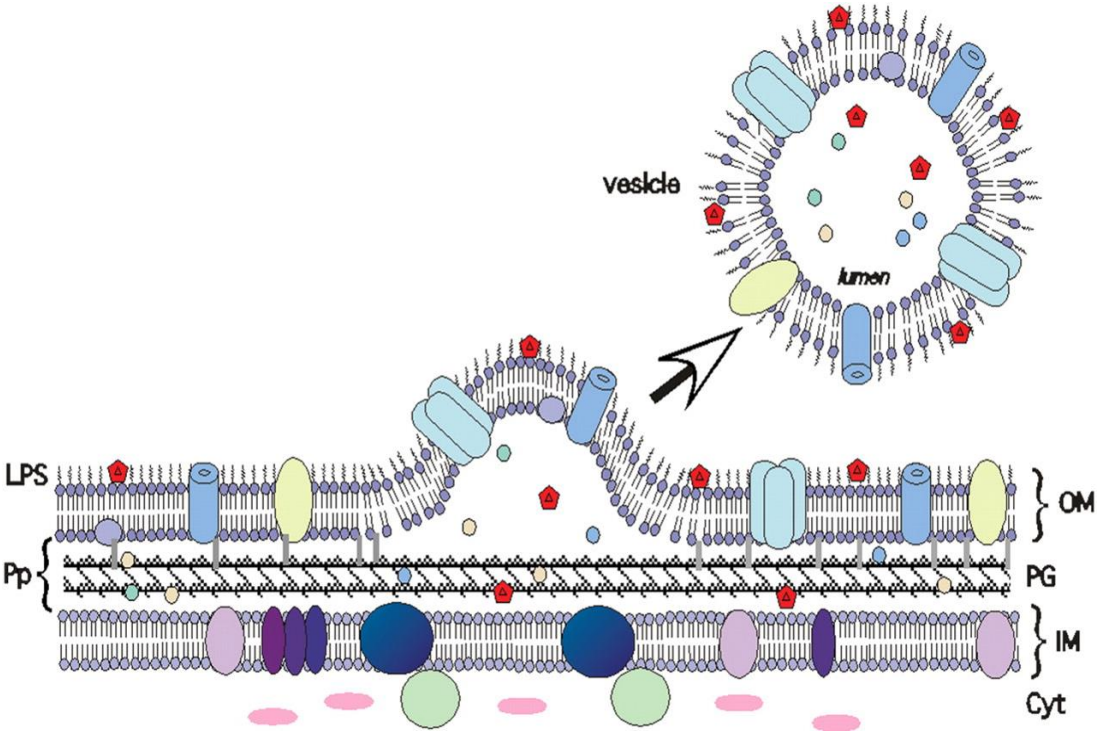

Figure 1.2 Model of vesicle biogenesis. OM vesicles are proteoliposomes consisting of OM phospholipids and LPS, a subset of OM proteins, and periplasmic (luminal) proteins. Proteins such as heat labile toxin (red) that adhere to the external surface of the bacteria are associated with the external surface of vesicles. Proteins and lipids of the IM and cytosolic content are excluded from OM vesicles. Vesicles are likely to bud at sites where the links between the peptidoglycan and OM are infrequent, absent, or broken. Lipopolysaccharide (LPS); periplasm (Pp); outer membrane (OM); peptidoglycan (PG); inner membrane (IM); Cytosol (Cyt). Reproduced from Kuehn and Kesty (2005).

OMVs are responsible for secreting products that enhance bacterial survival, such as delivering virulence factors to host cells (Kuehn and Kesty, 2005). OMVs from pathogenic bacteria contain toxins, adhesins and immunomodulatory compounds, which are responsible for increasing adhesion and invasion, causing cytotoxicity and modulating the host immune response (Kuehn and Kesty, 2005). OMVs also possess pores that are used to diffuse soluble secretion substances, such as ions, amino acids, and small sugars (Beveridge, 1999, Delcour, 2009). OMVs can also deliver quorum-signaling molecules, such as the PQS molecule from *Pseudomonas aeruginosa* (Mashburn and Whiteley, 2005).

It has been suggested that OMVs deliver their contents by lysis once near a target site, such as a Gram-positive bacteria, whilst when near another Gram-negative bacteria, OMVs can fuse to the outer membrane to release their contents directly inside the cell (Kadurugamuwa and Beveridge, 1999, Kulp and Kuehn, 2010).

Non-pathogenic bacteria can secrete OMVs containing protective compounds that can aid in reducing the level of toxic compounds in the surrounding environment (Kuehn and Kesty, 2005). Pathogenic bacteria can produce more OMVs than non-pathogenic bacteria (Horstman and Kuehn, 2002).

Lindmark *et al.* (2009) have demonstrated that *C. jejuni* strains 81-176 and 81116 can secrete CDT via OMVs, suggesting that OMVs are the main form of delivery of CDT. All subunits were detected in OMVs. Intestinal epithelial cells were treated with OMVs isolated from the *C. jejuni* 81-176 wild-type strain. It was observed that the cells entered into cell cycle arrest, demonstrating that OMVs deliver active CDT and cause cytolethal distending effects (Lindmark *et al.*, 2009). OMVs contain soluble and insoluble proteins. Secreting proteins inside OMVs can provide protection against extracellular proteases present in the bacterial environment (Kulp and Kuehn, 2010). Thus, OMVs can be active for longer time and can travel larger distances (Kulp and Kuehn, 2010).

Proteomic analysis of *C. jejuni* 11168H OMVs identified 151 proteins (Elmi *et al.*, 2012). A great number of identified proteins were: membrane-associated, cytoplasmic and periplasmic proteins, and virulence-associated proteins (Elmi *et al.*, 2012). Some of the virulence-associated proteins identified were the fibronectin binding proteins CadF and FlpA, CDT and three proteases (HtrA, Cj0511 and Cj1365c) (Elmi *et al.*, 2012, Elmi *et al.*, 2015). However, 26% of the proteins that were identified have as yet unknown functions (Elmi *et al.*, 2012). *C. jejuni* 11168H OMVs have a cytotoxic effect on Caco-2 cells (Elmi *et al.*, 2012). OMVs also induced an immune response from T84 cells with increasing the levels of IL-8, IL-6, TNF-α and hBD-3 (Elmi *et al.*, 2012).

In vitro assays demonstrated that *C. jejuni* 11168H proteases HtrA and Cj1365c can actively cleave E-cadherin and occludin, which are adherens junction and tight junction proteins respectively (Elmi *et al.*, 2015). The cleavage of these two proteins facilitates the bacterial invasion of human intestinal epithelial cells (Elmi *et al.*, 2015).

Presence of antibiotics and oxidative stresses are some mechanisms that can influence OMV formation and content, which can increase the chances of bacterial survival (Kuehn and Kesty, 2005). Antibiotic treatment can enhance OMV formation (Kulp and Kuehn, 2010). OMVs can bind to antimicrobials and inactivate them, and can increase antibiotic resistance when associated with biofilms (Kulp and Kuehn, 2010).

Sabra *et al.* (2003) demonstrated that when *P. aeruginosa* is exposed to oxidative stress, *P. aeruginosa* increases the number of OMVs, both attached to the cell surface and also released into the environment.

1.12 Biofilm formation

A biofilm is a community of bacteria that attaches to a surface (O'Toole *et al.*, 2000). This community of bacteria can belong to the same species or to different species (O'Toole *et al.*, 2000). In order to form a biofilm, free planktonic cells attach to a surface, then start attaching to one another forming microcolonies (O'Toole *et al.*, 2000). Once the bacteria is attached to a surface, the colony undergoes adaptation and starts producing extracellular polymers that facilitate attachment of other bacteria and forms a matrix of polymers (Donlan, 2001). The extracellular matrix is composed of polysaccharides, proteins, nucleic acids and dead cells (Parsek and Greenberg, 2005). Bacteria that have flagella, pili or fimbriae can attach more easily to surfaces (Donlan, 2001). The *C. jejuni* flagella has been demonstrated to be important for biofilm formation as non-motile wild-type strains and aflagellate mutants fail to attach to surfaces to form biofilms (Joshua *et al.*, 2006, Reuter *et al.*, 2010). Biofilms can be flat or can have several layers of cells encased in the extracellular matrix (Parsek and Greenberg, 2005). A mature biofilm structure decreases bacterial sensitivity to antimicrobial agents (Donlan, 2001).

Biofilms are more metabolically active at the surface, with low levels of activity and slow growth in the centre (Hoiby *et al.*, 2010). Biofilms produce endogenous ROS that can accumulate in the matrix, whilst external ROS can also increase oxidative stress levels in biofilm cells (Hoiby *et al.*, 2010). Accumulation of ROS in biofilms has been linked to mutations in *P. aeruginosa* (Hoiby *et al.*, 2010). These mutations can lead to the production of new enzymes that inactivate antibiotic compounds increasing the

associated biofilm antibiotic resistance (Hoiby *et al.*, 2010).

Quorum sensing is an intercellular communication system that acts as a global regulatory system in several different bacteria and which also has been shown as an important communication system for biofilm formation (Parsek and Greenberg, 2005). Changes in the environmental conditions can alter gene transcription, cell phenotype and biofilm growth rate (Donlan, 2001). Quorum sensing genes can regulate biofilm aggregation, maturation and architecture development (Parsek and Greenberg, 2005), Quorum sensing can also regulate the release of cells from the biofilm (Parsek and Greenberg, 2005). Expression of the *V. cholera vps* operon is regulated by quorum sensing and this operon encodes the secretion of exopolysaccharide responsible for cell aggregation to form biofilms (Zhu and Mekalanos, 2003). In *P. aeruginosa,* quorum sensing regulates the expression of 170 to 200 genes where some of them are involved in biofilm formation (Parsek and Greenberg, 2005). However, there are species where a quorum sensing system was not identified, but these species still have the capability of forming biofilms (Parsek and Greenberg, 2005).

Environmental factors also affect biofilm formation, such as nutrient starvation, osmotic changes, temperature variation, and changes in oxygen tensions (Reeser *et al.*, 2007). *C. jejuni* increases biofilm formation in nutrient-poor media, and decreases biofilm formation in high osmolarity environment and under aerobic environment (Reeser *et al.*, 2007). *C. jejuni* forms 3 different types of biofilm: aggregates in liquid culture, pellicles at liquid-gas interface, and attachment to surfaces (Joshua, 2005). Aggregates are bacteria that auto-agglutinate in liquid cultures and have similar structure to both biofilms attached to surfaces and to liquid-gas pellicles (Joshua, 2005).

1.13 Oxidative stress

Oxidative stress causes damage to cell compounds through the exposure to reactive oxygen species (ROS) (Storz and Imlay, 1999). ROS are generated by the incomplete reduction of oxygen as a by-product of aerobic metabolism (Imlay, 2008). The atomic oxygen structure makes oxygen susceptible to radical formation (Held, 2015). The addition of electrons to the oxygen molecule generates ROS compounds (Figure 1.3),

such as the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$) (D'Autreaux and Toledano, 2007).

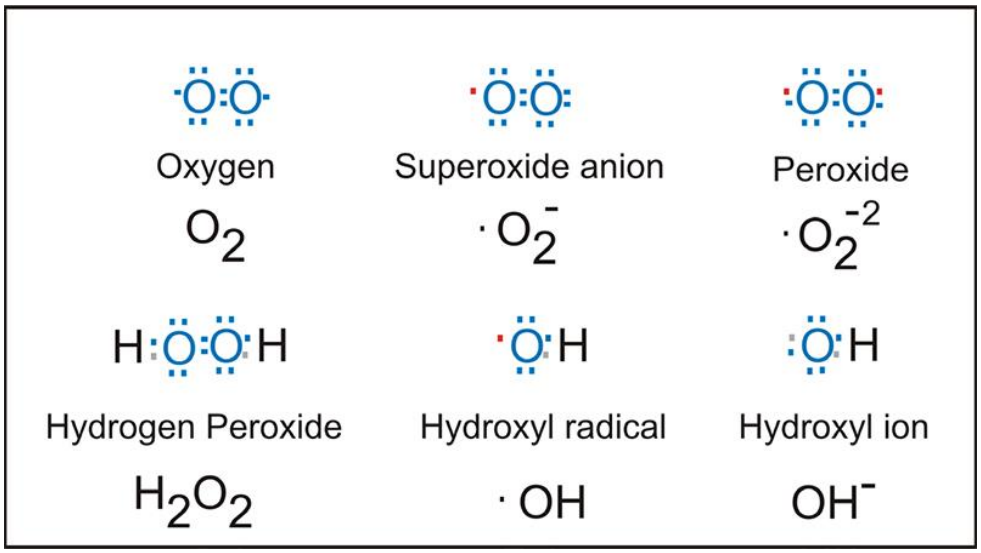

Figure 1.3 Electron structures of common reactive oxygen species. Each structure is provided with the name and chemical formula. The red • designates an unpaired electron. Reproduced from Held (2015).

ROS compounds are present in the environment, are produced by bacterial metabolism itself, and are also produced by the host immune response to counter infection (Dalton *et al.*, 1999). ROS are generated by the host's inflammatory immune response as part of a defence mechanism against bacterial infection that occurs within phagocytes (Simon *et al.*, 2000, Imlay, 2008). Therefore, it is essential for bacterial survival to have mechanisms to neutralise these toxic compounds because ROS accumulation in the bacterial cytoplasm and periplasm causes damage to lipids, proteins and DNA by oxidation (Dalton et al., 1999).

The generation of ROS at low levels can be beneficial to cells as it is important for cell signalling by reversible oxidation of proteins, starting phosphorylation cascades and regulating gene expression (Fisher, 2009). H_2O_2 and $O_2^{\bullet-}$ can penetrate the cell membrane through aquaporin channels and anion channels respectively (Fisher, 2009).

Fenton reaction is also important in the formation of ROS. Fenton (1894) discovered that some metals, such as iron and copper, have a catalytic power to transfer an electron to H_2O_2, then generating highly reactive oxygen radicals. Bacteria respiratory metabolism generates by-products, such as H_2O_2 and $O_2^{\bullet-}$. Fenton reaction is the H_2O_2 reaction with Fe^{2+} generating OH^- and $OH^{\bullet}$ as shown in equation 1. $O_2^{\bullet-}$, by-product generated from respiratory metabolism, can reduce the oxidised Fe_3^+ released by Fenton reaction, as shown in equation 2. $O_2^{\bullet-}$ can also catalyse the Harber-Weiss reaction, as shown in equation 3 (Figure 1.4).

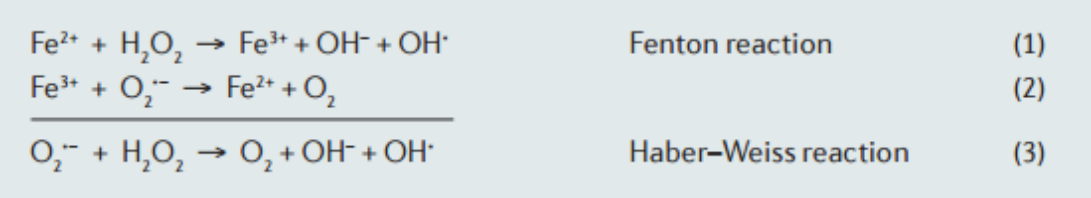

Figure 1.4 The Fenton reaction, free radical chemistry and metal poisoning. In vivo Fenton chemistry and other metal-catalysed free radical chain reactions are initiated by the inadvertent by-products of aerobic respiration, such as hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet-}$). Many in vitro experiments have indicated that H_2O_2 can oxidize Fe_2^+ to produce hydroxide (OH^-) and the highly reactive hydroxyl radical ($OH^{\bullet}$), which is called the Fenton reaction (see equation 1). In vitro work has demonstrated that $O_2^{\bullet-}$ can reduce the oxidized metal released by the Fenton reaction (see equation 2), and the net reaction, which is called the Haber–Weiss reaction, is catalytic (see equation 3). Reproduced from Lemire et al. (2013).

1.14 *C. jejuni* oxidative stress

Microaerobic bacteria normally do not grow in environmental oxygen concentrations or grow poorly (Krieg and Hoffman, 1986). *C. jejuni* is a microaerobic bacteria that has optimal growth in an atmosphere containing 5% O_2 and 10% CO_2 (Atack and Kelly, 2009). However, *C. jejuni* has evolved complex mechanisms to cope with high oxygen levels and toxic compounds in order to survive under unfavourable conditions (Imlay, 2008).

C. jejuni can survive under environmental conditions. However, *C. jejuni* lacks many important regulators of the oxidative stress response compared to other enterobacteria, such as SoxRS and OxyR (Imlay, 2008). These two regulators are responsible for activating gene expression involved in oxidative stress defence mechanisms (Imlay, 2008). However, *C. jejuni* possesses different regulators to counter oxidative stress, such as PerR, which is a functional but non-homologous substitute for OxyR (van Vliet *et al.,* 1999). Most of the *C. jejuni* regulators have shown to have roles in invasion and colonisation of chicks ceacum (Palyada et al., 2009). The complete role of all *C. jejuni* regulators is still poorly understood (Atack and Kelly, 2009), which shows how complex and inter-linked the *C. jejuni* oxidative stress defense mechanism is.

1.15 Oxidative stress defence mechanisms

1.15.1 Enzymes

C. jejuni has many enzymes that are responsible for countering oxidative stress to protect against accumulation of ROS (Atack and Kelly, 2009). These enzymes include catalase, superoxide dismutase, peroxiredoxins, cytochrome c peroxidises, methionine sulfoxide reductases (Atack and Kelly, 2009). They are responsible for neutralising ROS compounds generated during bacterial metabolism, encountered during survival in the environment and during colonisation of a host (Imlay, 2008).

1.15.1.1 Catalase

Catalase is the main enzyme responsible for reducing H_2O_2 into water and oxygen. *C. jejuni* expresses just one type of catalase (KatA), whilst many microorganisms have more than one catalase (Atack and Kelly, 2009). *C. jejuni* KatA is induced by H_2O_2 and O_2^- (Atack and Kelly, 2009). KatA is an iron-repressed protein regulated by PerR (van Vliet *et al.,* 2002). Low iron levels increase the level of *katA* expression, and mutation of *perR* also increases *katA* expression, but at extremely high levels (van Vliet *et al.,* 1999).

A *C. jejuni* 11168H *katA* mutant is extremely sensitive to H_2O_2 stress when compared

to the wild-type strain and cannot survive under even low concentrations of H_2O_2 (Gundogdu *et al.*, 2011). However, a *C. jejuni* M129 *katA* mutant was able to colonise chickens at the same level as the wild-type strain (Day *et al.*, 2000). A *C. jejuni* M129 *katA* mutant showed reduced intra-macrophage survival. However, KatA does not play a role in intra-epithelial survival, as the *katA* mutant survived at the same level as the wild-type strain (Day *et al.*, 2000). Paraquat is an organic compound that induces oxidative stress and increases *katA* expression in *C. jejuni* (Garenaux *et al.*, 2008).

Cj1386 has been demonstrated to be involved in the H_2O_2 stress response, as an 11168 *Cj1386* mutant was sensitive to H_2O_2 at the same level as a *katA* mutant (Flint *et al.*, 2012). The *Cj1386* gene, located downstream of *katA*, is independently transcribed and contributes to KatA activity (Flint *et al.*, 2012). A *Cj1386* mutant showed reduced catalase activity, but did not reduce *katA* expression (Flint *et al.*, 2012). It was suggested that Cj1386 is involved in haem trafficking to KatA and optimising the haem co-factor in active KatA (Flint *et al.*, 2012).

1.15.1.2 Superoxide dismutase

Superoxide dismutase (SOD) has an important role in defence against superoxide anions (O_2^-) converting two molecules of O_2^- into H_2O_2 and oxygen (Atack *et al.*, 2008, Atack and Kelly, 2009). There are three forms of SOD: with copper and Zinc as co-factor; with manganese as co-factor; and with iron as co-factor (Smith and Doolittle, 1992). *C. jejuni* has a single superoxide dismutase (SodB) and uses iron as co-factor (van Vliet *et al.*, 2002), whilst other bacteria normally express more than one SOD (Atack and Kelly, 2009). However, SOD is also known to be important in the *C. jejuni* capacity to invade and survive within epithelial cells (Atack and Kelly, 2009). A *C. coli* UA585 *sodB* mutant showed reduced growth in intracellular epithelial cells, decreased chick colonisation and increased sensitivity to superoxide anion generated by freeze-thaw process (Purdy *et al.*, 1999, Stead and Park, 2000). A *C. jejuni* 11168 *sodB* mutant showed reduced ability to survive to exposure to H_2O_2, cumene hydroperoxide and menadione (Palyada et al., 2009). A *C. jejuni* 11168 *sodB* mutant also exhibited increased sensitivity to paraquat (Garenaux *et al.*, 2008). However, the wild-type strain did not increase SOD expression under paraquat exposure (Garenaux *et al.*, 2008).

1.15.1.3 Alkyl hydroperoxide reductase

Alkyl hydroperoxide reductase (AhpC) is an enzyme that has a role in neutralising H_2O_2 in the cytoplasm (Atack and Kelly, 2009). AhpC also eliminates small hydroperoxides and organic hydroperoxides in *Salmonella typhimurium* (Parsonage *et al.*, 2008). However, the exact role of AhpC in *C. jejuni* is still unclear (Atack and Kelly, 2009). Most bacteria express AhpF and AhpC, where AhpF is a flavoprotein responsible for activation of AhpC by oxidation (Poole *et al.*, 2000). *C. jejuni* has AhpC, but lacks an AhpF orthologue (Atack and Kelly, 2009). In addition, it has been shown that iron represses *ahpC* expression (van Vliet *et al.*, 1999).

AhpC is responsible for removing low concentrations of H_2O_2 in *E. coli* and may play a similar role in *C. jejuni* (Seaver and Imlay, 2001, Atack and Kelly, 2009). AhpC has also been demonstrated to remove toxic hydroperoxide intermediates in *C. jejuni* (Baillon et al., 1999). A *C. jejuni* 81116 *ahpC* mutant is hypersensitive to cumene hydroperoxide and less aerotolerant than the wild-type strain, but did not show increased sensitivity to H_2O_2 (Baillon *et al.*, 1999). A *C. jejuni* 11168 *ahpC* mutant exhibits enhanced biofilm formation due to accumulation of ROS and lipid hydroperoxides (Oh and Jeon, 2014).

1.15.1.4 Thiol peroxidases

Thiol peroxidase (Tpx) is a peroxiredoxin that also catalyses the breakdown of peroxides, mainly H_2O_2. It is thought that Tpx acts in concert with KatA and AhpC to eliminate toxic H_2O_2 from the cytoplasm, thus providing maximum cell protection (Atack *et al.*, 2008). A *C. jejuni* 11168 *tpx* mutant showed reduced growth under aerobic conditions and also showed increased lipid peroxidation (Atack *et al.*, 2008). Tpx production was increased under aerobic conditions, which could be due to the increase of ROS levels under aerobic growth conditions (Atack *et al.*, 2008).

1.15.1.5 Bacterioferritin comigratory protein

Bacterioferritin comigratory protein (Bcp) is another peroxiredoxin present in *C. jejuni*. Bcp is responsible for reducing H_2O_2 and organic peroxides *in vitro* showing

some redundancy with Tpx function (Atack *et al.*, 2008). Bcp has a role as a general peroxidase enzyme breaking down different compounds and seems to complement Tpx activity (Atack *et al.*, 2008). A *C. jejuni* 11168 *bcp* mutant also showed similar phenotypic characteristics to the *C. jejuni* 11168 *tpx* mutant: reduced growth under aerobic conditions, increased Bcp production under aerobic conditions and increased lipid peroxidation due to the increase of ROS levels induced by the aerobic growth (Atack *et al.*, 2008). A *C. jejuni* 11168 *bcp tpx* double mutant exhibited severe growth defects under microaerobic conditions and did not grow under aerobic conditions (Atack *et al.*, 2008).

1.15.1.6 Cytochrome c peroxidase

C. jejuni contains two periplasmic cytochrome c peroxidases (CCPs) (Cj0020c and Cj0358 in the NCTC 11168 strain), whilst most bacteria possess only one (Atack and Kelly, 2009). Each CCP contains two c-type haems and are located in the periplasm (Atack *et al.*, 2008). The exact role of these two CCPs has not yet been elucidated. It is believed that CCPs are responsible for breaking down H_2O_2 present in the periplasm (van Vliet *et al.*, 2002). CCPs reduce H_2O_2 and the electrons generated bind to haem molecules. Therefore, CCPs avoid the generation of other reactive oxygen intermediates (Atack *et al.*, 2008).

Both CCPs are required for complete resistance to peroxide in *C. jejuni* 11168 (Atack and Kelly, 2009). Both CCPs also demonstrated to have peroxidase activity in *C. jejuni* 81-176 strain. However, mutation of either CCPs did not increase sensitivity to H_2O_2 compared to the wild-type strain (Atack and Kelly, 2009).

It is also suggested that the two CCPs may have a role in colonisation of intestinal epithelial cells, as *C. jejuni* 81-176 *Cj0020c* and *Cj0358* mutants both showed colonisation defects compared to the wild-type strain (Bingham-Ramos and Hendrixson, 2008).

1.15.1.7 DNA binding protein from starved cells

DNA binding protein from starved cells (Dps) plays an important role in protecting DNA against oxidative stress (Zhao et al., 2002). Dps is expressed under oxidative and nutritional stress conditions (Zhao et al., 2002).

A 81-176 *dps* mutant demonstrated increased sensitivity to H_2O_2 compared to the wild-type strain (Ishikawa *et al.*, 2003). Addition of an iron chelator reduced the sensitivity of the *dps* mutant to H_2O_2 and demonstrated that Dps provides protection against iron-mediated H_2O_2 stress (Ishikawa *et al.*, 2003). Conversely, Dps sequesters free iron molecules (Fe^{2+}) to prevent H_2O_2 formation of hydroxyl radicals via the Fenton reaction (Ishikawa *et al.*, 2003, Huergo *et al.*, 2013). Dps is up-regulated in the presence of Fe^{2+} or H_2O_2, then Dps binds to DNA to protect from hydroxyl radical damage (Huergo *et al.*, 2013).

Dps has different roles in *C. jejuni* besides protection against oxidative stress, such as roles in chick colonisation and biofilm formation (Theoret *et al.*, 2012). A *C. jejuni* 11168 *dps* mutant demonstrated reduced biofilm formation compared to the wild-type strain (Theoret *et al.*, 2012). *dps* expression is increased during chick colonisation in the wild-type strain, and a *dps* mutant showed reduced ability to colonise chicks (Theoret *et al.*, 2012).

1.15.1.8 Ferredoxin

Ferredoxins are small iron–sulfur non-haem proteins that function as electron carriers in different metabolic reactions (Bruschi and Guerlesquin, 1988). *C. jejuni* has a ferredoxin FdxA. *fdxA* gene sequence is located upstream *ahpC* in *C. jejuni* strain 81116 genome (van Vliet *et al.*, 2001). *fdxA* was also identified in the genome sequence of *C. jejuni* strain NCTC 11168 and identical to 81116 strain (van Vliet *et al.*, 2001). *fdxA* gene expression is induced by iron (van Vliet *et al.*, 2001), which is the opposite to *ahpC* expression. An 81116 *fdxA* mutant showed no difference in sensitivity to H_2O_2 compared to the wild-type strain (van Vliet *et al.*, 2001). Also an 81116 *fdxA* mutant did not show growth deficiency under iron-restrict media (van Vliet *et al.*, 2001). However, when this mutant was grown under aerobic conditions, the bacterial growth was severely affected, suggesting a role in the oxidative stress

defence mechanism (van Vliet *et al.*, 2001).

C. jejuni lacks AhpF homolog, which in other bacteria is responsible for reducing the oxidised form of AhpC (Baillon *et al.*, 1999, Parkhill *et al.*, 2000). The reduced form of AhpC is responsible for reducing alkyl hydroperoxides to alcohols (Baillon *et al.*, 1999). As *C. jejuni* does not have the AhpF homolog, it has been suggested that *C. jejuni* may utilise an alternative system (Baillon *et al.*, 1999). Ferredoxins are important reducing agents and could be used by *C. jejuni* to reduce AhpC (Baillon *et al.*, 1999).

1.15.1.9 Thioredoxin system

Thioredoxin system plays a role as antioxidant in the defence against oxidative stress (Lu and Holmgren, 2014). This system activates enzymes by electron reduction to thiol-dependent peroxidases (Lu and Holmgren, 2014).

Thioredoxin is a small protein capable of reducing protein disulphide bonds which are formed by the oxidation of two cysteine residues (Ritz *et al.*, 2000). Thioredoxin reduces the oxidized intracellular proteins and contributes to the maintenance of the redox status (Palyada *et al.*, 2004). Thioredoxins are responsible for maintaining cytoplasmic proteins in a reduced state, and thioredoxin reductases are responsible for reducing the thioredoxin using electrons from NADPH (Arner and Holmgren, 2000).

E. coli has two thioredoxins, and mutation of either thioredoxin increases the strain sensitivity to H_2O_2 (Ritz *et al.*, 2000). In *C. jejuni* 11168, *trxA* encodes a thioredoxin and *trxB* encodes a thioredoxin reductase (Holmes *et al.*, 2005). In an iron restricted environment, both *trxA* and *trxB* increased gene expression (Holmes *et al.*, 2005). *trxB* expression is co-regulated by Fur and PerR (Holmes *et al.*, 2005).

1.15.2 Regulators of oxidative stress

SoxR and OxyR are conserved regulators of the oxidative stress response in different bacterial groups, such as *E. coli* (Storz and Imlay, 1999). These two regulators respond to stresses $O_2^{\bullet-}$ and H_2O_2 (Storz and Imlay, 1999). However, *C. jejuni* lacks

homologues of SoxRS and OxyR (van Vliet et al., 1999). OxyR regulates almost 40 genes to protect *E. coli* against H_2O_2 stress (Chiang and Schellhorn, 2012). OxyR also regulates the responses to different stresses, such as heat stress, lipid peroxidation cell damage, and neutrophil killing (Chiang and Schellhorn, 2012). Expression of *katA* and *ahpC* homologues is normally regulated by OxyR (Chiang and Schellhorn, 2012). SoxRS is a two-component regulatory system important for controlling the response to superoxide stress (Chiang and Schellhorn, 2012). The expression of *soxS* is induced when SoxR is activated. SoxS regulates the expression of genes in response to oxidative stress (Chiang and Schellhorn, 2012).

C. jejuni has sensory systems to detect different forms of oxidative stress. Regulators can repress or increase enzyme production to control cytoplasmic levels of ROS (Atack and Kelly, 2009). The following regulators are found in *C. jejuni*: the peroxide-sensing regulator (PerR), the ferric uptake regulator (Fur), the carbon starvation regulator (CsrA), the two-component *Campylobacter* planktonic growth regulatory system (CprRS), and the reduced ability to colonise (RacRS) two-component regulatory system (Atack and Kelly, 2009; Van Vliet et al., 2002). Further regulators were discovered more recently in *C. jejuni*, such as the OmpR-type response regulator (CosR) (Hwang et al., 2011) and the two MarR-type response regulators RrpA and RrpB (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015).

1.15.2.1 Peroxide-sensing regulator (PerR)

PerR is a global transcriptional regulator that regulates at least 104 genes, a number of which are related with peroxide stress defence (Palyada *et al.*, 2009). PerR is considered a functional non-homologue of OxyR (van Vliet *et al.*, 1999). The mechanism by which PerR senses levels of peroxide has not been elucidated, but it is thought that PerR might sense peroxide stress by oxidation of the metal cofactor or by oxidation of the PerR protein itself (van Vliet *et al.*, 2002). *perR* transcription is negatively auto-regulated (Kim *et al.*, 2015) and PerR uses iron as co-factor to repress gene expression (Palyada *et al.*, 2009). Peroxide and superoxide reduce the level of *perR* transcription regardless of whether iron is present or absent (Kim *et al.*, 2015). PerR constitutively represses *katA* and *ahpC* (van Vliet et al., 1999). Therefore, a *C. jejuni* 11168 *perR* mutant has a constitutive expression of *katA* and *ahpC*, which

makes cells highly resistant to peroxide stress (Van Vliet et al., 1999). A *C. jejuni* 11168 *perR* mutant also shows reduced capacity to invade and colonise chicks, which suggests that PerR regulates genes related with different roles, not only with the oxidative stress response (Palyada et al., 2009). Putative PerR binding regions were found in the promoter region of *ahpC*, *katA* and *dps* (Kim *et al.*, 2015).

1.15.2.2 Ferric uptake regulator (Fur)

Fur is a regulator that controls the expression of genes responsible for the maintenance of the iron level within bacterial cells (Atack and Kelly, 2009). Fur controls genes responsible for iron uptake and also regulates levels of other metals, such as zinc, tungsten and molybdenum (Butcher *et al.*, 2012). Fur represses the expression of proteins involved in iron acquisition processes (Palyada *et al.*, 2009). Butcher *et al.* (2012) identified all the Fur binding sites throughout the *C. jejuni* NCTC 11168 genome. Fur is also involved in regulating both flagella and membrane biosynthesis, energy production, and stress responses besides controlling metal homeostasis (Butcher *et al.*, 2012). A *C. jejuni* 11168 *fur* mutant was shown to reduce chick colonisation compared to the wild-type strain (Palyada *et al.*, 2009). A *fur* mutant accumulates iron and can increase ROS formation, which can hinder chick colonisation (Palyada *et al.*, 2009). Flagella genes are regulated by Fur. Expression of flagella genes is important for chick colonisation. It is suggested that mutation of *fur* can cause a defect in the flagella (Palyada *et al.*, 2009). A *C. jejuni* 11168 *fur* mutant showed reduced chick colonisation (Palyada *et al.*, 2009).

There is an overlap between the Fur and PerR regulons (van Vliet *et al.*, 1999). Fur regulates *perR* expression, whereas PerR does not affect *fur* expression (van Vliet *et al.*, 1999), which demonstrates that regulators have multiple inter-linked levels as well as overlapping responses related to oxidative stress defence mechanisms (Palyada et al., 2009). Fur co-regulates several oxidative stress defence genes, such as *katA*, *fdxA*, *trxB*, *sodB* and *ahpC* (van Vliet *et al.*, 1999, Butcher *et al.*, 2012). However, it is not clear how this interaction between regulators occurs (Atack and Kelly, 2009). Fur-activated and Fur-inactivated forms have been shown to recognise two distinct consensus binding sequences. This finding has not been demonstrated in other bacteria (Butcher *et al.*, 2012).

1.15.2.3 *Campylobacter* oxidative stress regulator (CosR)

CosR has more recently been identified as a *Campylobacter* oxidative stress regulator belonging to OmpR family of transcriptional regulators (Hwang *et al.*, 2011). CosR is an essential protein for *C. jejuni*, as *cosR* mutants were not viable, indicating a lethal mutation (Hwang *et al.*, 2011). In order to investigate the CosR regulatory roles, antisense peptide nucleic acids were used to regulate *cosR* expression (Hwang *et al.*, 2011). Antisense regulation was used to knockdown or to overexpress *cosR* in *C. jejuni* 11168 (Hwang *et al.*, 2011). CosR regulates other essential genes, demonstrating important regulation of vital cell functions (Hwang *et al.*, 2012).

CosR was shown to be responsible for the negative regulation of the oxidative stress response proteins SodB, Dps, Rrc and LuxS and the positive regulation of AhpC and KatA (Hwang et al., 2011; Hwang et al., 2012). CosR regulates a number of genes involved in different mechanisms, such as energy production, transcription, protein synthesis, motility, secondary metabolite biosynthesis, and stress defence (Hwang *et al.*, 2012). Knockdown of *cosR* reduced the level of CosR in the *C. jejuni* cells, enhanced SodB activity, and increased resistance to both O_2^- and H_2O_2 (Hwang *et al.*, 2011). CosR knockdown in *C. jejuni* 11168 increases cellular motility (Hwang *et al.*, 2012). The CosR binding site does not overlap with the predicted PerR and Fur binding sites in the *katA* promoter, suggesting that CosR may not interfere with PerR and Fur binding to the *katA* promoter (Hwang *et al.*, 2012).

1.15.2.4 Carbon starvation regulator (CsrA)

CsrA is a post-transcriptional regulator shown to have a role in the oxidative stress resistance mechanism. A *C. jejuni* 81-176 *crsA* mutant was highly sensitive to atmospheric oxygen concentrations (Fields and Thompson, 2008). The same study also showed that the 81-176 *crsA* mutant has greater sensitivity to H_2O_2 (Fields and Thompson, 2008). CsrA is required for biofilm formation as the 81-176 *csrA* mutant exhibits decreased biofilm formation and also had reduced adhesion to and invasion of epithelial cells (Fields and Thompson, 2008).

1.15.2.5 Two-component regulatory systems

1.15.2.5.1 *Campylobacter* planktonic growth regulation (CprRS)

CprRS is a two-component regulatory system with a role controlling changes in physiology and metabolism involved in biofilm formation, stress tolerance, and colonisation (Svensson *et al.*, 2009). CprR is a response regulator and CprS is a sensor kinase that regulates CprR by phosphorylation (Svensson *et al.*, 2009). Mutation of *cprR* is lethal for bacterial survival, demonstrating that CprR is essential for *C. jejuni*, whilst a *cprS* mutant was viable (Svensson *et al.*, 2009). A *C. jejuni* 11168 *cprS* mutant displayed growth defects, enhanced biofilm formation, and bacterial aggregation (Svensson *et al.*, 2009). Mutation of *cprS* also reduced colonisation levels in one-day-old chicks (Svensson *et al.*, 2009). Oxidative stress proteins were up-regulated in the *C. jejuni* 11168 *cprS* mutant, such as KatA, TrxB and AhpC. However, the mutant exhibited increased sensitivity to paraquat and H_2O_2 (Svensson *et al.*, 2009).

1.15.2.5.2 Reduced ability to colonise (RacRS)

RacR is a two-component regulatory system that affects *C. jejuni* growth *in vitro* in a temperature-dependent manner (Bras *et al.*, 1999). RacR is a response regulator and RacS is a sensor kinase. A *C. jejuni* 81116 *racR* mutant entered stationary phase earlier than the wild-type strain. Furthermore, the *racR* mutant showed a more accentuated growth defect at 42ºC (Bras *et al.*, 1999). Mutation of *racR* also reduced the ability to colonise chickens (Bras *et al.*, 1999). RacR also affects expression of *Cj0358*, which encodes a CCP (Bras *et al.*, 1999). Both 81-176 *racR* and *racS* mutants showed great colonisation defects in chicks. However, both mutants showed only minor growth defects at 42ºC (Apel *et al.*, 2012). RacR regulates expression of the *racRS* operon and represses *dnaJ* (Apel *et al.*, 2012). Both *racR* and *racS* mutants reduced expression at 44ºC of *dnaJ*, *dnaK* and *groEL*, which encode heat shock response proteins (Apel *et al.*, 2012). Both mutants were less resistant to increased temperature of 44ºC (Apel *et al.*, 2012). However, no link with the oxidative stress response was suggested by Apel *et al.* (2012).

1.15.2.6 LuxS

Vibrio harveyi luxS is a gene belonging to a family of auto-inducer synthases responsible for production of the signal molecule auto-inducer 2 (AI-2) (Surette *et al.*, 1999). *V. harveyi luxS* regulates the bioluminescence using quorum sensing depending on AI-2 (Surette *et al.*, 1999).

LuxS has been shown to mediate quorum sensing communication, and to regulate acid and oxidative stress in *Streptococcus mutans* (Wen and Burne, 2004). LuxS also showed to have a role in the biofilm formation (Wen and Burne, 2004). In *P. aeruginosa*, quorum sensing contributes to regulation of oxidative stress response mechanism (Hassett *et al.*, 1999). Elvers and Park (2002) identified a *luxS* orthologue in the genome of NCTC 11168 strain. *C. jejuni* 11168 also demonstrated AI-2 activity (Cloak *et al.*, 2002, Elvers and Park, 2002).

A *C. jejuni* NCTC 11168 *luxS* mutant did not increase sensitivity to oxidative stress compared to the wild-type strain when first tested by Elvers and Park (2002). However, further analysis with a *C. jejuni* 81-176 *luxS* mutant demonstrated that the lack of *luxS* reduced growth under microaerobic conditions, and increased sensitivity to both H_2O_2 and cumene hydroperoxide compared to the wild-type (He *et al.*, 2008). 81-176 *luxS* mutant also demonstrated to stop the expression of AI-2 signal molecule (He *et al.*, 2008). LuxS is involved in the regulation of different cell aspects, such as cellular metabolism, flagella assembly, oxidative stress response, and efflux systems (He *et al.*, 2008). The expression of *ahpC* and *tpx* genes was down regulated in the 81-176 *luxS* mutant (He *et al.*, 2008).

1.15.2.7 Rubredoxin oxidoreductase / Rubrerythrin-like (rrc)

rrc encodes a protein homologous to the rubredoxin oxidoreductase/rubrerythrin protein in other bacteria, which have been shown to protect anaerobic microorganisms against oxidative stress (Yamasaki *et al.*, 2004). The presence of Rrc in *C. jejuni* cells decreased in the presence of H_2O_2 (Yamasaki *et al.*, 2004). *C. jejuni* grown under aerobic conditions also decreased the levels of Rrc (Yamasaki *et al.*, 2004). Rrc is sensitive to H_2O_2 as Rrc concentrations were rapidly decreased in presence of H_2O_2 and also under aerobic conditions (Yamasaki *et al.*, 2004). Although the exact function of Rrc is not known, it is suggested that Rrc has a regulatory role to control endogenous

oxidative stress (Yamasaki *et al.*, 2004).

1.15.2.8 RrpA and RrpB transcriptional regulators

Reannotation of *C. jejuni* NCTC 11168 genome sequence identified two genes (*Cj1546* and *Cj1556*) encoding putative transcriptional regulators that belonged to the multiple antibiotic resistance regulator (MarR) family of transcriptional regulators (Gundogdu *et al.*, 2007). Cj1546 and Cj1556 belong to the MarR family based on the presence of a Pfam motif PF01638 (Gundogdu *et al.*, 2007). PF01638 is a HxlR-like helix-turn-helix motif which includes proteins that play a role in regulating expression of genes involved in multiple antibiotic resistance phenotypes, oxidative stress, and production of pathogenic factors (Kumarevel, 2012).

The two MarR-type response regulators Cj1546 and Cj1556 have demonstrated to play a role in the peroxide stress resistance (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). We have thus designated Cj1546 and Cj1556 as RrpA and RrpB (regulator of response to peroxide) respectively. A *C. jejuni* 11168H *rrpB* mutant showed decreased ability to survive to aerobic and peroxide stress (Gundogdu *et al.*, 2011). RrpB was shown to be important in cell invasion as the 11168H *rrpB* mutant exhibited decreased capacity to invade and colonise human intestinal cells (Gundogdu *et al.*, 2011). Microarray analysis indicated that 73 genes were up-regulated and 18 genes were down-regulated in the 11168H *rrpB* mutant compared to the wild-type strain (Gundogdu *et al.*, 2011). Some oxidative stress genes were down-regulated, such as *ahpC*, *katA* and *perR*, whilst *sodB* was up-regulated (Gundogdu *et al.*, 2011). RrpB was the first MarR-type regulator characterised in *C. jejuni* (Gundogdu *et al.*, 2011). The second MarR transcriptional regulator RrpA showed 43.6% identity and 58.4% similarity to RrpB (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015).

1.16 Aims and objectives

The aim of this research project was to investigate the mechanisms of regulation of the *C. jejuni* oxidative stress responses, specifically the roles of the two recently identified MarR-like transcriptional regulators RrpA and RrpB. A further aim was to investigate genomic and phenotypic differences amongst *C. jejuni* strains isolated in Brazil.

Specific objectives:

1. Investigation into the role of RrpA and RrpB in the *C. jejuni* oxidative stress response.
2. Investigation into the role of RrpA and RrpB in biofilm formation.
3. Investigation of the distribution of *rrpA* and *rrpB* amongst *C. jejuni* strains.
4. Investigate the phenotypes of 43 Brazilian *C. jejuni* strains with respect to hydrogen peroxide stress and biofilm formation.
5. Perform whole genome sequencing for the 43 Brazilian *C. jejuni* strains.
6. Perform bioinformatics analysis on the 43 Brazilian *C. jejuni* strains with respect to important virulence genes and the distribution of *rrpA* and *rrpB*.

2 Material and Methods

2.1 Bacterial strains

The *C. jejuni* strains used in this study are described in Appendix 1.

2.2 Growth conditions

C. jejuni strains were grown on blood agar (BA) plates (Sigma-Aldrich, Poole, UK) containing 7% (v/v) of horse blood (TCS Biosciences, Botolph Claydon, UK) and *Campylobacter* Selective Supplement (Skirrow, Oxoid, Basingstoke, UK). Strains were resuscitated from glycerol stocks stored at -80°C. Vials were thawed on ice, the contents transferred to BA plates, and incubated for 48 h at 37ºC under microaerobic conditions (85% Nitrogen, 10% Carbon Dioxide and 5% Oxygen) in a Variable Atmospheric Incubator (VAIN) (Don Whitley Scientific, Shipley, UK).

Strains were re-streaked every 72-96 h on fresh BA plates. A maximum of 10 passages were performed before a new line was resuscitated. Bacteria were re-streaked onto BA plates and grown for 24 h to be used in assays. Mutants were grown on BA plates supplemented with kanamycin (50 µg/ml) or chloramphenicol (10 µg/ml) as required (Sigma-Aldrich).

Assays were performed from 24 h BA plates or from broth cultures. Broth cultures were prepared in 25 cm^2 tissue culture flasks (Thermo Scientific, Massachusetts, USA) using either 10 ml Brucella broth (Sigma-Aldrich) or 10 ml Mueller Hinton broth (Oxoid) inoculated with a bacterial suspension (see Section 2.3) prepared from a 24 h BA plate to a starting OD_{600} of 0.1. Broths were grown for 16 h with shaking at 75 rpm on a shaker (Platform Shaker STR6, VWR-Jencons, East Grinstead, UK) at 37ºC under microaerobic conditions.

Glycerol stocks were prepared from 24 h BA plates. Cells were harvested and re-suspended in 10% (v/v) glycerol, 10% (v/v) foetal calf serum in Brucella broth. Aliquots (50 µl) of this suspension were transferred to 0.6 ml tubes (Starlab, Hamburg, Germany) and snap-frozen using dry-ice in 100% (v/v) ethanol. Tubes were stored at -80°C.

2.3 Preparation of a bacterial cell suspension at a specific OD_{600}

Bacterial cells from a 24 h BA plate were re-suspended in 1 ml Phosphate-buffered saline (PBS) (Sigma-Aldrich). 100 µl of this suspension was diluted in 900 µl PBS. This volume was transferred to a cuvette (Fisher Scientific, Loughborough, UK), and the OD_{600} was recorded using a spectrophotometer (S200UV/Vis Spectrophotometer, VWR-Jancons, Leighton Buzzard, UK). 1 ml PBS was used as blank. The OD_{600} obtained was used in the following dilution formula:

OD_{600} (Initial) x **Volume** (Initial) = **OD_{600}** (Final) x **Volume** (Final)

2.4 Assays

2.4.1 Motility assays

C. jejuni strains were grown on BA plates for 24 h. Colonies were re-suspended in 1 ml PBS and adjusted to an OD_{600} of 1.0. Then 5 µl of this suspension was inoculated into the centre of a motility agar plate using a P10 Gilson pipette (Anachem Ltd, Luton, UK). Motility agar consisted of Brucella broth supplemented with 0.4% (w/v) bacteriological agar (Oxoid). Plates were incubated at 37ºC under microaerobic conditions without inverting. The diameter of the motility ring was measured at 24, 48 and 72 h and images were recorded using a GeneGenius Bio Imaging System (Syngene, Cambridge, UK).

2.4.2 Protein quantification

The quantification of protein concentration was performed using a BCA Protein Assay Kit (Thermo Scientific, Rockford, USA). This kit assesses the total protein concentration compared to a protein standard. The reagents in the kit were mixed following the manufacture's guidelines, which reduces the cupric ion (Cu^{2+}) to

cuprous ion (Cu^+) in an alkaline medium by bicinchoninic acid (BCA). This reaction is called the biuret reaction, which produces a blue colour. In the second step, the two BCA molecules are chelated with a Cu^+ ion, which results in an intense purple colour with absorbance at 562 nm.

The protein standard utilised was bovine serum albumin (BSA) prepared in different concentrations in PBS to generate a standard curve with concentrations ranging from 25 to 2000 µg/ml. The average OD_{562} reading of the blank replicates was subtracted from all other individual standards and samples. The average OD_{562} reading for each concentration replicate of the BSA standards was plotted against concentration in µg/ml. The standard curve generated was used to determine the protein concentration of the samples. Samples were then diluted with PBS to the desired concentration before further assays were performed.

2.4.3 Outer membrane vesicle (OMV) isolation

C. jejuni strains were grown on BA plates for 24 h under microaerobic conditions. Brucella broth (50 ml) was pre-incubated in 150 cm^2 tissue culture flasks (Corning Incorporated, New York, USA) at 37ºC with shaking at 75 rpm under microaerobic conditions overnight. For each strain, two flasks were prepared. Plate colonies were re-suspended in 1 ml PBS, and the OD_{600} was recorded. The pre-incubated Brucella broth was inoculated to an initial OD_{600} of 0.1 and incubated at 37ºC with shaking at 75 rpm under microaerobic conditions. Bacterial cultures were grown for 16 h, up to mid-log to late-log phase. Cultures were transferred to 50 ml tubes (Corning) and centrifuged using a Centrifuge 5810 R (Eppendorf, Stevenage, UK) at 4,000 rpm for 30 min at 4ºC. The supernatant was then filtered through a 0.22 µm syringe filter (Merk Millipore Ltd, Tullagreen, Ireland). Sterile supernatants were transferred to an Amicon Ultra-15 centrifugal filter unit (Merck Millipore Ltd) with a 10 kDa cut off and centrifuged at 4,000 rpm for 30 min at 4ºC. This step was repeated until the whole supernatant was filtered. The concentrated samples were transferred to ultra clear centrifuge tubes (Beckman Coulter, Brea, USA) and ultra-centrifuged at 45,000 rpm for 3 h at 4°C using a TLS 55 rotor in an Optima TL Ultracentrifuge (Beckman Instruments, Palo Alto, USA). The supernatant was discarded and the pellet re-suspended in 200 µl of sterile PBS. Aliquots of 50 µl were prepared and stored at -

20ºC. One aliquot of each sample was diluted 1:10 and the protein concentration was quantified (see Section 2.4.2).

2.4.4 Preparation of Whole Cell lysates

2.4.4.1 Blood agar plates

Strains were harvested from 24 h BA plates and re-suspended in 1 ml PBS in 1.5 ml tubes (Starlab). The suspensions were placed on ice in a Bioruptor® sonicator (Diagenone, Seraing, Belgium). Samples were sonicated at 60 kHz for 30 seconds with 30 seconds intervals for 15 min. Samples were then centrifuged at 13,000 rpm for 15 min at 4°C. Supernatants were transferred to new 1.5 ml tubes and stored on ice. Whole cell lysate concentrations were quantified (see Section 2.4.2).

2.4.4.2 Minimum Essential Media alpha (MEMα)

Strains were grown on BA plates for 24 h. Two 25 cm^2 tissue culture flasks containing 10 ml of MEMα were pre-incubated overnight at 37ºC, shaking at 75 rpm under microaerobic conditions. Colonies were re-suspended in 1 ml PBS and broths were inoculated to an OD_{600} of 0.05. Flasks were incubated for 48 h at 37°C, with shaking at 75 rpm under microaerobic conditions. After this incubation period, 20 ml of each sample were centrifuged in a 50 ml flask (Corning). Pellets were re-suspended in 1 ml PBS in a microcentrifuge tube and sonicated as described in Section 2.4.4.1. Supernatants were removed and stored on ice. Whole cell lysate concentrations were quantified (see Section 2.4.2).

2.4.5 Growth curves

Strains were grown for 24 h on BA plates. 25 cm^2 tissue culture flasks containing 10 ml Brucella broth were pre-incubated at 37ºC under microaerobic conditions with shaking at 75 rpm. A bacterial suspension was prepared in 1 ml PBS. The Brucella broth in the flasks was inoculated to an initial OD_{600} of 0.1. Flasks were re-incubated at 37ºC with shaking at 75 rpm under microaerobic conditions. OD_{600} readings were

performed at 0, 3, 6, 9, 16 and 24 h. At each time point, an aliquot was taken for serial dilutions (10^{-1} to 10^{-6}) so colony forming units (CFUs) could be counted. 10 µl of each dilution was plated on BA plates in duplicate and incubated at 37ºC for 48 h under microaerobic conditions.

Growth curves were also performed in presence of sodium taurocholate (ST). ST was prepared at a concentration of 10% (w/v) (200 mM) in MilliQ water and filter sterilised using a 0.22 µm syringe filter. ST was added to broths to a final concentration of 0.1% (w/v) (2 mM) or 0.2% (w/v) (4 mM) and incubated for further 15 min at 37ºC under microaerobic conditions before inoculating with bacterial suspension.

2.4.6 Oxidative stress assays

C. jejuni strains were grown for 24 h on BA plates, then cells were harvested and re-suspended in 1 ml PBS. The OD_{600} of each bacterial suspension was recorded and adjusted to an OD_{600} of 1.0. This concentration of bacterial cells was prepared in 1 ml for the control samples and in 900 µl for the test samples. Calculations were made using the dilution calculation formula (see Section 2.3).

Bacterial suspensions were exposed to three different types of oxidative stress; hydrogen peroxide (H_2O_2, Sigma-Aldrich), cumene hydroperoxide (Sigma-Aldrich), and menadione (Sigma-Aldrich). During the oxidative stress assays, cell suspensions were incubated at 37°C under microaerophilic conditions. As a positive control, the strains were also incubated at 37°C under microaerophilic conditions without exposure to any oxidative stress.

Strains were also grown in presence of ST prior to the oxidative stress assay. A solution of 10% ST was prepared, sterilised with 0.22 µm syringe filter, then added to BA plates to a final concentration 0.1 % (w/v) or 0.2 % (w/v). Strains were grown on BA plates containing either 0.1% or 0.2% ST for 24 h. Bacterial cells were re-suspended in 1 ml PBS and the OD_{600} adjusted to 1. Bacterial suspensions were then exposed to H_2O_2 stress as described below in Section 2.4.6.1.

2.4.6.1 Hydrogen peroxide stress assays

1 M H_2O_2 was used to prepare the following concentrations: 250, 500 and 1000 mM. 100 µl of one of these concentrations was added to 900 µl of bacterial suspension, giving a final concentration of 25, 50 or 100 mM H_2O_2, respectively. Bacterial suspensions were also tested using a final concentration of 200 mM H_2O_2, where 200 µl of the 1 M H_2O_2 was added to 800 µl of each bacterial suspension. Bacterial suspensions were exposed to different H_2O_2 concentrations at 37°C for 15 min under microaerobic conditions. Serial dilutions were prepared (10^{-1} to 10^{-6}), then 10 µl of each dilution was pipetted onto a BA plate in duplicate and incubated for 48 h. CFUs were counted.

2.4.6.2 Cumene hydroperoxide stress assays

Cumene hydroperoxide was diluted to a concentration of 0.5% (v/v) and 100 µl added to 900 µl of bacterial suspension. The final concentration of cumene hydroperoxide was 0.05% (v/v). Bacterial suspensions were incubated at 37°C for 15 min under microaerobic conditions. Serial dilutions were prepared (10^{-1} to 10^{-6}), then 10 µl of each dilution was pipetted onto a BA plate in duplicate and incubated for 48 h. CFUs were counted.

2.4.6.3 Menadione stress assays

Menadione was prepared to a 1 M concentration, then 100 µl was added to 900 µl of bacterial suspension. Bacterial suspensions were exposed to a final concentration of 100 mM menadione. Bacterial suspensions were incubated at 37°C for 1 h under microaerobic conditions. Serial dilutions were prepared (10^{-1} to 10^{-6}), then 10 µl of each dilution was pipetted onto a BA plate in duplicate and incubated for 48 h. CFUs were counted.

2.4.7 Catalase activity assays

Catalase activities were quantified using a Catalase Activity Assay Kit (Sigma-Aldrich). This kit quantifies the antioxidant activity of the catalase enzyme through a colourimetric assay. Whole cell lysates were prepared as described in Section 2.4.4. All samples were normalised to a final concentration of 100 ng/µl protein.

The color chromogen reagent containing 0.25 mM 4-aminoantipiryne and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid was re-suspended in 150 ml of assay buffer (50 mM potassium phosphate buffer, pH 7.0). This solution was prepared, aliquoted and stored at -20°C. A solution of peroxidase (0.69 mg/ml) was freshly prepared. Then 20 ml color chromogen reagent was thawed before use and mixed with 20 µl of peroxidase solution.

The compound 3,5-dichloro-2-hydroxybenzenesulfonic acid present in the color reagent oxidises 4-aminoantipyrine in the presence of H_2O_2 and horseradish peroxide producing a red quinoneimine dye (N-(4-antipyryl)-3-chloro-5-sulfonate-*p*-benzoquinone-monoimine). The red quinoneimine dye was detected using a SpectraMax M3 microplate reader (Molecular Devices, Sunnyvale, USA) at an absorbance of 520 nm.

A 3% (w/v) solution of H_2O_2 was provided with the kit. However, the assay requires the concentration of H_2O_2 to be exactly 200 mM. Therefore, the H_2O_2 concentration was quantified using Beer's Law ($\varepsilon^{mM} = 0.0436$). The H_2O_2 solution was diluted 100 fold and the absorbance at 240 nm recorded, the reading was applied to the Beer's Law formula and the concentration corrected to 200 mM using the assay buffer.

Beer's Law:

$$\text{mM } H_2O_2 = \frac{A_{240}}{0.0436}$$

A H_2O_2 solution of 10 mM was also prepared to obtain a standard curve of H_2O_2 concentration against the absorbance of the red quinoneimine dye. The 10 mM H_2O_2 solution was diluted to the following concentrations in mM: 0, 0.0125, 0.025, 0.05 and

0.075. The readings of the known H_2O_2 concentrations were plotted as a standard curve and used to calculate the H_2O_2 concentration from the sample readings.

Catalase assay reactions were set up as follows:

Sample

10 µl	Whole cell lysate (100 ng/µl)
65 µl	Assay Buffer
25 µl	200 mM H_2O_2 solution

Blank

75 µl	Assay Buffer
25 µl	200 mM H_2O_2 solution

All reactions were incubated at room temperature for 1 min. The reaction was then stopped with 900 µl stop solution (15 mM sodium azide). A 10 µl aliquot of each reaction was transferred to a new tube, 1 ml of the color reagent was added, then incubated at room temperature for 15 min to allow colour development. The reactions were then transferred to cuvettes and the absorbance recorded at 520 nm. The absorbance readings were used with the following formula to obtain the H_2O_2 concentrations.

Calculation:

$$\text{Catalase Activity} = \frac{\triangle \mu\text{mol } (H_2O_2) \text{ x } 100}{V \text{ x } t} \times 10$$

$\triangle$ µmol of H_2O_2 = µmol of H_2O_2 in Blank - µmol of H_2O_2 in Sample

t = catalase reaction duration in min

V = Sample volume in ml

100 = dilution of aliquot from catalase reaction in colorimetric reaction

10 = convert ng to µg (Total amount of protein used in each reaction was 100 ng/µl. Values were multiplied by 10 so results are presented as H_2O_2/min/µg)

Catalase activity is presented as µmol H_2O_2/min/µg. One unit of catalase activity is defined as 1 µmol of H_2O_2 decomposed to water and hydrogen per minute at 25°C and at pH 7.0.

2.4.8 Superoxide dismutase activity assay

Superoxide dismutase (SOD) activities were quantified using a Superoxide Dismutase Assay kit (Sigma-Aldrich). SOD breaks downs the superoxide anion (O_2^-) into H_2O_2 and molecular oxygen. This kit utilises Dojindo's highly water-soluble 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1). Dojindo's WST-1 has sulfonate groups added to the phenyl ring to improve water solubility. When WTS-1 is reduced by a O_2^-, this generates a water-soluble formazan dye, which absorbs at 450 nm. SOD removes O_2^- inhibiting the formazan dye formation. SOD activity represents the percentage of inhibition rate of the formazan dye.

For assay monitoring, a WST-1 inhibition curve was generated with different concentrations of SOD. SOD was diluted with dilution buffer to the following concentrations: 200 U/ml, 100 U/ml, 50 U/ml, 20 U/ml, 10 U/ml, 5 U/ml, 1 U/ml, 0.1 U/ml, 0.05 U/ml, 0.01 U/ml, 0.001 U/ml.

Strains were grown on BA plates for 24 h and whole cell lysates prepared as described previously (see Section 2.4.4). Whole cell lysates were normalised at a concentration of 100 ng/µl. For each assay, 20 µl of whole cell lysate was used.

Assay reaction:

Sample

20 µl	Whole cell lysate (100 ng/µl)
200 µl	WST-1 working solution
20 µl	Enzyme working solution

Blank 1

20 µl	ddH_2O
200 µl	WST-1 working solution
20 µl	Enzyme working solution

Blank 2

20 µl	Whole cell lysate (100 ng/µl)
200 µl	WST-1 working solution
20 µl	Dilution buffer

Blank 3

20 µl	ddH_2O
200 µl	WST-1 working solution
20 µl	Dilution buffer

Reactions were prepared in 96 well plates (Corning), then incubated at 37°C for 20 min. Absorbance was measured at 450 nm using a SpectraMax M3 microplate reader.

Calculation:

$$\text{SOD activity} = \left\{ \frac{[\,(A_{450}\text{blank 1} - A_{450}\text{blank 3}) - (A_{450}\text{sample} - A_{450}\text{blank 2})]}{(A_{450}\text{blank 1} - A_{450}\text{blank 3})} \right\} \times 100$$

2.4.9 Biofilm formation

Strains were re-streaked on BA plates and grown for 24 h at 37ºC under microaerobic conditions. 10 ml Mueller Hinton broth was pre-incubated in a 25 cm^2 tissue culture flask at 37ºC with shaking at 75 rpm under microaerobic conditions overnight. Bacterial cells were re-suspended in 1 ml PBS and the OD_{600} was measured. Mueller Hinton broths were inoculated to a final OD_{600} of 0.1. Broths were incubated for 5 h at 37ºC with shaking at 75 rpm under microaerobic conditions. The OD_{600} was readjusted to 0.1 with fresh Mueller Hinton broth and 1 ml of this suspension was added to each well in a 24 well plate (Corning). The plates were incubated at stationary at 37ºC for 72 h under either aerobic or microaerobic conditions. The plates were washed twice with PBS, dried at 37ºC for 20 min, then stained with 1.2 ml of 1% (w/v) crystal violet (Sigma-Aldrich) for 15 min. Wells were then washed three times with PBS followed by addition of 1 ml destaining buffer containing 10% (v/v) acetic acid / 30% (v/v) methanol. Plates were placed in a shaker at 400 rpm for 15 min for destaining. Absorbance was measured at 595 nm using a SpectraMax M3 microplate reader.

2.4.10 Biofilm formation in the presence of chicken juice

2.4.10.1 Chicken juice preparation

Chicken juice biofilm assays were performed based on the methodology of Brown *et al.* (2014). Frozen chicken pieces were defrosted at room temperature. Chicken juice was obtained from the exudate released from defrosted chicken pieces. The exudate was centrifuged to remove debris at 4,000 rpm for 20 min at 4ºC. The supernatant was

diluted with sterile water 1:1, then filter sterilised using a 0.22 µm syringe filter. 1 ml aliquots were prepared and stored at -20°C. This chicken juice is referred as undiluted chicken juice. Stored chicken juice was thawed on ice prior to use.

2.4.10.2 Biofilm formation with diluted chicken juice

Strains were re-streaked on BA plates and prepared as described in Section 2.4.9. After the 5 h growth in Mueller Hinton broth under microaerobic conditions, the OD_{600} was readjusted to 0.1 with fresh Mueller Hinton broth. Then 1 ml of this suspension was added to each well of a 24 well plate with four replicates for each strain. Chicken juice was diluted with sterile MilliQ water 1:100 and 5% (v/v) of diluted chicken juice was added to each well. Plates were incubated at 37ºC for 72 h under either aerobic or microaerobic conditions. Wells were washed, stained with crystal violet and destained as described in Section 2.4.9. Absorbance was measures at 595 nm using a SpectraMax M3 microplate reader.

2.4.10.3 Biofilm formation with undiluted chicken juice

Strains were re-streaked on BA plates and prepared as described in Section 2.4.9. After the 5 h growth in Mueller Hinton broth under microaerobic conditions, the OD_{600} was readjusted to 0.1 with fresh Mueller Hinton broth. Then 1 ml of this suspension was added to each well of a 24 well plate with four replicates for each strain. 5% (v/v) of undiluted chicken juice was added to each well. The plates were incubated at 37ºC for 72 h under either aerobic or microaerobic conditions. After the incubation period, wells were washed 3 times with sterile PBS. Brucella broth was supplemented with 0.05% (v/v) 2,3,5-triphenyltetrazolium chloride solution (TTC) (Sigma-Aldrich) (Brown *et al.*, 2014), then 1.2 ml was added to each well and the plates were re-incubated at 37ºC for 72 h under microaerobic conditions. TTC can differentiate between metabolically active and inactive cells. Active cells reduce TTC to a red compound 1,3,5-triphenylformazan (TPF) due to enzymatic activity associated with cellular metabolism. After incubation, the plates were washed twice with PBS followed by the addition of 1 ml elution buffer containing 20% (v/v) acetone and 80% (v/v) ethanol to each well. Plates were placed in a shaker at 400 rpm for 15 min at

room temperature for homogenisation. Absorbance was measured at 500 nm using a SpectraMax M3 microplate reader.

2.4.11 *Galleria mellonella* haemolymph collection

G. mellonella larvae were obtained from TruLarv (Biosystems Technology, Exeter, UK) kept at room temperature. Strains were grown on BA plates for 24 h. Bacterial cells were re-suspended in 1 ml PBS and the OD_{600} adjusted to 0.1. Larvae were injected with 10 µl of the bacterial suspension in the right foremost leg using a micro-injection syringe (Hamilton, Bonaduz, Switzerland) and incubated at 37ºC for 16 h. After incubation, the larvae were chilled on ice for 15 min. The larval surface was cleaned with 70% (v/v) ethanol using a tissue. A small cut was made at the side of the body with sterilised scissors and the haemolymph drained into a sterile microcentrifuge tube. 10 µl of the heamolymph was then serially diluted (10^{-1} to 10^{-6}). 10 µl of each dilution were plated on BA plates. Plates were incubated for 48 h at 37ºC under microaerobic conditions then CFUs were counted.

2.5 Molecular methods

2.5.1 DNA isolation

C. jejuni strains were grown on BA plates for 24 h and genomic DNA was isolated using a PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, USA). Briefly, a loopful of bacterial cells was re-suspended in 1 ml PBS in a 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm for 1 min. The supernatant was discarded and the pellet re-suspended in 180 µl PureLink Genomic Digestion Buffer. 20 µl of Proteinase K (20 mg/µl) was added to lyse the cells and vortexed. Tubes were incubated in a heat block at 55ºC for 30 min with occasional further vortexing. Then 20 µl RNase A (20 mg/µl) was added, vortexed and incubated at room temperature for 2 min. 200 µl PureLink Genomic Lysis/Binding buffer was added and vortexed. 200 µl of absolute ethanol was added to the lysate and vortexed for 5 sec to yield a homogenous solution. The lysate solution was added to a PureLink spin column and centrifuged at 10,000 rpm for 1 min. The column was then placed in a new collection tube. 500 µl of Wash Buffer 1 was added to the column and centrifuged at 10,000 rpm for 1 min. The column

was then placed in another new collection tube. 500 µl of Wash Buffer 2 was added to the column and centrifuged at 13,000 rpm for 3 min. The spin column was then placed in a new 1.5 ml microcentrifuge tube. 50 µl of MilliQ water was added to the centre of the column membrane, incubated at room temperature for 1 min then centrifuged at 13,000 rpm for 90 seconds. DNA concentrations were quantified using a NanoDrop (see Section 2.5.2.1). Genomic DNA samples were stored at -20ºC.

2.5.2 DNA quantification

2.5.2.1 NanoDrop ND-1000 spectrophotometer

Quantification of genomic DNA was performed using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA). Quantity and quality of the isolated DNA was assessed based on Ultra Violet (UV) absorbance. Nucleic acids absorb UV light in a specific pattern when exposed to 260 nm wavelength. The light that passes through the sample is measured and calculation of the concentration is made using the Beer-Lambert Law. The formula is based on the average extinction coefficient for double-stranded DNA (0.020 µg ml^{-1} cm^{-1}) that is automatically performed by the NanoDrop. The DNA quality is evaluated based on the ratio between the absorbance readings at 260 nm and 280 nm, which indicates the sample purity. A ratio of 1.8 is considered as good quality DNA. Lower ratio indicates the presence of proteins or other contaminants that absorb at 280 nm.

Quantification of RNA was also performed using the NanoDrop. The calculation formula using the Beer-Lambert Law is based on the average extinction coefficient for single-stranded RNA (0.025 µg ml^{-1} cm^{-1}). Quality of RNA was also calculated based on the ratio between the absorbance readings at 260 nm and 280 nm. A ratio of 2.0 is considered as good quality RNA.

The NanoDrop was blanked with MilliQ water, after this 1.5 µl of each DNA sample was measured. Each sample was pipetted onto the NanoDrop pedestal, which contains one end of a fibre optic cable. The arm was closed on top of the pedestal, which contains the other end of the fibre optic cable. The liquid sample makes contact with both ends of the fibre optical cables. The pedestal automatically adjusts the optimal

path length and a pulse of light passes through the sample. Readings were displayed in a computer screen and recorded.

2.5.2.2 Qubit

The Qubit dsDNA HS Assay kit (Life Technologies, Carlsbad, USA) uses a fluorometer that is highly selective for double-stranded DNA and quantifies the DNA accurately. The fluorescent dye only emits a signal when bound to double-stranded DNA. Qubit generates a concentration curve based on the two standards of known concentration (0 ng/µl and 10 ng/µl). Sample fluorescence readings are plotted on the concentration curve and values displayed on the Qubit screen. Qubit reagent was diluted in Qubit buffer (1:200) to prepare the working solution. 190 µl of working solution was mixed with 10 µl of standards and 198 µl of working solution was mixed with 2 µl of each DNA sample. Tubes were mixed and incubated for 2 min at room temperature. Tubes were inserted into the Qubit 2.0 fluorometer and readings were displayed on the Qubit screen.

2.5.3 DNA quality control

DNA quality was analysed using a BioAnalyzer High Sensitivity DNA Kit (Agilent Technologies, Wokingham, UK) according to the manufacturer's instructions. Briefly, 15 µl High Sensitivity DNA dye concentrate was mixed with High Sensitivity DNA gel matrix and vortexed for 10 sec. The solution was transferred to a spin filter column and centrifuged for 10 min at 6,000 rpm. 9 µl of the gel-dye mix was loaded onto a new high sensitivity DNA chip. The chip was inserted into the chip priming station and closed. A 1 ml syringe filled with air was depressed down to the lowest position of the plunger, held there for 60 sec, the plunger was released for 5 sec and then slowly pulled back to the 1 ml position. The chip was removed from the priming station and 9 µl of the gel-dye mix was pipetted into each well marked as G. 5 µl High Sensitivity DNA marker was pipetted into each sample well and also into the ladder well. 1 µl High Sensitivity DNA ladder was then pipetted into the ladder well. 1 µl of each DNA sample was pipetted into one sample well. An extra 1 µl High Sensitivity DNA marker

was pipetted into each unused sample well. The DNA chip was placed in the IKA vortex mixer at 2,400 rpm for 1 min. The DNA chip was then inserted in the Agilent BioAnalyzer. The 2100 Expert software was selected for high sensitivity DNA analysis.

2.5.4 Primers

Oligonucleotide primers were designed based on the *C. jejuni* NCTC 11168 genome sequence. Primers were checked for self-dimers and hairpin loops using Oligoanalyzer 3.1 software on the Integrated DNA Technologies website (http://eu.idtdna.com/analyzer/applications/oligoanalyzer/).

Primers were supplied by Invitrogen and reconstituted with sterile MilliQ water to 100 pmol/µl to produce 100 µM stock solutions. Concentrations of 15 pmol/µl of each primer were used in PCR reactions.

Primers utilised for quantitative real time PCR (qPCR) were further diluted to 10 pmol/µl to produce 10 µM stock solutions. Primers used in this study are described in Table 2.1.

Table 2.1 Oligonucleotide primers used in this study

Primer	**Sequence**
rrpA – Forward degenerate	CCNCCNAARGTNGARTAC
rrpA – Reverse degenerate	GTAYTCNACYTTNGGNGG
rrpB – Forward degenerate	TGYCCNATHGARACNACN
rrpB – Reverse degenerate	GTAYTCNACYTTNGGNGG
cosR – Forward degenerate	GTNATHGARGAYGARATH
cosR – Reverse degenerate	YTTYTTNGGRAARCARAA
rrpB – Forward specific	AGATTTGGAGAGTTAAG
rrpB – Reverse specific	CTAAAGATGTAAGTGAGTAC
perR – Forward specific	CATGAGTTAAAAGCTACTCCGC
perR – Reverse specific	GCAATTATCAACATAAGCAC
chl^R – Forward out	CGATTGATGATCGTTGTA
sodB – Forward specific	TACCTTATGATACCAATGC
sodB – Reverse specific	CAGGGTGAAGTTCATTTGC
ahpC – Forward specific	CCAGCGGTATTAGGAAAC

ahpC – Reverse specific	CAAGATATTCAGCCACGCC
gyrA – Forward specific	GGTCGTTATCACCCACATGGAG
gyrA – Reverse specific	CCTACAGCTATACCAC
gyrA qPCR – Forward	CGCACAGGGCGTGGTCGCGTG
gyrA qPCR – Reverse	ACACGGCCGATTTCACGCAC
katA qPCR – Forward	GTAGCAGGTGAAGCAGGTGC
katA qPCR – Reverse	CCTACCAAGTCCCAGTTTCC

2.5.5 Polymerase Chain Reaction

Each reaction was prepared as follows:

Forward primer (100 pmol/µl)	0.15 µl
Reverse primer (100 pmol/µl)	0.15 µl
Genomic DNA (10 – 100 ng/µl)	1 µl
MyTaq Red DNA Polymerase (Bioline, London, UK)	24 µl

Tubes were inserted into a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad, Hemel Hempstead, UK) and PCR performed using the following program:

Standard PCR program:

Step 1: Denaturing at 94°C for 15 sec
Step 2: Annealing at 50°C for 1 min
Step 3: Extension at 72°C for 1 min
} 34 cycles

Step 4: Final extension at 72°C for 7 min

2.5.6 Analysis of PCR products using agarose gel electrophoresis

PCR reactions used MyTaq Red DNA Polymerase which contains a buffer with a red dye for direct gel loading (without the need to add loading buffer). Amplified PCR products were analysed on a 0.7% (w/v) agarose gel in 1X Tris-acetate-EDTA (TAE) electrophoresis buffer supplemented with 0.5 µg/ml ethidium bromide (Fisher Scientific). Briefly, 10 µl amplified PCR products were loaded into wells on the agarose gel. 5 µl of Hyperladder 1 kb marker was loaded into the first well of each gel. Gel electrophoresis was performed in a AGE electrophoresis tank (Bio-Rad) at 120 V for 40 min in 1X TAE buffer. PCR products were visualised and images recorded using a GeneGenius Bio Imaging System.

2.5.7 Design of degenerate oligonucleotide primers for screening *rrpA*, *rrpB* and *cosR* genes

RrpA, RrpB and CosR are recently identified regulators of the *C. jejuni* oxidative stress response. Degenerate primers were designed to amplify each of these genes based on multiple amino acid alignments of RrpA, RrpB and CosR.

The NCTC 11168 genome sequence was used with the Basic Local Alignment Search Tool (BLAST) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) in order to perform gene comparisons with all other *C. jejuni* genomes already sequenced in the database. This website allows searching for regions of local similarity within sequences from different strains and calculates the statistical significance of matches.

Gene and amino acid sequences from different *C. jejuni* strains were copied into a

Microsoft Word document. These files were used to perform alignments using the ClustalW2 website (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Nucleotide alignments were performed as well as amino acid alignments. Amino acid alignments proved to be the best option when searching for areas of patch homology (Appendix 2). Patch homology regions with a minimum of 6 amino acids were selected for each gene and degenerate primers were designed. Primers were checked for self-dimers and hairpin loops. Different PCR programs were used for each set of primers (Table 2.2).

Table 2.2 PCR programs used for *rrpA*, *rrpB* and *cosR* degenerate primers.

	cosR	***rrpA***	***rrpB***
Step 1: Denaturing	94°C for 15 sec	94°C for 15 sec	94°C for 15 sec
Step 2: Annealing	50ºC for 1 min	50ºC for 1 min	50ºC for 1 min
Step 3: Extension	72°C for 1 min	72°C for 30 sec	72°C for 1 min
Number of cycles	34	34	25
Step 4: Final extension	72°C for 7 min	72°C for 7 min	72°C for 7 min

2.5.8 Construction of a *C. jejuni* 11168H *rrpB perR* double mutant

rrpB gene was PCR amplified, purified and then ligated into a plasmid pGEM-T Easy vector. *rrpB* gene was digested with a restriction enzyme to allow the insertion of a chloramphenicol (chl^R) cassette. This construct was obtained from Gundogdu *et al.* (2011) study, where it was used for the generation of 11168H *rrpAB* double mutant.

The 11168H *perR* mutant was grown on a BA plate for 24 h. Colonies were re-suspended in 10 ml ice cold EBF buffer. EBF buffer consists of 1% (w/v) sucrose and 6% (v/v) glycerol in MilliQ water and sterilised by filtration using 0.22 µm syringe

filter. The cell suspension was centrifuged for 10 min at 4,000 rpm and the supernatant was discarded. The pellet was re-suspended in 1 ml ice cold EBF buffer, transferred to a 1.5 ml microcentrifuge tube and centrifuged for 2 min at 13,000 rpm. This step was repeated and the pellet then re-suspended in 250 µl of ice cold EBF. 50 µl of this cell suspension was transferred to a new microcentrifuge tube and 5 µl of plasmid (1 – 5 µg) containing the disrupted *rrpB* gene was added to the cells then mixed by pipetting. This mixture was incubated on ice for 10 min and then transferred to a cold electroporation 2 mm gap cuvette (Bio-Rad). Electroporation was performed using a GenePulser Xcell (Bio-Rad) with the following settings: 2.5 kV, 25 µFD and 200 Ω. Immediately after the electroporation, 100 µl SOC Medium (Invitrogen) at room temperature was added to the cells to aid bacterial recovery. Bacterial cells were plated onto BA plates and incubated at 37ºC under microaerobic conditions for 2 days. Colonies were then harvested and re-suspended in 500 µl PBS. Aliquots of 100 µl or 200 µl were plated on BA plates containing 50 µl/ml kanamycin and 10 µl/ml chloramphenicol. Plates were incubated at 37ºC under microaerobic conditions for up to 5 days. Single colonies were re-streaked on fresh BA kanamycin/chloramphenicol plates. A loop of each re-streaked colony was re-suspended in 100 µl MilliQ water in a 0.6 µl microcentrifuge tube, vortexed and incubated at 95ºC for 10 min to lyse the bacterial cells, then centrifuged at 13,000 rpm for 5 min. Boilate supernatants were used for PCR screening using *rrpB* gene specific primers. *rrpB* gene specific forward and chl^R forward-out primers were also used to determine the orientation of the chl^R cassette within the cloned *rrpB* gene. Glycerol stocks were prepared as described in Section 2.2.

2.5.9 Genomic analysis at the Institute of Food Research

A total of 4,232 *Campylobacter* genome sequences (3,746 *C. jejuni* and 486 *C. coli*) were obtained from Genbank (www.ncbi.nlm.nih.gov) and from the *Campylobacter* pubMLST database (pubmlst.org/Campylobacter). The multilocus sequence type (MSLT) and clonal complex (CC) were determined using the scheme provided by the *Campylobacter* pubMLST website.

The genomes were phylogenetically clustered using FFPry feature frequency profiling with a word length of 18 (van Vliet and Kusters, 2015), which compares the

occurrence of purine/pyrimidine words in each separate genome sequence, and subsequently converts the information into a distance table followed by construction of a tree using the Neighbour-Joining method. Genomes were provisionally annotated using Prokka (Seemann, 2014) and searched for the presence of RrpA and RrpB using BLAST. Presence of the corresponding genes was also assessed at the DNA level using BLASTN+ and the MIST program (Kruczkiewicz *et al.*, 2013) and BLAST+ (v2.28). Both DNA and amino acid comparisons were performed using the *rrpA* and RrpA sequences from *C. jejuni* NCTC 11168, *C. jejuni* 81116, *C. jejuni* 414 and *C. coli* 76639, and the *rrpB* and RrpB sequences from *C. jejuni* NCTC 11168. Conservation of flanking genes was assessed using the Prokka-annotated genomes analysed with the pangenome analysis software package Roary (Page *et al.*, 2015) and the gene numbers obtained from the provisional Prokka annotation of the 4,232 *C. jejuni* and *C. coli* genome sequences.

2.5.10 RNA isolation

Bacteria were re-streaked on BA plates and grown for 24 h. 10 ml Brucella broth was pre-incubated overnight with shaking at 75 rpm under microaerobic conditions. Bacterial cells were re-suspended in 1 ml PBS. The pre-incubated Brucella broth was inoculated to an initial OD_{600} of 0.1 and incubated for 17 h at 37ºC with shaking at 75 rpm under microaerobic conditions. 4 ml of the culture was added to 8 ml of RNA protect Bacterial Reagent (Qiagen, Manchester, UK), vortexed for 5 sec and incubated for 5 min at room temperature for RNA stabilisation. The stabilised culture was then centrifuged at 4,000 rpm for 10 min at 4ºC and the supernatant discarded. The pellet was re-suspended in 200 µl Tris-EDTA (TE) buffer/lysozyme mix (one part of lysozyme solution (1 mg/ml) diluted in 9 parts of TE buffer) and mixed using a P1000 Gilson pipette for 1 min. This suspension was then incubated at room temperature for 10 min and vortexed every 2 min. RLT buffer from a RNeasy Mini Kit (Qiagen) was mixed with 14.4 M β-mercaptoethanol (10 µl per 1 ml RLT buffer) (Stratagene, Amsterdam, The Netherlands). This mix was added to the suspension and vortexed vigorously. 500 µl of 100 % (v/v) ethanol was added to the suspension and mixed by pipetting. A 700 µl aliquot of this suspension was transferred to an RNeasy Mini spin column and centrifuged for 15 sec at 10,000 rpm. The flow through was discarded and

this step repeated. 350 µl RW1 buffer was then added to the column and centrifuged for 15 sec at 10,000 rpm and the flow through discarded. 70 µl RDD buffer (Qiagen) was mixed with 10 µl DNase I (1 U/µl), and 80 µl of this mix was added to the centre of the RNeasy mini spin column and incubated at room temperature for 15 min. 350 µl of RW1 buffer was added to the spin column, centrifuged for 15 sec at 10,000 rpm and the flow through discarded. 500 µl of RPE buffer was added to the spin column, centrifuged for 15 sec at 10,000 rpm and the flow through discarded. Another 500 µl of RPE buffer was added to the spin column and centrifuged for 2 min at 10,000 rpm. The column was transferred to a new 2 ml collection tube and centrifuged at 13,000 rpm for 1 min to allow removal of any residual RPE buffer. The spin column was then placed in a 1.5 ml microcentrifuge tube. 50 µl RNase-free water was added to the centre of the spin column directly onto the membrane and then centrifuged at 13,000 rpm for 1 min to elute the RNA. The concentration of the RNA was quantified using a NanoDrop (see Section 2.5.2.1). RNA samples were stored at -80ºC.

2.5.11 RNA normalisation

Based on the NanoDrop concentration, all RNA samples were normalised to the same concentration. RNase-free water was used to dilute RNA samples.

2.5.12 DNase treatment

The TURBO DNA-*free* Kit (Fisher Scientific) contains a highly efficient DNase I compared to traditional DNase I. This kit was used to completely remove any traces of DNA present in the RNA samples.

The reactions were prepared as follows:

10x TURBO DNase Buffer	5 µl
TURBO DNase	1 µl
RNA sample (2 µg)	x µl
Nuclease-free water up to	50 µl

Samples were incubated for 30 min at 37ºC. 5 µl of DNase Inactivation Reagent was then added to each reaction and incubated at room temperature for 5 min with occasional mixing for re-dispersion of the DNase Inactivation Reagent. Tubes were centrifuged at 10,000 rpm for 90 sec. Supernatants were transferred to clean tubes. This step removes the DNase from the RNA samples. The final concentration obtained for each sample was 40 ng/µl.

2.5.13 Preparation of cDNA from total RNA

The SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) was used to convert total RNA to cDNA. 100 ng of each RNA sample was used in each RT-PCR reaction to be converted to cDNA.

RNA sample (40 ng/µl)	2.5 µl
Random hexamers (50 ng/µl)	1 µl
10 mM dNTP mix	1 µl
RNase-free water	5.5 µl

Samples were incubated at 65ºC for 5 min, then incubated on ice for 1 min. Then 10 µl of the cDNA synthesis mix was prepared at the following concentration per sample:

10X RT buffer	2 µl
25 mM $MgCl_2$	4 µl
0.1 M DTT	2 µl
RNase OUT (40 U/µl)	1 µl
SuperScript III RT (200 U/µl)	1 µl

No RT reactions were also prepared as a control to analyse the reverse transcriptase enzyme efficiency and as a control for contaminating genomic DNA. Samples were

prepared in duplicate, one without the addition of SuperScript III RT. 10 µl of cDNA synthesis mix was added to each RNA sample, mixed and incubated in the DNA Engine Tetrad 2 Peltier Thermal Cycler using the following program:

Step 1: 25ºC for 10 min

Step 2: 50°C for 50 min

Step 3: 85°C for 5 min

Reactions were then incubated in ice for 1 min. Reactions were briefly centrifuged, then 1 µl RNase H added and incubated for 20 min at 37ºC to degrade the remaining RNA. cDNA samples were stored at -20ºC.

2.5.14 Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was used to investigate *katA*, *ahpC* and *sodB* expression semi-quantitatively using *gyrA* as a control housekeeping gene. Two reactions were set up for each cDNA sample, one using *katA* primers and the other using *gyrA* primers. RT-PCR reactions were performed in three biological replicates. PCR products were analysed as described previously (see Section 2.5.6). Images were recorded using a GeneGenius Bio Imaging System and band intensities were quantified using ImageJ software (NIH Image, Bethesda. USA). A rectangle was drawn around the amplified band using the ImageJ software to record the pixel intensity. The selected area was copied and used to read the other bands amplified so all the bands were quantified using exactly the same area. All PCR reactions were analysed using the same PCR program described in Section 2.5.4, using the same number of amplification cycles and scanning settings. A graph of each band was generated with a peak indicating the intensity of each band. The area of each peak was marked, values for each peak were generated indicating the area in square pixels and the percentage of the size of all other peaks. *katA* cDNA PCR product ratios were calculated and normalised against *gyrA* cDNA PCR product ratios for each sample. Gene expression levels are described as relative intensity (vs. *gyrA*).

2.5.15 qPCR

The cDNA obtained from the total RNA was diluted 1:10 with nuclease-free water as the reverse transcription buffer can inhibit PCR amplification. Power SYBR Green PCR Master Mix (Fisher Scientific) was used to label the double stranded DNA during target amplification. This mix contains SYBR Green I dye, DNA Polymerase, dNTPs with dUTP, passive reference Rox dye and optimised buffer. Samples were prepared in duplicate in MicroAmp Fast Optical 96-Well Plates (Applied Biosystems), then sealed with Optical Adhesive Covers (Applied Biosystems) and analysed using a 7500 Real-Time PCR System (Applied Biosystems).

Reactions were prepared as follows:

SYBR Green Master Mix	10 µl
Primer forward (10 µM)	1 µl
Primer reverse (10 µM)	1 µl
Nuclease-free water	4 µl
cDNA	4 µl

Cycling conditions:

Denaturation: 95ºC for 3 min

Amplification: 95ºC for 15 sec
50ºC for 20 sec
} 40 cycles

Melting curve: 95ºC for 15 sec
50ºC for 1 min
95ºC for 30 sec
50ºC for 15 sec

A Melting curve was performed due to the non-specific binding capability of the SYBR Green dye, which can bind to any double stranded DNA. Multiple melting points indicate DNA contamination. Multiple melting points can also indicate primer dimmers.

2.5.16 Whole genome sequencing

The Laboratory Coleção de Campylobacter from Instituto Oswaldo Cruz (Oswaldo Cruz Foundation) from Rio de Janeiro kindly provided 43 *C. jejuni* strains isolated from chicken meat, human infections and the environment. Whole genome sequencing was performed following Nextera protocol.

Genomic DNA was isolated from all strains as described in Section 2.5.1. Quantification of the genomic DNA was performed initially using a NanoDrop (see Section 2.5.2.1). Genomic DNA was diluted to 1 ng/µl and the concentration further analysed using the Qubit (see Section 2.5.2.2). Genomic DNA was then further diluted to 0.2 ng/µl for library preparation.

2.5.16.1 Fragmentation and tagging

DNA samples were fragmented and tagged using Nextera XT transposome. This step allowed the samples to be fragmented and simultaneously tagged with adapter sequences to the ends of the fragments. Briefly, 10 µl Tagment DNA buffer and 5 µl DNA at 0.2 ng/ µl was used for each sample in a 96-well plate. 5 µl Amplicon Tagment mix was added to each sample and mixed. The plate was sealed and centrifuged at 280 x g for 1 min at room temperature. The plate was inserted into a DNA Engine Tetrad 2 Peltier Thermal Cycler and incubated at 55ºC for 5 min, then cooled to 10ºC. When the samples reached 10ºC, the seal was removed and 5 µl Neutralize Tagment buffer was added to each well and mixed. The plate was re-sealed and centrifuged at 280 x g for 1 min at room temperature. The plate was incubated for 5 min at room temperature. Quality of samples was then analysed using the BioAnalyzer (see Section 2.5.3).

2.5.16.2 Amplification

DNA samples were amplified using PCR and indexes required for cluster formation. Each DNA sample was tagged with two specific index primers provided in the Nextera XT DNA kit. Briefly, 15 µl Nextera PCR master mix was added to each well. 5 µl of specific index 2 primer was added to each well, followed by 5 µl of specific index 1 primer and mixed. The plate was sealed and centrifuged at 280 x g for 1 min at room temperature. Then the following PCR program was used:

Step 1: 72ºC for 3 min

Step 2: 95ºC for 30 sec

Step 3: 95ºC for 10 sec
55ºC for 30 sec
72ºC for 30 sec
} 12 cycles

Step 4: 72ºC for 5 min

Step 5: Hold at 10ºC

2.5.16.3 PCR clean-up

DNA samples were cleaned using microbeads to purify the library and remove short library fragments. Briefly, the 96-well plate was centrifuged at 280 x g for 1 min at room temperature. 50 µl of each DNA sample was transferred to a new 96-well plate. The microbeads were equilibrated to room temperature and mixed for 30 sec to be evenly dispersed. 30 µl AMPure XP beads were added to each well and mixed by pipetting 10 times. The plate was then incubated at room temperature for 5 min and placed on a magnetic stand for 2 min. The supernatant was discarded and the microbeads were washed with freshly prepared 80% (v/v) ethanol. 200 µl of 80% (v/v) ethanol was added to each well and incubated on the magnetic stand for 30 sec. The supernatant was removed and a second wash performed. The plate was kept on the magnetic stand for 15 min to air-dry. The plate was then removed from the magnetic stand and 52.5 µl Resuspension buffer was added to each well and mixed by pipetting

10 times. The plate was incubated at room temperature for 2 min and re-placed on the magnetic stand. 50 µl of the supernatant was carefully transferred to a new 96-well plate.

2.5.16.4 Library normalisation

This step normalises the concentration of each DNA sample to ensure equal library representation in the pooled samples. Briefly, 20 µl of the supernatant was transferred to a new 96-well plate. 800 µl of re-suspended Library normalisation beads were mixed with 4.4 ml Library normalisation additives. 45 µl of the mixture was added to each well. The plate was sealed and placed on a shaker at 1800 rpm for 30 sec. The plate was then placed on the magnetic stand for 2 min. 80 µl of the supernatant was removed and discarded. The beads were washed with 45 µl Library normalisation wash, then the plate re-sealed and placed on a shaker at 1,800 rpm for 5 min. The plate was placed on a magnetic stand for 2 min. The supernatant was removed and discarded. The plate was removed from the stand and a further washing process was performed. The plate was then removed from the stand and 30 µl 0.1 M NaOH was added to each well. The plate was sealed and placed on a shaker at 1,800 rpm for 5 min. Then, 30 µl Library normalisation storage buffer was added to each well and samples re-suspended and shaken for another 5 min. The plate was placed on the magnetic stand for 2 min. 30 µl of the supernatant was transferred to a new 96-well plate, sealed and centrifuged at 1,000 x g for 1 min.

2.5.16.5 Library pooling

During this step, DNA samples were pooled with equal volumes of normalised library for cluster generation and sequencing. Briefly, 5 µl of each library was transferred to a PCR tube and mixed. 24 µl of the pooled library was transferred to another tube and 576 µl Hybridization buffer was added and mixed. This mix was incubated at 96ºC for 2 min. After the incubation, each tube was mixed by inversion and placed in an ice-water bath for 5 min. A MiSeq cartridge was defrosted and the library content was loaded into the cartridge.

2.5.16.6 Genomic sequencing and analysis

Genome sequencing was performed using Illumina MiSeq 2 x 150 bp paired-end sequencing. Initial data quality was assessed in FastQC (Andrews, 2010). The sequencing reads were quality controlled using Trimmomatic (v0.32) (leading' and 'trailing' setting of 3, a 'slidingwindow' setting of 4:20 and a 'minlength' of 36 nucleotides) (Bolger *et al.*, 2014). Reads were mapped using BWA-MEM (v0.7.7-r441) against the genome sequence of either the *C. jejuni* NCTC 11168 (AL111168) or *C. jejuni* 414 strains. Assembly was performed using VelvetOptimiser (v2.2.5) using n50 optimization (Zerbino and Birney, 2008, Gladman and Seemann, 2012). Contigs were ordered against *C. jejuni* 414 (CM000855) using ABACAS (v1.3.1) (Assefa *et al.*, 2009). Annotation of genomes was performed with RATT (Otto *et al.*, 2011) using *C. jejuni* NCTC 11168 (AL111168). Genomes were visualised using Artemis and ACT software (Carver *et al.*, 2012).

2.5.17 Search for pathogenicity genes

Genomes were analysed for specific genes (protein sequences) using the program Blastall (Altschul *et al.*, 1990). Gene sequences were obtained from either the *C. jejuni* NCTC 11168 or 414 strain. Searched genes included *rrpA, rrpB, cadF, cdtA, cdtB, cdtC, ciaB flpA, dnaJ, flaA, flaC, hipO, ilpA, peb1A, pldA,* and *racR* based on the genome sequence of *C. jejuni* NCTC 11168 strain. T6SS-associated genes searched were *tssA, tssB, tssC, tssD, tssE, tssF, tssG, tssH, tssI, tssJ, tssK, tssL,* and *tssM* based on the genome sequence of *C. jejuni* 414 strain.

To perform searches, the selected genes were set as the database and the newly sequenced whole genome was converted into amino acid sequence form. To set the query sequence as the database, the formatdb command was used as follows:

formatdb -i file -oT -pF -VT

The option -i denotes the input file. The option -oT was set to true allowing creation of indexing for potential downstream applications. The option -pF was set to false denoting the input sequence is a nucleotide sequence. The option -VT was set to true so as to allow warning messages to appear if sequence identifiers were not unique.

Using the blastall program, blastx was used to perform the comparison. Blastx compares the six-frame translation products of a nucleotide query sequence against a protein database. In this case, the newly generated genome sequences were converted in this manner and the gene to be searched acted as the protein database. To perform the blast search, the blastall command was used as follows:

blastall -p blastx -i inputfile -d RacR_p.txt –o outputfile -b2 -a1 -v2 -e0.01 –FF

The option -b was set to 2 which selected the number of alignments to show. The option -a was set to 1 which selected the number of processors to use. The option –v was set to 2 which selected the number of descriptions to display. The option –e allowed the Expectation value to be set (in this case -e0.01) and the value allowed the filtering of non-significant matches. The option -F was set to 'false' (-FF) so there was no filtering of low-complexity subsequences. Blastall results were considered significant where Expectation values were less than 1e-15 and similarity was greater than 50%.

2.5.18 Multilocus sequence typing

Housekeeping genes are normally expressed and conserved. However, variation in housekeeping gene nucleotide sequences can occur. Nucleotide differences were assigned an allele number and then to a unique allelic profile, which were then assigned to a sequence type (ST). Sequences of housekeeping genes were analysed and assignments were performed using the script from Torsten Seemann which scans contig files against PubMLST typing schemes (Jolley and Maiden, 2010). This method uses a web-accessible database system (https://github.com/tseemann/mlst) called The Bacterial Isolate Genome Sequence Database (BIGSDB) for identification of MLST of different bacterial specimens. Similarities of *C. jejuni* isolates were made by comparing the different allelic profiles. The housekeeping genes used for MLST were *aspA, glnA, gltA, glyA, pgm, tkt* and *uncA*. The MLST script used was:

mlst – scheme campylobacter *.dna > mlst.csv

2.5.19 RNA sequencing

RNA samples were obtained as described in Section 2.5.9 and treated with DNase I to remove any traces of DNA in the samples (see Section 2.5.11).

2.5.19.1 Ribosomal RNA (rRNA) removal

The Ribo-Zero rRNA Removal Kit (Illumina) was used to remove rRNA from total RNA samples. Briefly, for each sample 225 µl of magnetic beads were transferred to a 1.5 ml tube and placed on a magnetic stand for 1 min. The supernatant was discarded and beads were re-suspended in 225 µl of RNA-free water and mixed by pipetting. This washing step was repeated one more time and the supernatant discarded. Beads were re-suspended in 65 µl of Magnetic Bead Suspension Solution and mixed. 1 µl of RiboGuard RNase Inhibitor was added to the beads. The following reaction was prepared to remove ribosomal RNA from total RNA samples:

Ribo-Zero Reaction Buffer	4 µl
RNA sample	1 µg
Ribo-Zero rRNA Removal Solution	8 µl
RNase-free water up to	40 µl

Reactions were incubated at 68ºC for 10 min, then incubated at room temperature for 5 min. Each treated sample was transferred to a 1.5 ml microcentrifuge tube containing the prepared magnetic beads, mixed thoroughly by pipetting and then vortexexd for 10 sec. Samples were incubated at room temperature for 5 min, vortexed for 10 sec and then incubated at 50ºC for 5 min. Tubes were then placed in the magnetic stand for 1 min. The supernatant was removed carefully and transferred to a new 1.5 ml microcentrifuge tube.

2.5.19.2 Sample purification

Sample purification was performed using the Agencourt RNAClean XP Kit (Beckman Coulter, Brea, USA). Briefly, beads were vortexed for dispersion. 160 µl of the beads solution was added to each RNA sample prepared as described in Section 2.5.19.1, and thoroughly mixed by pipetting. Tubes were incubated at room temperature for 15 min, then placed on the magnetic stand for 5 min. Supernatants were discarded. 200 µl of freshly prepared 80% ethanol was added to each sample without disturbing the beads. After 30 sec, the supernatant was discarded and this step repeated. Tubes were air dried for 15 min at room temperature. Then, 11 µl of RNA-free water was added and the beads gently re-suspended. Tubes were incubated at room temperature for 2 min, then placed in the magnetic stand for 5 min. The supernatant was then removed to a collection tube.

2.5.19.3 RNA fragmentation

Reactions for RNA fragmentation were prepared in a 0.6 ml microcentrifuge tube as described:

Ribo-Zero-treated RNA	9 µl
RNA Fragmentation Solution	1 µl
cDNA Synthesis Primer	2 µl

Tubes were incubated at 85ºC for 5 min, then placed on ice.

2.5.19.4 cDNA synthesis

The following master mix was prepared for each reaction:

cDNA Synthesis PreMix	3 µl
100 mM DTT	0.5 µl
StarScript Reverse Transcriptase	0.5 µl

The 4 µl from the master mix were transferred to each sample prepared in Section 2.5.19.3. Tubes were incubated in thermocycler as follows:

Step 1: 25ºC for 5 min

Step 2: 42ºC for 20 min

Step 3: 37ºC Paused

1 µl of Finishing Solution was added to each reaction. Tubes were returned to the thermocycler and incubated as follows:

Step 4: 37ºC for 10 min

Step 5: 95ºC for 3 min (Inactivation of finishing solution)

Step 6: 25ºC Paused

2.5.19.5 Terminal tagging

For each sample the following reaction was prepared:

Terminal Tagging Premix	7.5 µl
DNA Polymerase	0.5 µl

The 8 µl reaction mixture was mixed gently by pipetting. Tubes were returned to the thermocycler and incubated as follows:

Step 7: 25º for 15 min

Step 8: 95ºC for 3 min

Step 9: Tubes were then cooled on ice.

2.5.19.6 cDNA purification

cDNA samples were purified using the Agencourt RNAClean XP Kit as follows: 45 µl of beads were added to each microcentrifuge tube prepared in Section 2.5.19.5 and mixed by pipetting. Samples were transferred to 1.5 ml microcentrifuge tubes and incubated at room temperature for 15 min. Tubes were placed in a magnetic stand for 5 min. The supernatant was discarded and 200 µl 80% (v/v) ethanol was added to each sample. Tubes were incubated at room temperature for 30 sec and the supernatant was discarded. This step was repeated once more. Tubes were air dried for 15 min at room temperature. 24.5 µl of nuclease-free water was added and the beads re-suspended by pipetting. Tubes were incubated for 2 min at room temperature then transferred to the magnetic stand for 5 min. 22.5 µl of the clear supernatant was transferred to a 0.6 µl tube.

2.5.19.7 Indexing and library amplification

This step generated the second strand of cDNA. Adaptor sequences and indexes were also added to the fragments for sequencing. ScriptSeq Index PCR Primers (Illumina) were used, a unique index primer was used for each sample.

FailSafe PCR PreMix E	25 µl
Forward PCR Primer (10 µM)	1 µl
ScriptSeq Index PCR Primer (10 µM)	1 µl
FailSafe PCR Enzyme (1.25 U)	0.5 µl

PCR program used: Step 1: 95ºC for 1 min

Step 2: 95ºC for 30 sec
55ºC for 30 sec
68ºC for 3 min
} 15 cycles

Step 3: 68ºC for 7 min

2.5.19.8 Library purification

The AMPure XP system was used to purify the libraries. Briefly, 50 µl of beads were added to each PCR tube prepared in Section 2.5.19.7 and mixed by pipetting. Samples were transferred to 1.5 ml microcentrifuge tubes and incubated at room temperature for 15 min. Tubes were placed in the magnetic stand for 5 min. The supernatant was discarded. Beads were washed twice with 200 µl 80% ethanol for 30 sec and supernatant discarded. Tubes were air dried for 15 min at room temperature. Beads were re-suspended with 20 µl nuclease-free water by gentle pipetting and incubated at room temperature for 2 min. Tubes were placed in the magnetic stand for 5 min. The clear supernatant was transferred to a new collection tube.

2.5.19.9 Assess library quality

Library qualities were assessed using BioAnalyzer as described in Section 2.3.5.

2.5.19.10 RNA sequencing and analysis

Library pooling and loading into MiSeq cartridge was performed as described in 2.5.16.5. RNA sequencing (RNA seq) was performed using Illumina MiSeq 2 x 75 bp paired-end sequencing. To allow quantification of RNA-Seq data, Rockhopper software was used (McClure *et al.*, 2013). Rockhopper allows input of Fastq files and based on built in genomes, allows determination of differential gene expression values. The software includes the reference genome NCTC 11168 and this was used as the reference. Rockhopper also conveniently allows the incorporation of biological replicates. For reference based analysis Rockhopper aligns creates a Burrows-Wheeler index based on the full-text minute space. After index creation for the reference genome, an exact alignment is attempted for each read and, if unsuccessful, an inexact alignment to the set of replicons is attempted by aligning the seed regions to the genome and extending these alignments with an optimized dynamic program. This dynamic program determines the optimal alignment using an algorithm based on the error probabilities of each sequencing read (McClure *et al.*, 2013).

For normalisation, the software allows for comparison of data from different samples and experiments, by normalising each set of data by upper quartile normalization (McClure *et al.*, 2013). Rockhopper quantifies transcript abundance based on RPKM (Reads Per Kilobase of transcript per Million mapped reads), which is a measure that sums the number of reads for a gene and divides by the gene's length and the total number of reads. However, Rockhopper divides RPKM by the upper quartile of gene expression.

For differential expression, Rockhopper uses local regression to obtain an estimate of gene expression variances. Then, for each transcript, Rockhopper performs a statistical test for the null hypothesis, which is that the expression of the transcript is the same in different conditions. The Negative Binomial distribution is used as the statistical model to compute a *p*-value indicating the probability of observing a transcript's expression levels in the different conditions by chance. Rockhopper generates *q*-values using Benjamini-Hochberg procedure to control the false discovery rate caused by multiple statistical analysis (McClure *et al.*, 2013).

2.6 Statistical analysis

All experimental assays consist of at least three biological replicates performed in duplicate. Error bars represent the standard error of mean. Statistical analyses were performed using GraphPad Prism software (v4.02). Variables were compared for significance using a student's *t*-test.

3 Investigation into the role of RrpA and RrpB in the *C. jejuni* oxidative stress response

3.1 Introduction

The re-annotation of the *C. jejuni* NTCT 11168 genome identified two previously unidentified transcriptional regulators RrpA and RrpB that belong to the MarR family of transcriptional regulators due to the presence of a Pfam motif PF01638 (Gundogdu *et al.*, 2007). PF01638 is a HxlR-like helix-turn-helix motif which is part of the DNA binding site of MarR-like transcriptional regulators (Kumarevel, 2012). MarR-like transcriptional regulators control many different biological functions, including resistance to multiple antibiotics, expression of virulence factors and resistance to different oxidative stresses (Kumarevel, 2012). The two MarR-type response regulators RrpA and RrpB have been shown to play a role in the peroxide and aerobic stress responses (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). Mutation of *rrpA* or *rrpB* in the 11168H wild-type strain resulted in decreased ability to survive under aerobic conditions and increased sensitivity to peroxide stress (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). Mutation of *rrpB* in the 11168H wild-type strain was shown to affect the expression of 73 genes based on microarray data (Gundogdu *et al.*, 2011). Some of the genes affected by the *rrpB* mutation were related to oxidative stress defence mechanisms, such as *ahpC*, *katA*, *perR* and *sodB* (Gundogdu et al., 2011). Further analysis showed that RrpA has 43.6% identity and 58.4% similarity to RrpB (Gundogdu *et al.*, 2015).

3.2 Results

3.2.1 Construction of a 11168H *rrpB perR* double mutant

A plasmid containing the cloned 11168H *rrpB* gene disrupted by the insertion of a chloramphenicol resistance (chl^R) cassette was obtained from Dr. Ozan Gundogdu (Gundogdu *et al.*, 2015) and electroporated into the 11168H *perR* mutant. 11168H *rrpB perR* double mutants were selected on BA kanamycin/chloramphenicol plates as described in Section 2.5.8.

The construction of an 11168H *rrpB perR* double mutant was confirmed by PCR. *rrpB* and *perR* gene specific primers were used to confirm that both genes had been mutated through the insertion of antibiotic resistance cassettes. The expected size for *rrpB* amplification is 163 bp and the size expected for *perR* amplification is 411 bp. The size of the kanamycin (kan^R) cassette is 1,426 kilobase (kb). Therefore, the size expected for *perR* plus the kan^R cassette is 1,837 kb. The size of the chl^R cassette is 804 bp. Therefore, the size expected for *rrpB* plus the chl^R cassette is 967 bp.

The *rrpB* gene specific forward primer was also used with a chl^R forward-out primer to investigate if the chl^R cassette was inserted in the correct orientation. The size expected for the amplification using *rrpB* forward and chl^R forward-out is 489 bp (Figure 3.1).

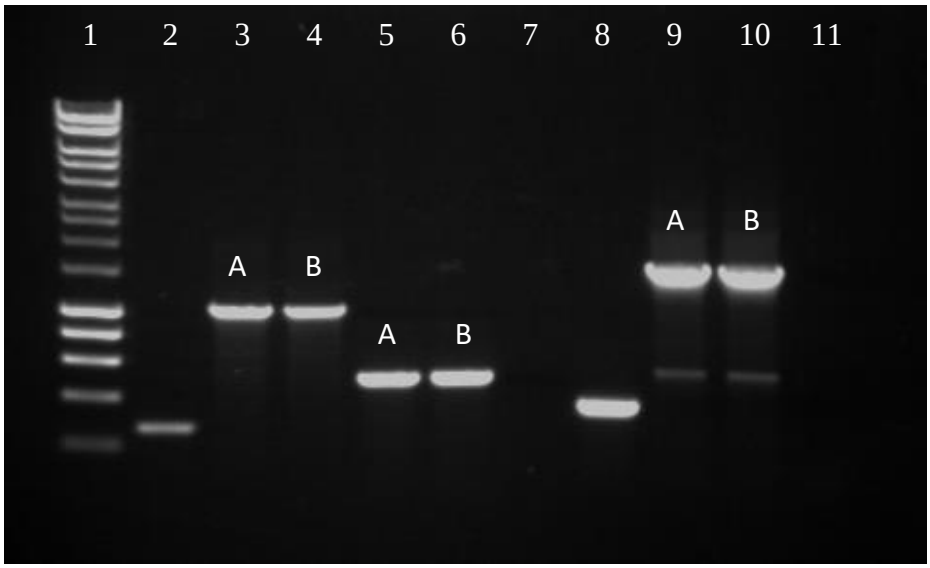

Figure 3.1 Confirmation of the construction of a 11168H *rrpB perR* mutant using PCR. A and B are genomic DNA from 2 different clones. Lane 1: ladder; Lanes 2 and 8: 11168H genomic DNA (positive controls); Lanes 2, 3 and 4: *rrpB* specific primers; Lanes 5, 6 and 7: *rrpB* forward and chl^R forward out primers; Lanes 8, 9, 10 and 11: *perR* specific primers; Lanes 7 and 11: negative controls.

3.2.2 Oxidative stress assays

The survival of the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant and *rrpAB* double mutant were tested under different oxidative stress conditions in order to investigate the roles of RrpA and RrpB. *rrpA* and *rrpB* complements were also tested under H_2O_2 stress conditions. As controls, different mutants with known defects in oxidative stress responses (*katA*, *ahpC*, *sodB* and *perR*) were also tested under the different oxidative stress conditions.

3.2.2.1 Hydrogen peroxide stress

The 11168H wild-type strain exhibited resistance to 25 mM H_2O_2, but was highly sensitive to both 50 mM and 100 mM H_2O_2. The *katA* mutant does not express catalase, the main bacterial enzyme that neutralises hydrogen peroxide, so was highly sensitive to all concentrations of H_2O_2 used. The *sodB* mutant exhibited increased resistance to H_2O_2 compared to the wild-type strain, surviving exposure to 50 mM H_2O_2. The *ahpC* mutant also exhibited increased resistance compared to the wild-type strain, demonstrating resistance to 100 mM H_2O_2 (Figure 3.2). Controls were performed incubating each strain under the same conditions, but without exposure to H_2O_2.

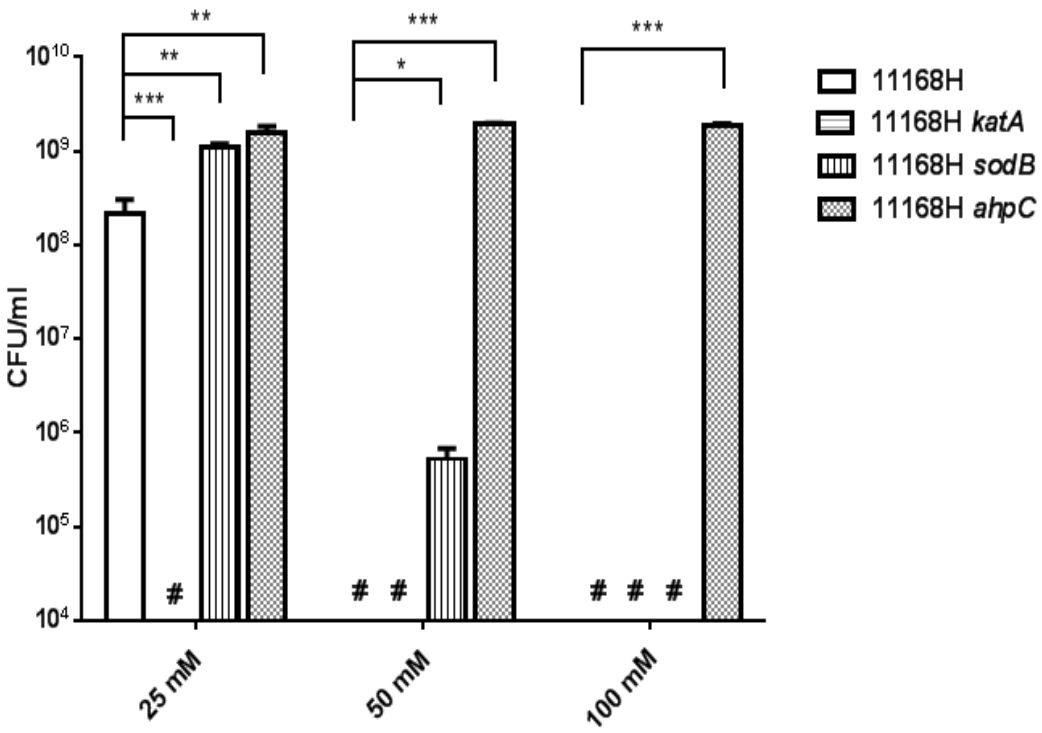

Figure 3.2 Sensitivity of the 11168H wild-type strain, *katA*, *sodB* and *ahpC* mutants to H_2O_2 stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 25, 50 and 100 mM H_2O_2 for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$; # no growth.

The sensitivity of the *rrpA* mutant, *rrpB* mutant and *rrpAB* double mutant to H_2O_2 was also investigated. The *rrpA* and *rrpB* mutants exhibited increased sensitivity to H_2O_2 compared to the wild-type strain. *rrpA* and *rrpB* complements exhibited restoration of the 11168H wild-type phenotype. However, the *rrpB* complement did not resist exposure to 25 mM H_2O_2 at the same level as the wild-type strain. The *rrpAB* double mutant exhibited increased resistance to H_2O_2 compared to the wild-type strain, even resisting exposure to 100 mM H_2O_2 (Figure 3.3).

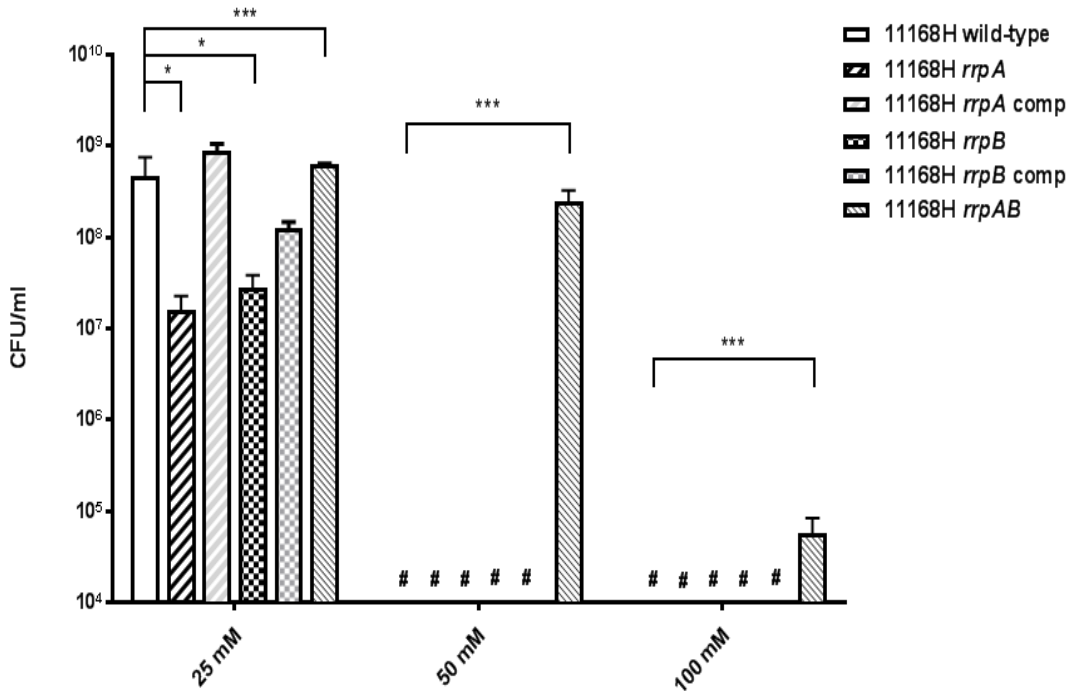

Figure 3.3 Sensitivity of the 11168H wild-type strain, *rrpA*, *rrpB* and *rrpAB* mutants, *rrpA* and *rrpB* complements to H_2O_2 stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 25, 50 and 100 mM H_2O_2 for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted onto duplicate in BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = $p < 0.05$; *** = $p < 0.001$; # no growth.

PerR is a regulator of the *C. jejuni* oxidative stress response and is known to repress both *katA* and *ahpC* expression (Palyada et al., 2009). The 11168H *perR* mutant was shown to be highly resistant to H_2O_2. The 11168H *rrpB perR* double mutant was also shown to be highly resistant to H_2O_2 (Figure 3.4).

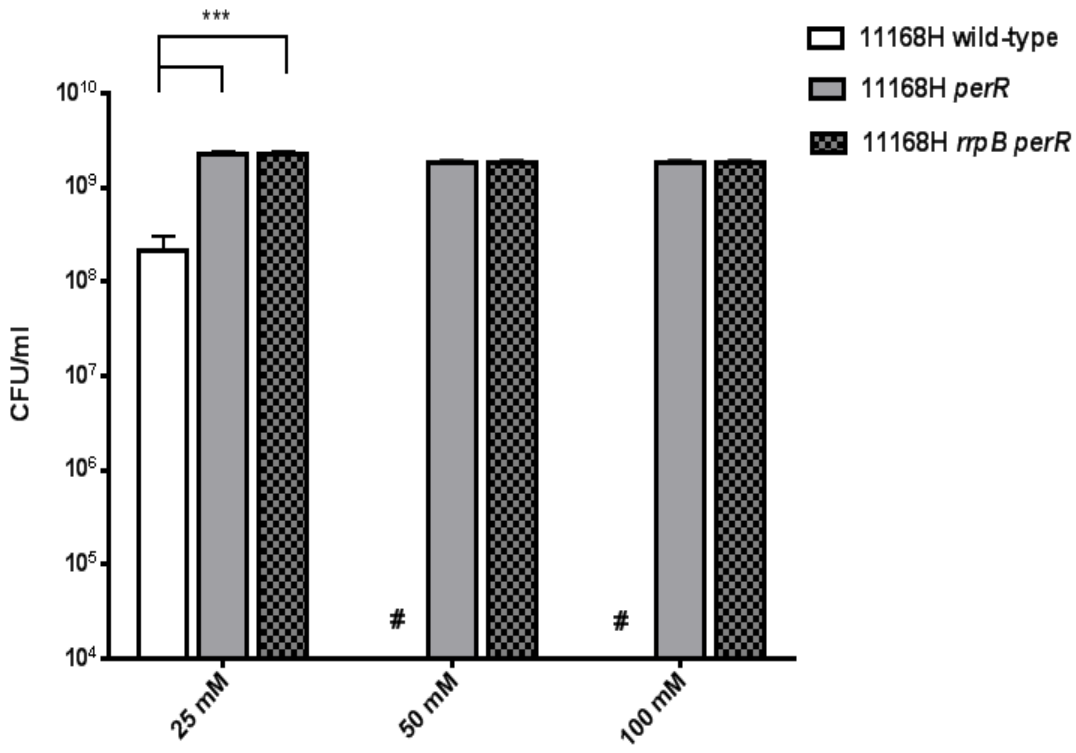

Figure 3.4 Sensitivity of the 11168H wild-type strain, *perR* and *rrpB perR* mutants to H_2O_2 stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 25, 50 and 100 mM H_2O_2 for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. *** = $p < 0.001$; # no growth.

Different *C. jejuni* wild-type strains and their respective *rrpA* and/or *rrpB* mutants were also investigated for H_2O_2 stress resistance. The strains analysed were: 81-176 wild-type strain, 81-176 *rrpA* mutant, 81-176 *rrpB* mutant, 81116 wild-type strain, 81116 *rrpA* mutant, M1 wild-type strain and M1 *rrpA* mutant. The sensitivity of these strains to H_2O_2 was compared to the 11168H wild-type strain as well as the 11168H *rrpA* and *rrpB* mutants. The 81-176 *rrpA* and *rrpB* mutants demonstrated increased sensitivity to 25 mM H_2O_2 compared to the wild-type strain. This result was similar to the phenotype demonstrated by the 11168H *rrpA* and *rrpB* mutants (Figure 3.5).

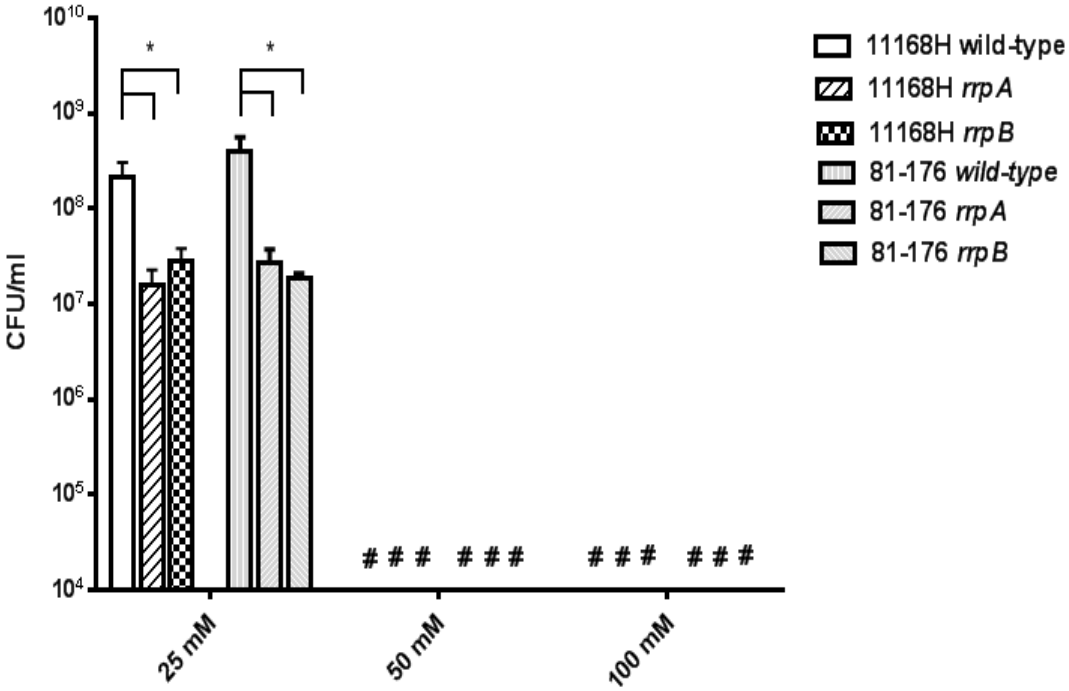

Figure 3.5 Sensitivity of the 11168H and 81-176 wild-type strains and respective *rrpA* and *rrpB* mutants to H_2O_2 stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 25, 50 and 100 mM H_2O_2 for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = $p < 0.05$; # no growth.

The genomes of the 81116 and M1 wild-type strains only possess an *rrpA* gene. When these wild-type strains were exposed to H_2O_2, both demonstrated an increased resistance compared to the 11168H and 81-176 wild-type strains. The 81116 *rrpA* and M1 *rrpA* mutants demonstrated no significant differences in the level of resistance to H_2O_2 compared to the respective wild-type strains (Figure 3.6), indicating that the mutation of *rrpA* in these two wild-type strain backgrounds does not affect sensitivity to H_2O_2 stress.

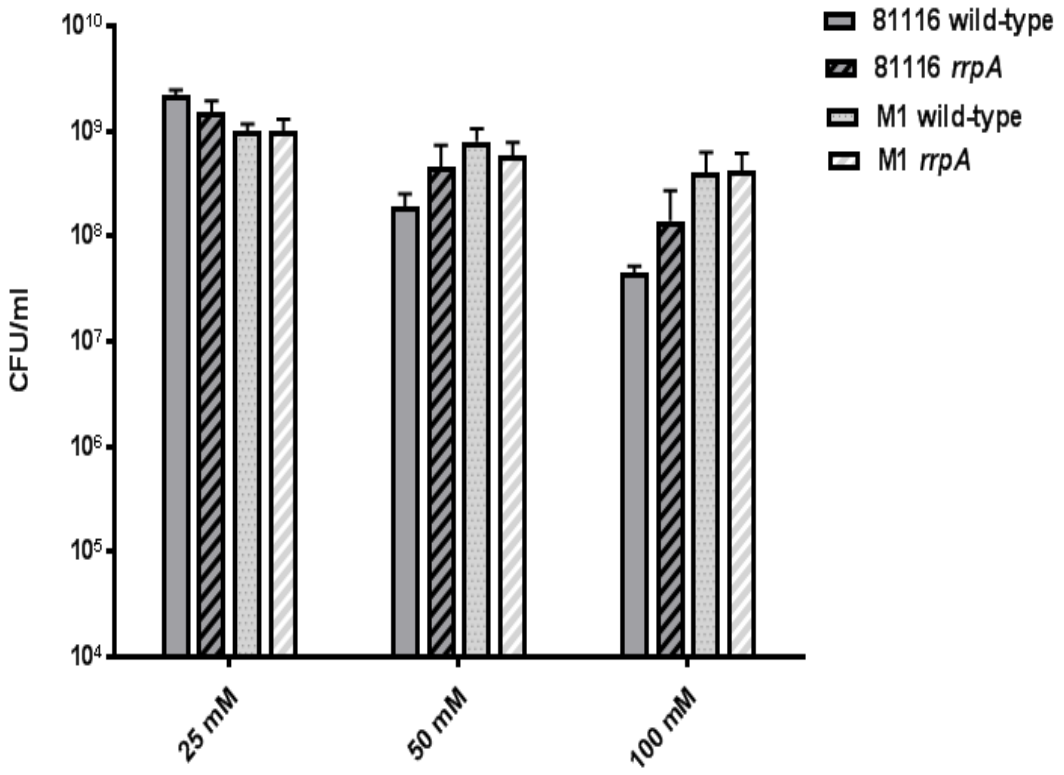

Figure 3.6 Sensitivity of the 81116 and M1 wild-type strains and respective *rrpA* mutants to H_2O_2 stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 25, 50 and 100 mM H_2O_2 for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates.

3.2.2.2 Menadione stress

The 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant and *rrpAB* double mutant were exposed to menadione stress to investigate sensitivity to this superoxide generator. 11168H *sodB*, *katA*, *ahpC* and *perR* mutants were also investigated as controls.

The *katA* mutant demonstrated a similar level of sensitivity to menadione as the wild-type strain. The *sodB* mutant was highly sensitive to menadione, due to the fact that *sodB* encodes the main enzyme that eliminates superoxides. The *ahpC* mutant

demonstrated increased sensitivity to menadione compared to the wild-type strain (Figure 3.7). Controls were performed incubating each strain under the same conditions, but without exposure to menadione.

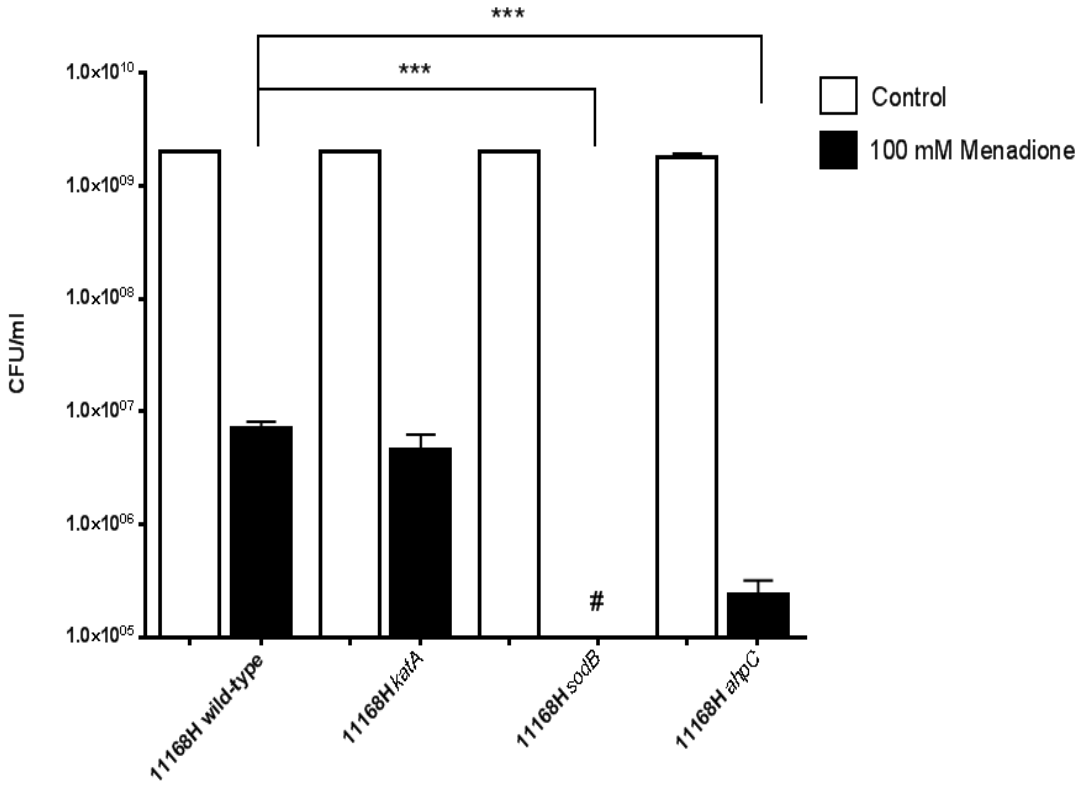

Figure 3.7 Sensitivity of the 11168H wild-type strain, *katA*, *sodB* and *ahpC* mutants to menadione stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM menadione for 1 h under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. *** = $p < 0.001$; # no growth.

The *rrpA* mutant and *rrpB* mutant were exposed to menadione stress. However, no differences in sensitivity were observed compared to the wild-type strain. The *sodB* mutant was included as a control (Figure 3.8).

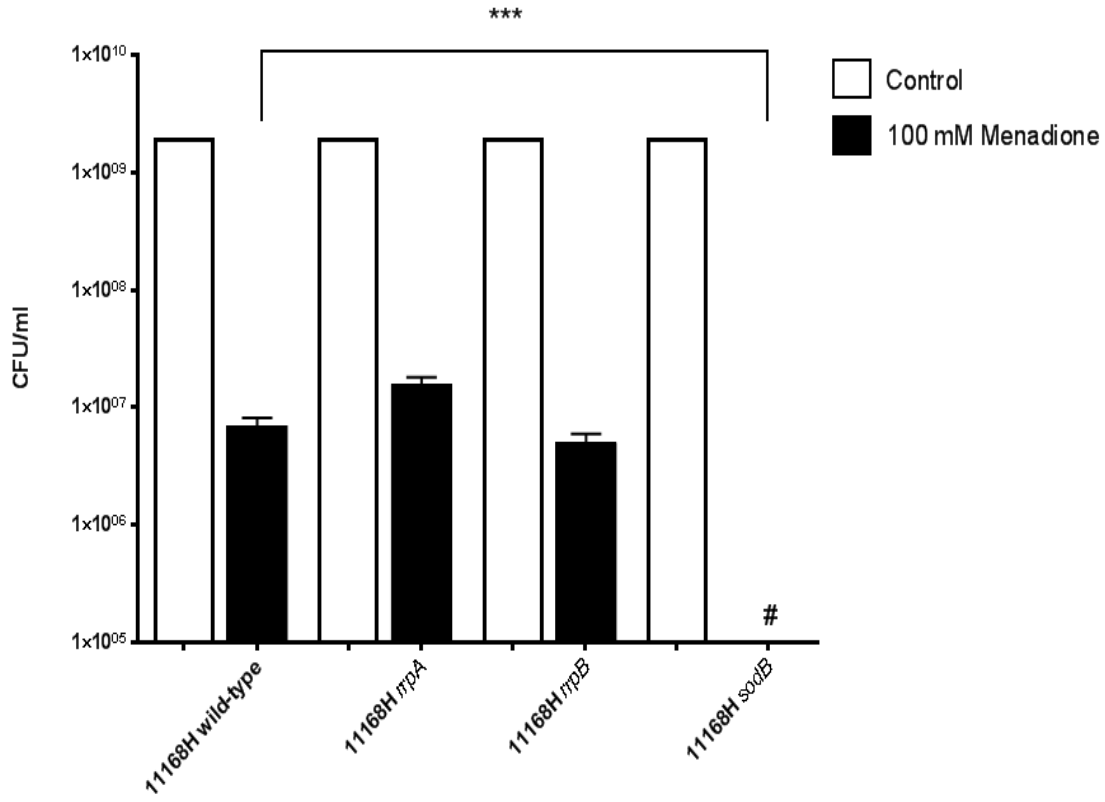

Figure 3.8 Sensitivity of the 11168H wild-type strain, *rrpA*, *rrpB* and *sodB* mutants to menadione stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM menadione for 1 h under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. *** = $p < 0.001$; # no growth.

The *rrpAB* double mutant exhibited increased resistance to menadione stress compared to the wild-type strain. The *perR* mutant exhibited the same level of sensitivity as the wild-type strain (Figure 3.9).

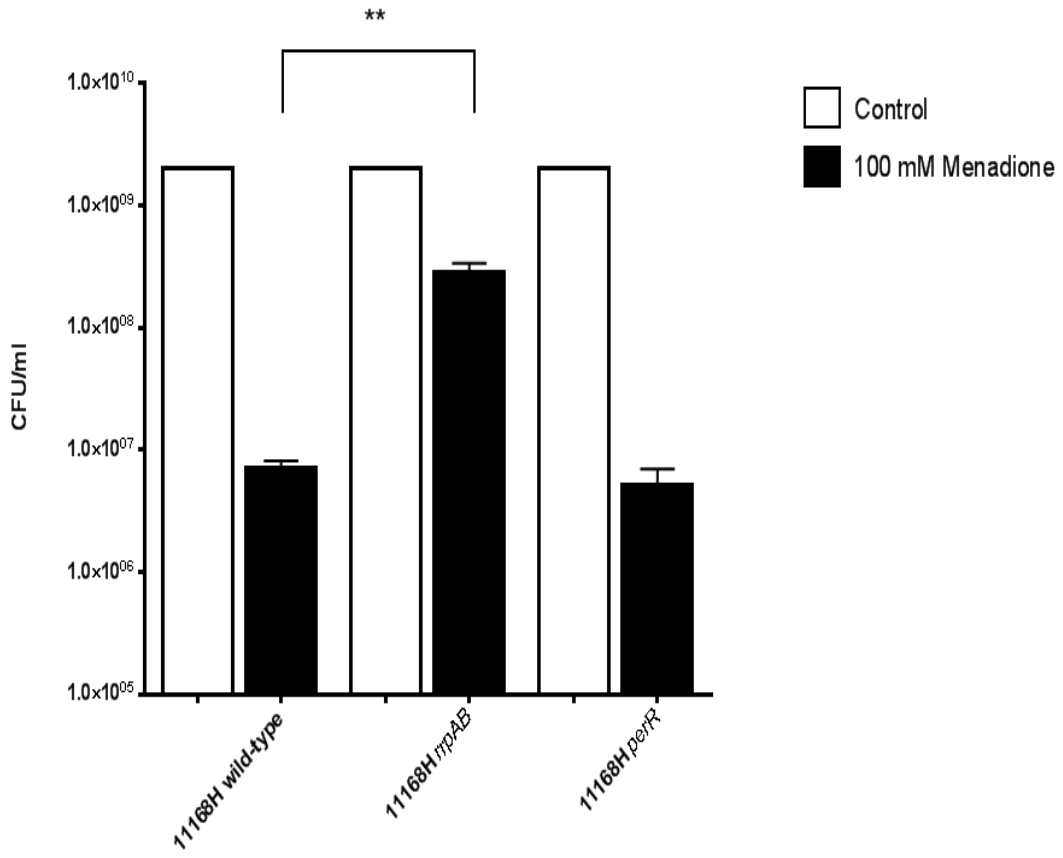

Figure 3.9 Sensitivity of the 11168H wild-type strain, *rrpAB* and *perR* mutants to menadione stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM menadione for 1 h under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. ** = $p < 0.01$.

The following non-11168H *C. jejuni* strains were also tested for sensitivity to menadione stress: 81-176 wild-type strain, 81-176 *rrpA* mutant, 81-176 *rrpB* mutant, 81116 wild-type strain, 81116 *rrpA* mutant, M1 wild-type strain and M1 *rrpA* mutant. The 81-176 wild-type strain demonstrated resistance to menadione stress, at a similar level as the control. The 81-176 *rrpA* mutant demonstrated no significant difference in sensitivity to menadione stress compared to the wild-type strain, whilst the *rrpB* mutant demonstrated a small but significant increase in sensitivity. The 11168H *sodB* mutant was included as a negative control (Figure 3.10).

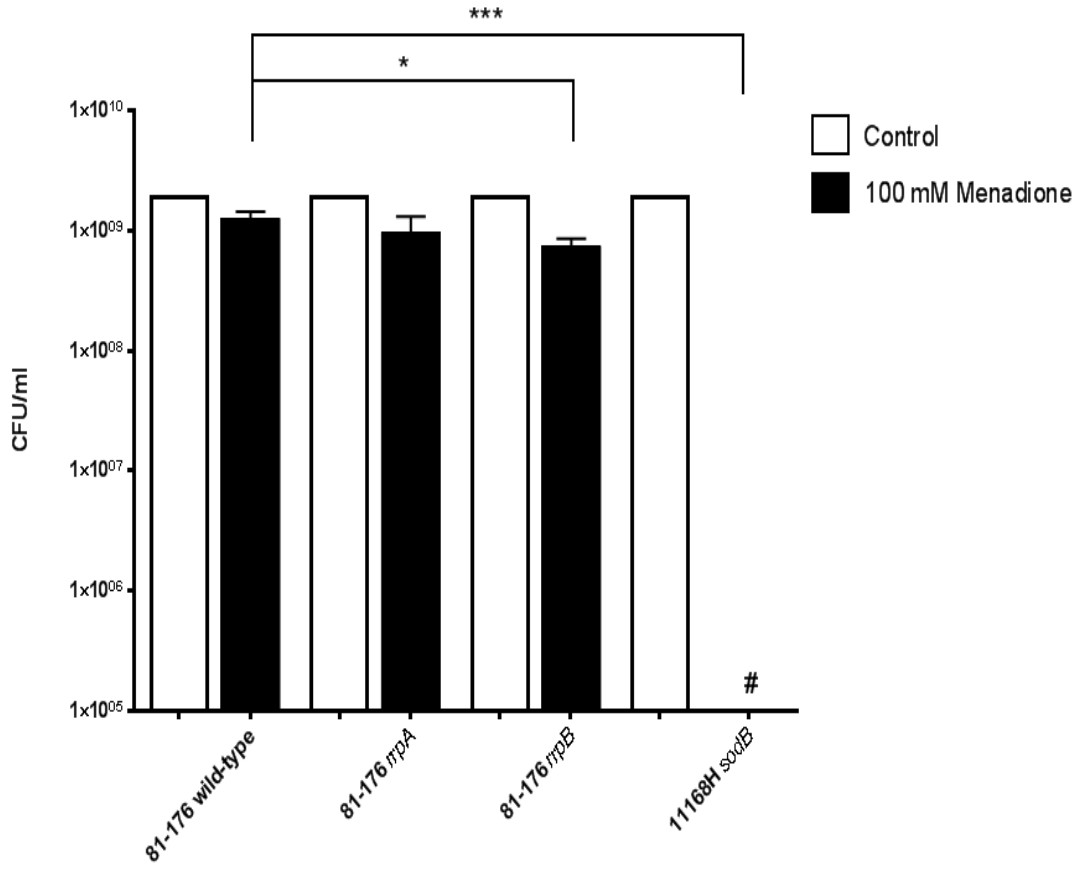

Figure 3.10 Sensitivity of the 81-176 wild-type strain, *rrpA* and *rrpB* mutants and 11168H *sodB* mutant to menadione stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM menadione for 1 h under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = $p < 0.05$; *** = $p < 0.001$; # no growth.

The 81116 *rrpA* mutant did not demonstrate any significant difference in sensitivity to menadione stress compared with the 81116 wild-type strain. Similarly, the M1 *rrpA* mutant did not demonstrate any significant difference in sensitivity to menadione stress compared to the M1 wild-type strain (Figure 3.11).

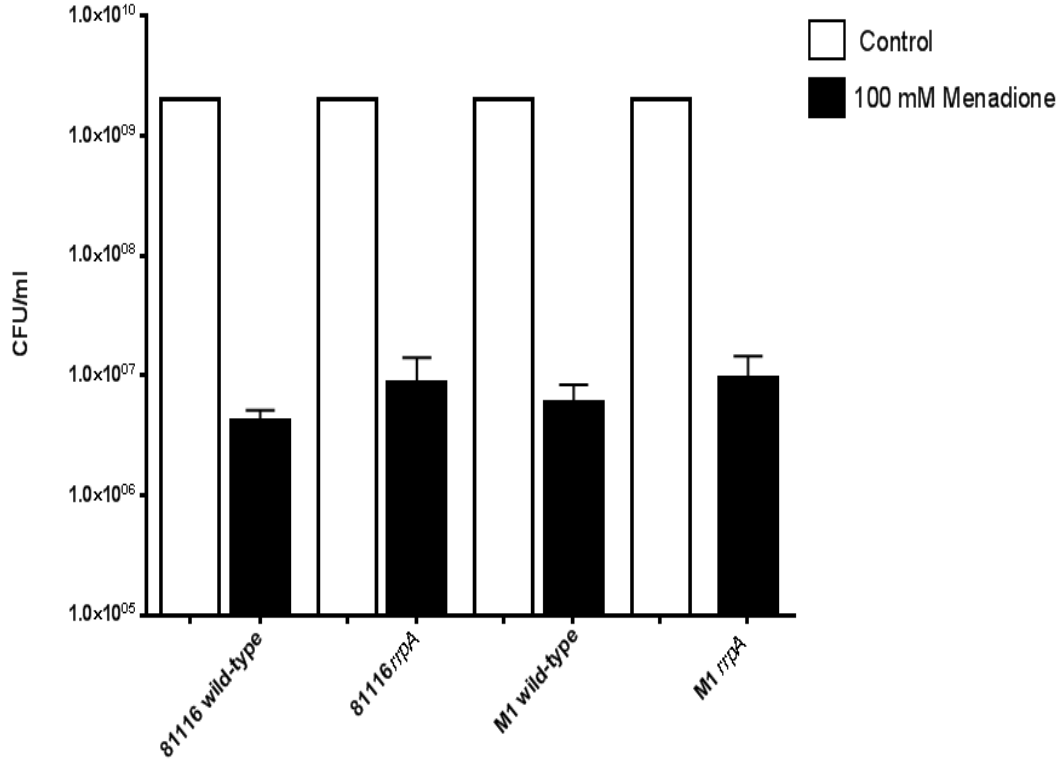

Figure 3.11 Sensitivity of the 81116 and M1 wild-type strains and respective *rrpA* mutants to menadione stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM menadione for 1 h under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates.

3.2.2.3 Cumene hydroperoxide stress

The ability of the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant and *rrpAB* double mutant to survive oxidative stress was further investigated by exposure to cumene hydroperoxide stress. The *katA*, *ahpC* and *perR* mutants were also included as controls.

Controls were performed by incubating each strain under the same conditions and with the same concentration of ethanol as the concentration present in the cumene

hydroperoxide solution. AhpC is known to break down organic peroxides. Therefore, the *ahpC* mutant was used as negative control in this assay.

The *katA* mutant exhibited increased resistance to cumene hydroperoxide compared to the wild-type strain. The *sodB* mutant also exhibited increased resistance to cumene hydroperoxide compared to the wild-type strain. However, the *ahpC* mutant demonstrated increased sensitivity to cumene hydroperoxide compared to the wild-type strain (Figure 3.12).

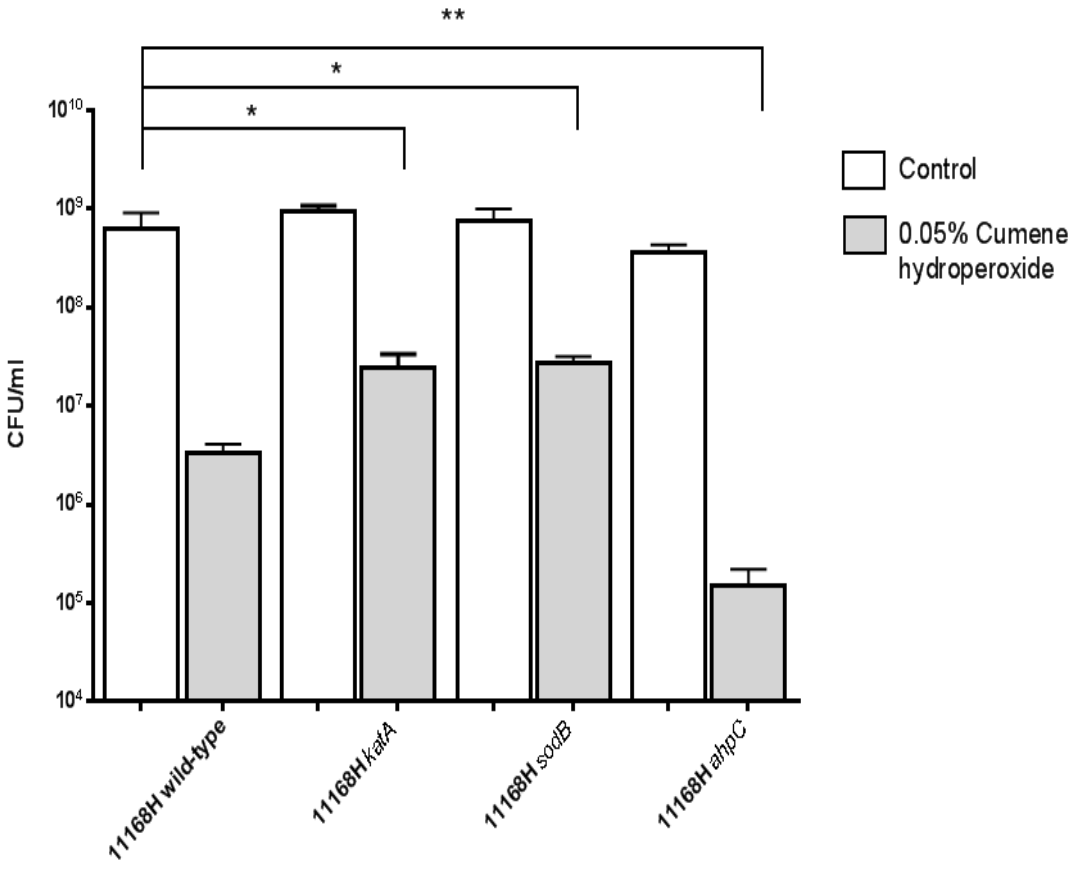

Figure 3.12 Sensitivity of the 11168H wild-type strain, *katA*, *sodB* and *ahpC* mutants to cumene hydroperoxide stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM cumene hydroperoxide for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = $p < 0.05$; ** = $p < 0.01$.

The *rrpA* mutant and *rrpB* mutant did not show any significant differences in sensitivity to cumene hydroperoxide compared to the wild-type strain (Fig 3.13). The *perR* mutant also did not show any significant difference in sensitivity to cumene hydroperoxide compared to the wild-type strain. However, the *rrpAB* double mutant demonstrated increased resistance to cumene hydroperoxide stress (Fig 3.14).

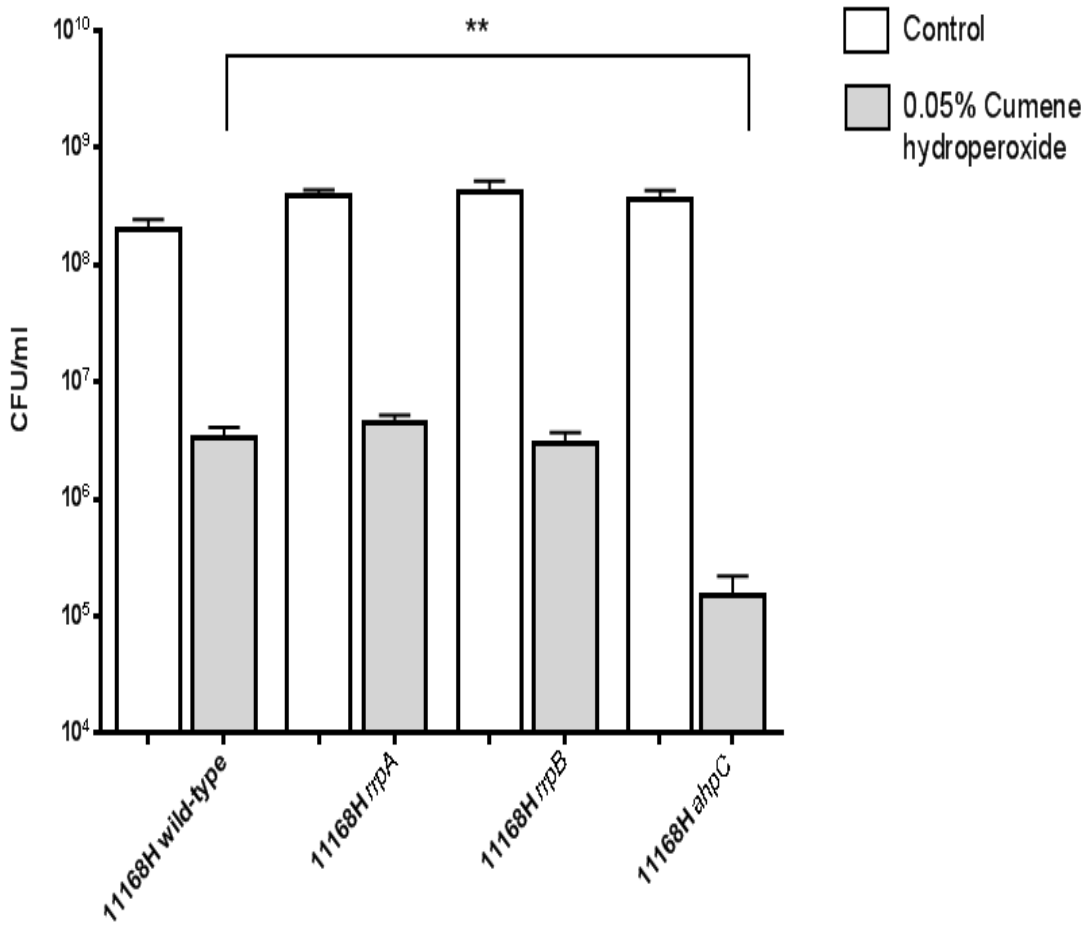

Figure 3.13 Sensitivity of the 11168H wild-type strain, *rrpA*, *rrpB* and *ahpC* mutants to cumene hydroperoxide stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM cumene hydroperoxide for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. ** = $p < 0.01$.

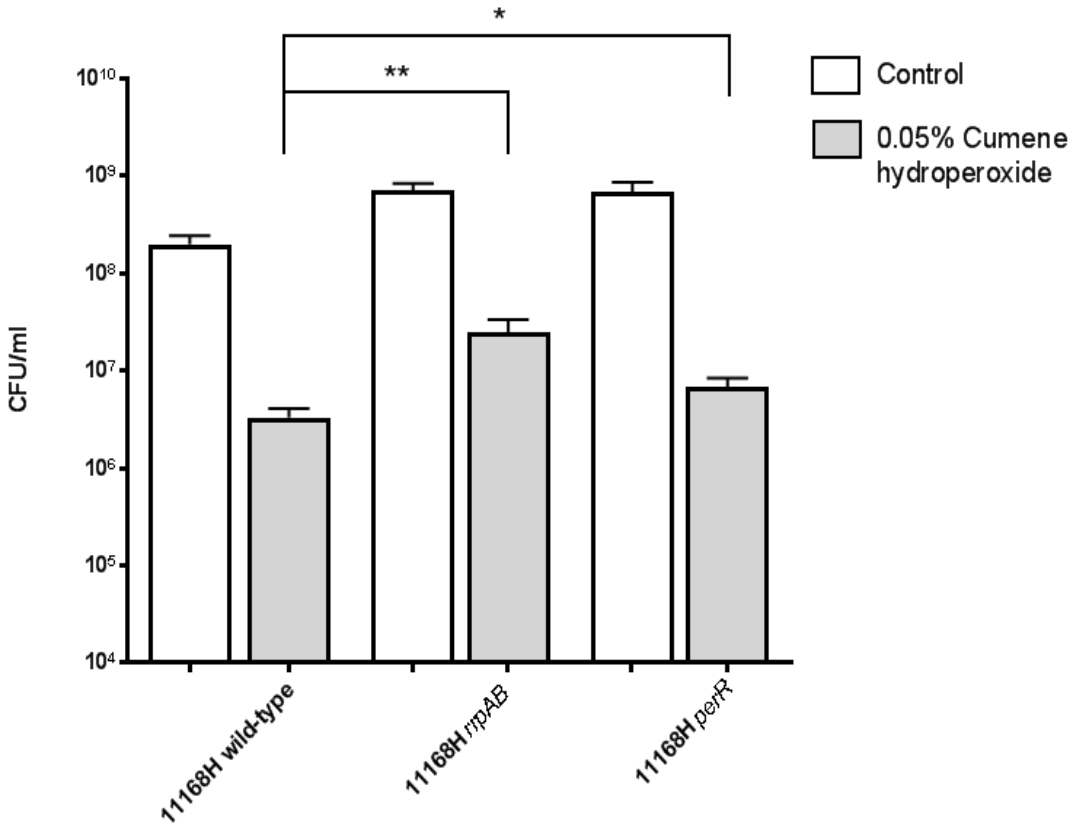

Fig 3.14 Sensitivity of the 11168H wild-type strain, *rrpAB* and *perR* mutants to cumene hydroperoxide stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM cumene hydroperoxide for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. ** = $p < 0.01$.

The *C. jejuni* 81-176 wild-type strain, *rrpA* and *rrpB* mutants were also tested for sensitivity to cumene hydroperoxide stress. Both the 81-176 *rrpA* and *rrpB* mutants exhibited the same level of sensitivity as the 81-176 wild-type strain (Figure 3.15).

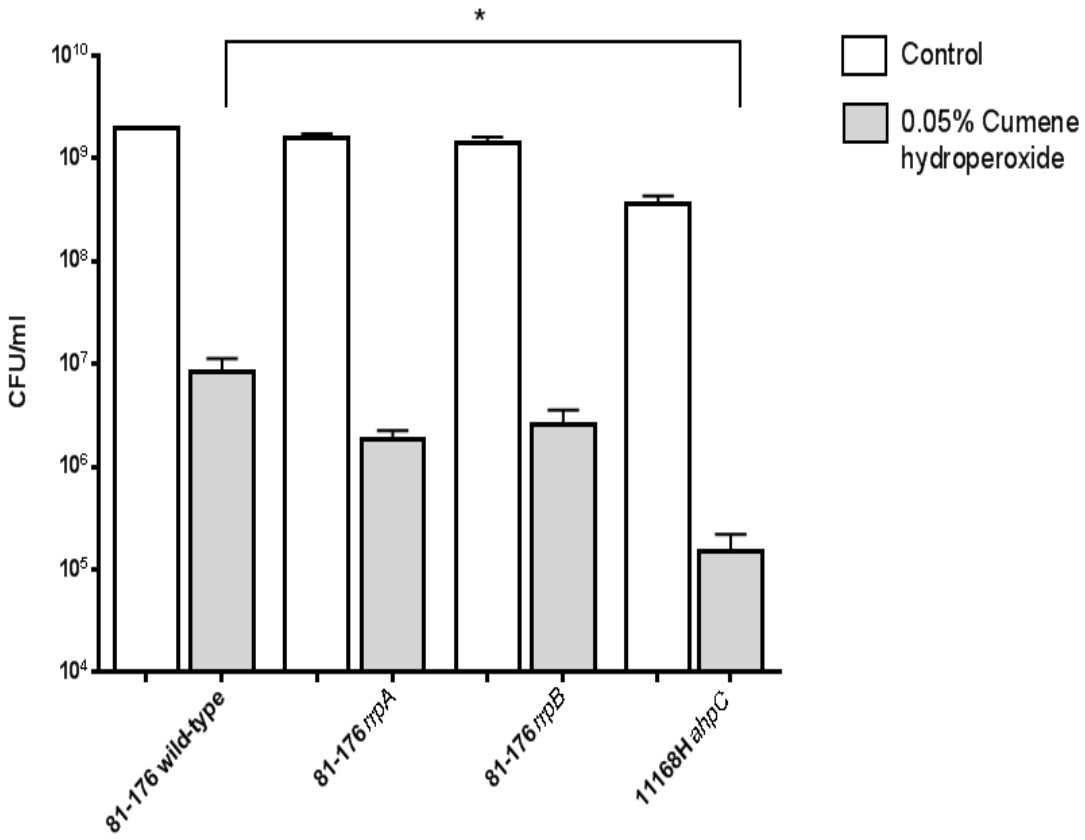

Figure 3.15 Sensitivity of *C. jejuni* 81-176 wild-type strain, *rrpA* and *rrpB* mutants and 11168H *ahpC* mutant to cumene hydroperoxide stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM cumene hydroperoxide for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = $p < 0.05$.

The *C. jejuni* 81116 wild-type strain, 81116 *rrpA* mutant, M1 wild-type and M1 *rrpA* mutant were also tested for sensitivity to cumene hydroperoxide stress. Surprisingly the 81116 wild-type strain exhibited increased sensitivity to cumene hydroperoxide compared to the 81116 *rrpA* mutant. However, the M1 wild-type strain and M1 *rrpA* mutant exhibited similar levels of sensitivity to cumene hydroperoxide (Figure 3.16).

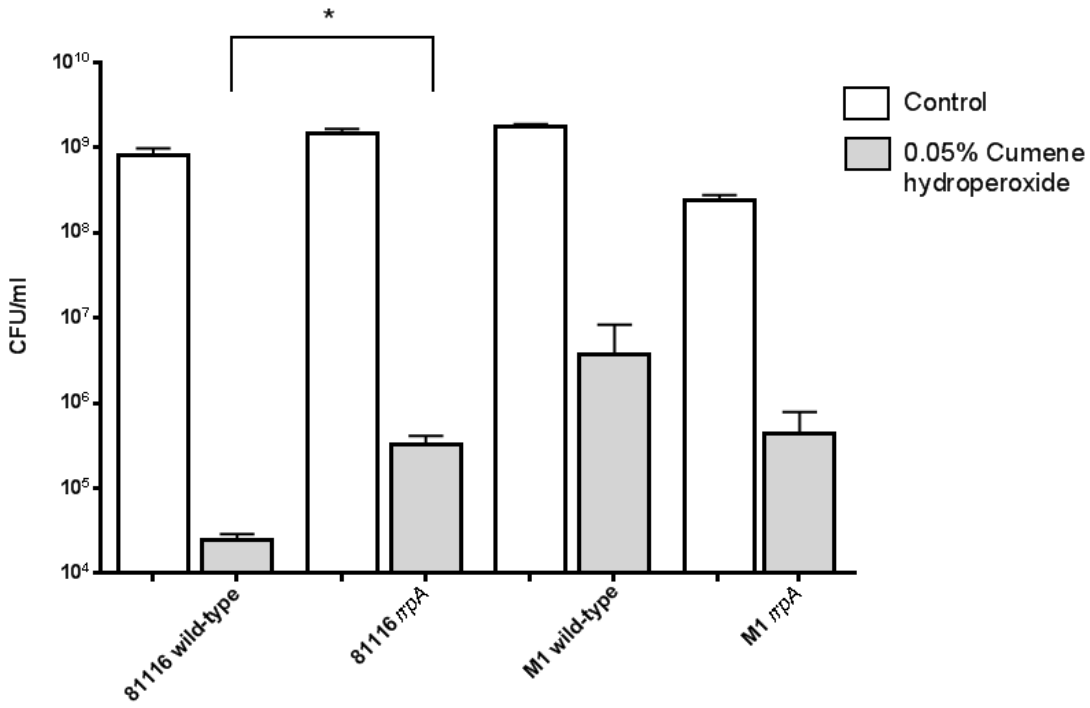

Figure 3.16 Sensitivity of *C. jejuni* 81116 and M1 wild-type strains and respective *rrpA* mutants to cumene hydroperoxide stress. Bacterial suspensions (OD_{600} 1.0) were exposed to 100 mM cumene hydroperoxide for 15 min under microaerobic conditions at 37ºC. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = $p < 0.05$.

3.2.3 Catalase activity assays

3.2.3.1 Catalase activity associated with *C. jejuni* whole cell lysates from BA plates

Strains were grown on BA plates and whole cell lysates prepared as described in Section 2.4.4.1. Catalase activity assays were performed as described in Section 2.4.7. Whole cell lysates were normalised to 100 ng/µl and used to quantify the catalase activity. Different catalase activity levels were observed comparing the 11168H wild-type strain with the different mutants. The *rrpA* mutant and *rrpB* mutant both exhibited reduced catalase activity compared to the wild-type strain. The *rrpA* complement and *rrpB* complement exhibited wild-type levels of catalase activity. The *rrpAB* double

mutant exhibited catalase activity slightly higher, but not significantly so, compared to the wild-type strain. The *katA* mutant exhibited no catalase activity and the *perR* mutant exhibited an extremely high level of catalase activity (Figure 3.17).

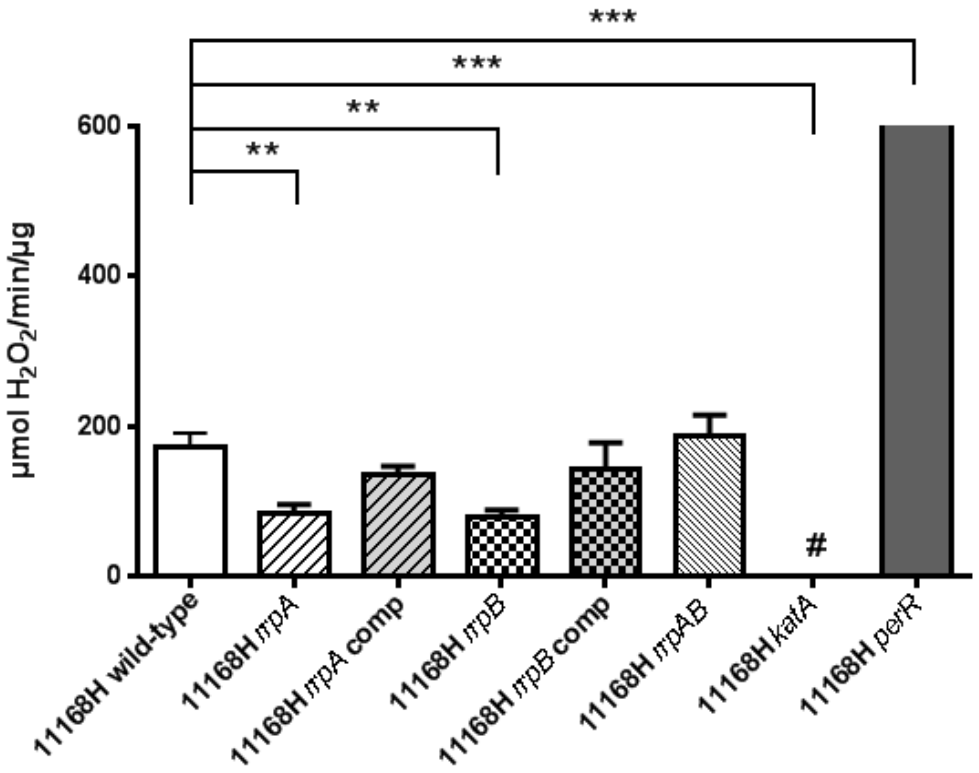

Figure 3.17 Catalase activity assay for the 11168H wild-type strain, *rrpA* mutant, *rrpA* complement, *rrpB* mutant, *rrpB* complement, *rrpAB* double mutant, *katA* mutant and *perR* mutant. Whole cell lysates were prepared from 24 h BA plates. Bacterial suspensions were sonicated and centrifuged. Protein concentrations were normalised to 100 ng/µl. Data represents at least four biological replicates. ** = $p < 0.01$; *** = $p < 0.001$; # no activity.

3.2.3.2 Catalase activity associated with *C. jejuni* whole cell lysates from MEMα broth

Catalase activity assays were also performed using whole cell lysates prepared from *C. jejuni* strains grown in MEMα broth to mimic low iron growth conditions, which should increase *katA* expression. The assay was performed as described in Section

2.4.4.2. Catalase activity assays were performed as described in Section 2.4.7. Whole cell lysates were again normalised to 100 ng/µl. The *rrpA* mutant and *rrpB* mutant both exhibited reduced catalase activity compared to the wild-type strain. The *rrpAB* double mutant did not exhibit any difference in catalase activity levels compared to the wild-type strain. The *katA* mutant exhibited a very low level of catalase activity whilst the *perR* mutant exhibited an extremely high level of catalase activity (Figure 3.18).

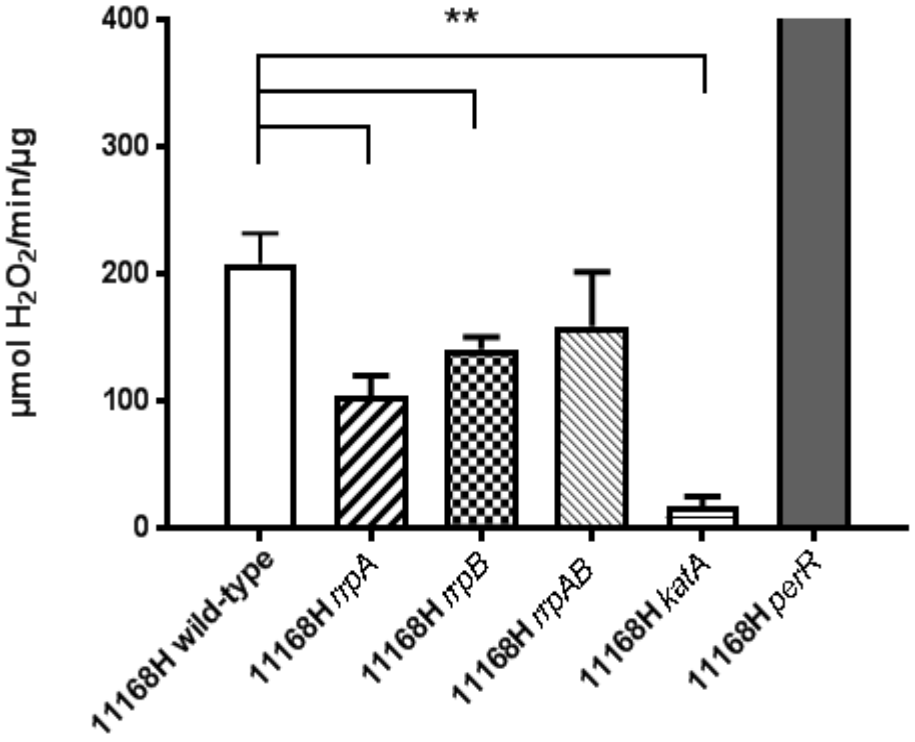

Figure 3.18 Catalase activity assay for the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant, *rrpAB* double mutant, *katA* mutant and *perR* mutant under low iron conditions. Whole cell lysates were prepared from 48 h MEMα broth cultures. Cell suspensions were sonicated and centrifuged. Protein concentrations were normalised to 100 ng/µl. Data represents at least four biological replicates. ** = $p < 0.01$.

3.2.4 Superoxide dismutase activity assays

SOD activity assays were performed to investigate whether RrpA and/or RrpB also affect the expression of SodB. Bacterial suspensions were prepared from 24 h BA plates and sonicated as described in Section 2.4.4.1. Protein concentrations were normalised to 100 ng/µl. SOD activity assays were performed as described in Section 2.4.8.

SOD activity represents the percentage of inhibition of the rate of reduction of the formazan dye compared to the blank control. Formazan dye is reduced by superoxide anions and this reaction can be inhibited by SOD. Reduction of formazan dye can be measured using a colorimetric method. Blank control 1 will give the highest reading as this contains no SOD to inhibit the reduction of formazan dye.

Neither the *rrpA* mutant, *rrpB* mutant nor *rrpAB* double mutant exhibited any significant differences in SOD activity compared to the 11168H wild-type strain. The *perR* mutant and *katA* mutant also did not exhibit any significant differences in SOD activity compared to the 11168H wild-type strain. The *sodB* mutant was used as a control due to the lack of SOD expression. The *sodB* mutant did not exhibit any SOD activity. However, the *ahpC* mutant demonstrated a significant increase in SOD activity compared to the wild-type strain (Figure 3.19).

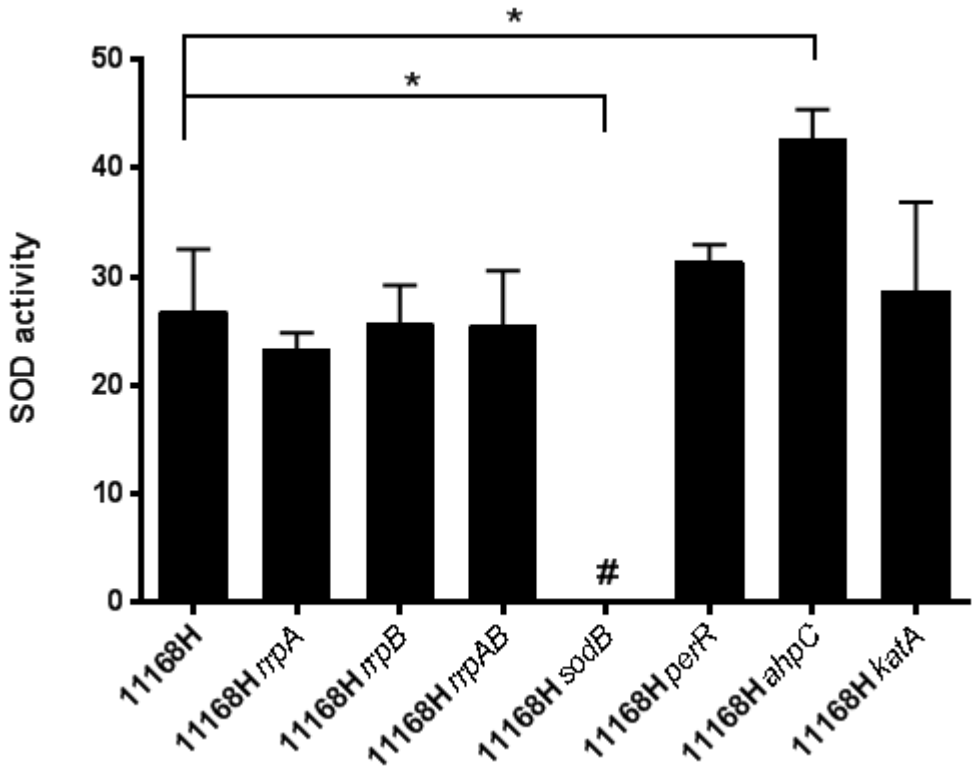

Figure 3.19 Superoxide dismutase activity assay for the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant, *rrpAB* double mutant, *sodB* mutant, *perR* mutant, *ahpC* mutant and *katA* mutant. Whole cell lysates were prepared from 24 h BA plates. Cell suspensions were sonicated and centrifuged. Protein concentrations were normalised to 100 ng/µl. Data represents at least four biological replicates. * = $p < 0.05$; # no activity.

3.2.5 RT-PCR analysis of *katA*, *sodB* and *ahpC* expression

RT-PCR was used as a semi-quantitative method to measure the relative intensity of *katA*, *ahpC* and *sodB* expression using *gyrA* as reference gene. *gyrA* encodes DNA gyrase which is constitutively expressed in *C. jejuni* (Joslin and Hendrixson, 2009). *gyrA* has been used as endogenous control in RT-PCR analysis by many different *Campylobacter* researchers (Bingham-Ramos and Hendrixson, 2008, Joslin and Hendrixson, 2009, Weingarten *et al.*, 2009).

Total RNA was isolated from all strains at the late log phase. mRNA was converted to cDNA as described in Section 2.5.9 to Section 2.5.13. The cDNA was used as a template for PCR amplification as described in Section 2.5.14. *katA*, *ahpC* and *sodB* expression levels were calculated based on the endogenous control *gyrA* expression and presented as relative intensities.

Both the *rrpA* and *rrpB* mutants exhibited reduced *katA* expression compared to the wild-type strain. The *rrpAB* double mutant demonstrated an increased level of *katA* expression compared to the wild-type strain. However, this was not statistically significant (Fig 3.20).

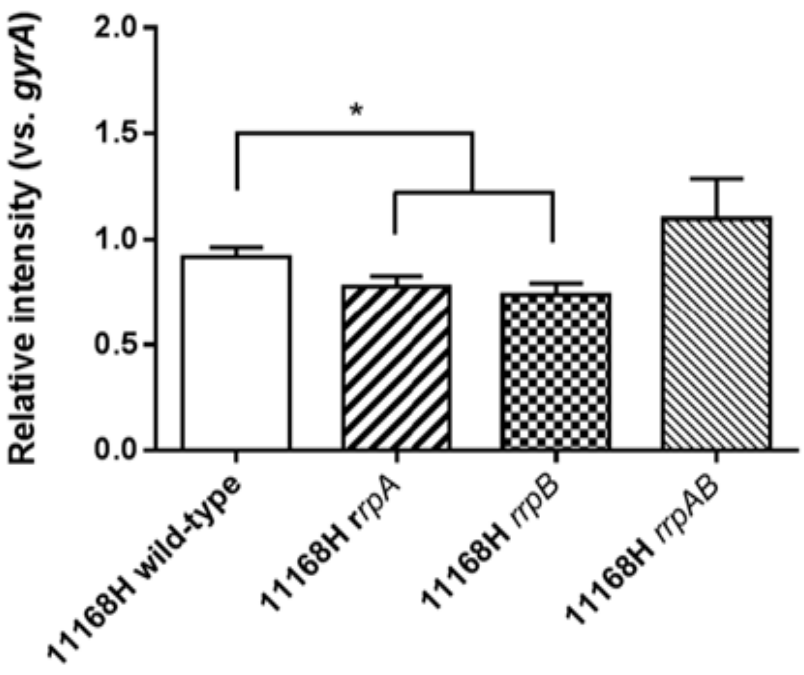

Figure 3.20 RT-PCR analysis of *katA* transcription in the 11168H wild-type strain, *rrpA*, *rrpB* and *rrpAB* mutants. RNA was isolated and converted to cDNA by RT-PCR. cDNA was used as a template in PCR reactions to amplify *katA*. *katA* expression was assessed relative to *gyrA* expression. Reactions were analysed on an agarose gel and relative band intensities were measured using ImageJ software. Data represents at least three biological replicates. * = $p < 0.05$.

No significant changes in *sodB* expression were observed in the *rrpA* mutant, *rrpB* mutant or *rrpAB* double mutant compared to the wild-type strain (Figure 3.21).

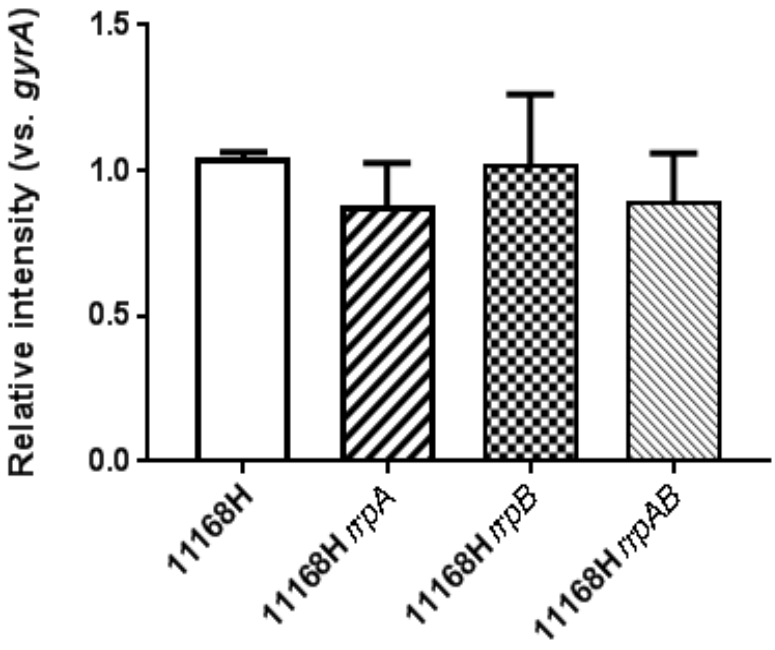

Figure 3.21 RT-PCR analysis of *sodB* transcription in the 11168H wild-type strain, *rrpA*, *rrpB* and *rrpAB* mutants. RNA was isolated and converted to cDNA by RT-PCR. cDNA was used as a template in PCR reactions to amplify *sodB*. *sodB* expression was assessed relative to *gyrA* expression. Reactions were analysed on an agarose gel and relative band intensities were measured using ImageJ software. Data represents at least three biological replicates.

The expression of *ahpC* was reduced in both the *rrpA* mutant and the *rrpB* mutant compared to the wild-type strain. However, the reduction in *ahpC* expression in the *rrpA* mutant was not significant compared to the wild-type strain (Figure 3.22). No significant changes in *ahpC* expression were observed in the *rrpAB* double mutant compared to the wild-type strain.

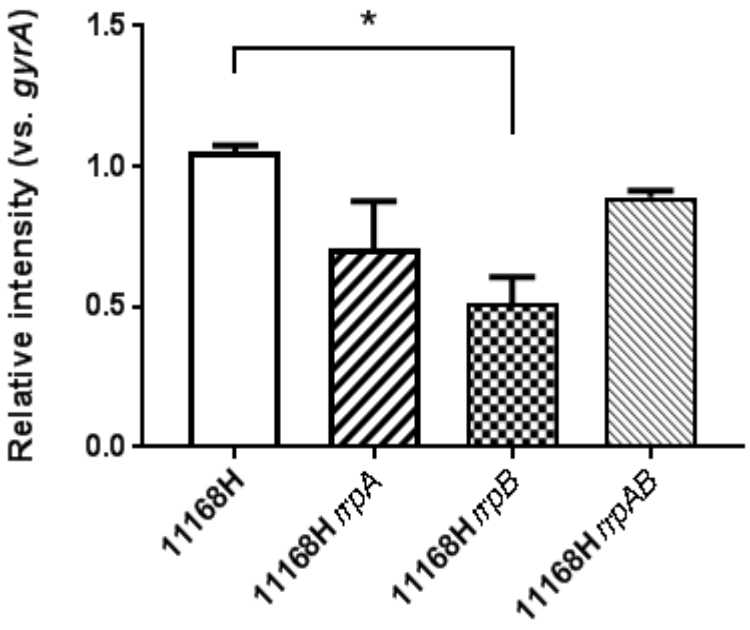

Figure 3.22 RT-PCR analysis of *ahpC* transcription in the 11168H wild-type strain, *rrpA*, *rrpB* and *rrpAB* mutants. RNA was isolated and converted to cDNA by RT-PCR. cDNA was used as a template in PCR reactions to amplify *ahpC*. *ahpC* expression was assessed relative to *gyrA* expression. Reactions were analysed on an agarose gel and relative band intensities were measured using ImageJ software. Data represents at least three biological replicates. * = $p < 0.05$.

3.2.6 qPCR

qPCR results appeared to indicate that *katA* expression levels are reduced in both the *rrpA* mutant and *rrpB* mutant compared to the wild-type strain. The *rrpAB* double mutant appears to have increased levels of *katA* expression. However, only one biological replicate is shown because most replicates demonstrated Ct readings at late cycles, which can indicate background amplification not related to target amplification. Therefore, no statistical analyses have been performed (Figure 3.23).

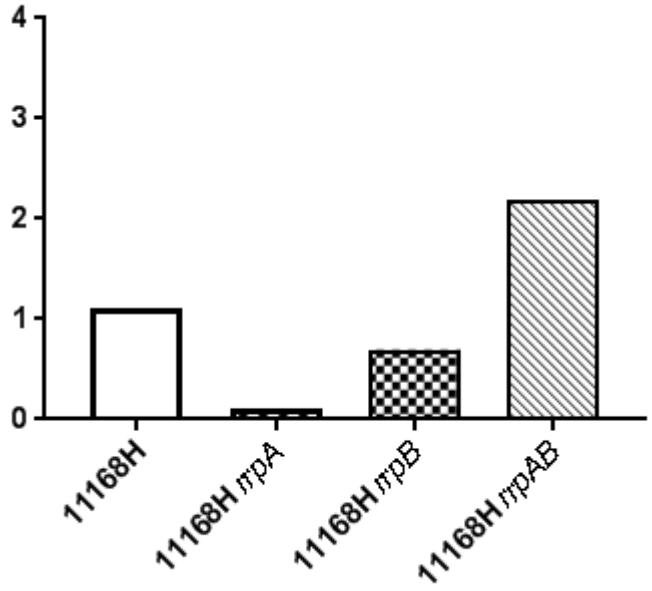

Figure 3.23 qPCR results for *katA* expression. RNA was isolated from each sample and converted to cDNA using two-step RT-PCR. *katA* relative expression levels are shown as fold changes and were normalised using *gyrA* as a control. Data presented is from one biological replicate only.

3.2.7 RNA sequencing

RNA sequencing data was analysed using Rockhopper software as described in Section 2.5.19.10. *q*-values less than 0.01 were considered significant indicating differential gene expression between the 11168H wild-type strain and either the *rrpA* mutant, the *rrpB* mutant or the *rrpAB* double mutant (Appendix 5).

Genes encoding proteins with a role in the *C. jejuni* oxidative stress response and with significant differential expression compared to the wild-type strain are listed in Table 3.1.

katA expression was reduced in the *rrpA* mutant compared to the 11168H wild-type strain. However, no changes in *katA* expression were observed in the *rrpB* mutant or in the *rrpAB* double mutant. None of the mutants demonstrated significant changes in the expression of *sodB* and *ahpC*. However, in the *rrpB* mutant, the expression of *fur* and *trxA* were up-regulated. The *rrpB* mutant and the *rrpAB* double mutant showed increased expression of *rrpB.*

Table 3.1 Differential gene expression in the *rrpA* mutant, *rrpB* mutant or *rrpAB* double mutant compared to the 11168H wild-type strain.

Strain	Gene	Product	Expression 11168H	Expression mutant
rrpA mutant	*katA*	catalase	125	67
rrpB mutant	*rrpB*	transcriptional regulator	38	337
	fur	ferric uptake regulator	66	136
	trxA	thioredoxin	294	525
rrpAB double mutant	*rrpB*	transcriptional regulator	38	86

Values indicate the number of reads of a transcript normalized using RPKM.

Results displayed are preliminary. Further investigation of RNA-seq data is in progress using different software for more in-depth analysis. These analyses could not be finished in time to be presented here.

3.3 Discussion

RrpA and RrpB have been identified as two new regulators of the *C. jejuni* oxidative stress response. The *C. jejuni* oxidative stress response is a complex and not fully understood defence mechanism. To investigate the roles of RrpA and RrpB, different oxidative stress assays were performed on the 11168H wild-type strain, *rrpA* and *rrpB* mutants and the *rrpAB* double mutant. Changes in gene expression in these three different mutants were also investigated.

Even though *C. jejuni* is a microaerobic microorganism, this bacterium is capable of surviving under high concentrations of oxygen and is found ubiquitously within the environment (Atack and Kelly, 2009). *C. jejuni* utilises a range of electron acceptors and electron donors for respiration, which allows *C. jejuni* to grow under different conditions and different oxygen availability (Woodall *et al.*, 2005). *C. jejuni* has multiple respiratory mechanisms which allow adaptation for growth under oxygen-limited conditions (Woodall *et al.*, 2005). *C. jejuni* utilises primarily only 5 specific amino acids as a carbon source and consumes hemin as a primary source of iron (Woodall *et al.*, 2005, Hofreuter *et al.*, 2008). *C. jejuni* has a complex and highly branched respiratory chain, being able to utilise different electron donors to generate energy (Weerakoon *et al.*, 2009).

C. jejuni fumarate metabolism is up-regulated under the oxygen restricted conditions of the chicken gut environment (Woodall *et al.*, 2005). Anaerobic conditions are stressful for *C. jejuni* and this bacterium is unable to grow under anaerobic conditions (Sellars *et al.*, 2002). Furthermore, DNA synthesis can only occur in the presence of oxygen (Sellars *et al.*, 2002). There is also variation in the metabolism amongst different *C. jejuni* strains. Some strains, such as 81-176, have additional respiratory functions compared to others, such as NCTC 11168. Additional features, such as an additional potassium uptake system in 81-176, may contribute to a more efficient ability to colonise human and animal intestinal epithelial cells (Hofreuter *et al.*, 2006).

Oxygen is a reactive molecule that diffuses easily through cell membranes, as a consequence, organisms need mechanisms to defend themselves against oxygen toxicity (Fridovich, 1998). Oxygen levels higher than the microorganism's natural intestinal habitat will cause cell damage and cell death (Imlay, 2013). Cell damage will occur due to the formation of ROS by partially reduced oxygen (Imlay, 2013).

Addition of electrons to the oxygen molecule generates superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$) (Fridovich, 1998, Imlay, 2013). These compounds are also generated continuously by bacterial cell respiration (Imlay, 2013).

C. jejuni has evolved complex mechanisms to cope with diverse environments and hosts. *C. jejuni* has the ability to counter different toxic oxidative compounds, generated by bacterial metabolism or from the external environment. These mechanisms are fundamental for bacterial survival under hostile environments (Palyada *et al.*, 2009).

C. jejuni expresses important enzymes to control the level of ROS within the cell. Some of the major scavengers are KatA, SodB and AhpC (Atack and Kelly, 2009). However, *C. jejuni* also expresses other different enzymes that complement the role of the major scavengers, hence optimising the control of ROS in the cell. The following *C. jejuni* enzymes also have a role in oxidative stress: Tpx, Bcp, CCPs, Dps, FdxA and Trx system (van Vliet and Ketley, 2001, Palyada *et al.*, 2004, Atack *et al.*, 2008, Atack and Kelly, 2009, Huergo *et al.*, 2013). However, most of these enzymes have not had their roles in the *C. jejuni* oxidative stress response completely clarified.

11168H mutants lacking one of the three major oxidative stress scavengers were used to confirm the role of KatA, SodB and AhpC in the oxidative stress response. These mutants were first exposed to H_2O_2 stress. KatA is the main enzyme that controls intracellular levels of H_2O_2 (Atack and Kelly, 2009). The *katA* mutant lacks this enzyme and, therefore, is not able to survive exposure to H_2O_2. The *sodB* and *ahpC* mutants were more resistant than the wild-type strain after exposure to H_2O_2. However, the *ahpC* mutant was more resistant than the *sodB* mutant. Both *sodB* and *ahpC* mutants may have compensatory mechanisms that increase *katA* expression and the elimination of H_2O_2. There is such an example of functional compensation due to a mutation in *Mycobacterium tuberculosis*. *M. tuberculosis* expresses KatG, which is the only catalase and the main enzyme that breaks down H_2O_2 in this bacterium (Sherman *et al.*, 1996). Mutation of *katG* leads to bacterial adaptation and overexpression of *ahpC* (Sherman *et al.*, 1996). In *C. jejuni*, the mutation of *sodB* and *ahpC* may result in increased *katA* expression.

C. jejuni lacks the classical regulators found in other enteropathogenic bacteria, but has functional substitutes, such as PerR (van Vliet *et al.*, 1999). PerR is a peroxide stress regulator substitute of OxyR, which is a common regulator of peroxide stress response genes in other Gram-negative bacteria (van Vliet *et al.*, 1999). *C. jejuni* possesses a number of different regulators of oxidative stress and their role in gene regulation frequently overlaps. Regulators known to have overlapping roles in oxidative stress regulation are PerR, Fur and CosR (van Vliet *et al.*, 1999, Palyada *et al.*, 2009, Hwang *et al.*, 2012). However, how exactly these regulators interact to fine tune the regulation of the oxidative stress defence mechanisms in *C. jejuni* is still not fully understood (Atack and Kelly, 2009).

RrpA and RrpB were identified as putative regulators of the *C. jejuni* oxidative stress response (Gundogdu *et al.*, 2007). Both regulators have now been shown to have a role in both the peroxide and aerobic stress responses (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). 11168H *rrpA* and *rrpB* mutants were exposed to different concentration of H_2O_2 and were shown to be more sensitive to H_2O_2 compared to the wild-type strain, suggesting that RrpA and RrpB may regulate *katA* expression. 11168H *rrpA* and *rrpB* complements restored the phenotype of the wild-type strain. However, the 11168H *rrpAB* double mutant demonstrated an increased resistance to H_2O_2 stress, suggesting that absence of both regulators generates a more resistant strain.

katA expression is regulated by PerR, which constitutively represses *katA* (van Vliet *et al.*, 2002). Mutation of *perR* generates a strain highly resistant to H_2O_2 with extremely high levels of KatA activity (van Vliet *et al.*, 1999). *C. jejuni* has a complex oxidative stress regulation system and PerR is not the only regulator of *katA*. CosR also regulates *katA* expression. However, CosR regulates *katA* positively and also regulates several other important oxidative stress genes (Hwang *et al.*, 2011, Hwang *et al.*, 2012). Fur is another regulator of *katA* expression. It is suggested that Fur represses *katA* expression and that Fur and PerR are the main regulators of oxidative stress response in *C. jejuni* (Palyada *et al.*, 2009). Furthermore, several of the oxidative stress genes regulated by Fur are co-regulated by PerR (Palyada *et al.*, 2009). *katA* is co-regulated by PerR, Fur and CosR (van Vliet *et al.*, 1999, Hwang *et al.*, 2012).

Absence of either RrpA or RrpB increased the sensitivity of the 11168H strain to H_2O_2, suggesting that absence of either regulator results in a decrease in KatA levels. To further investigate if RrpA and RrpB affect KatA expression levels, catalase activity assays were performed for the 11168H wild-type, *rrpA* and *rrpB* mutants and complements, as well as the *rrpAB* double mutant.

The catalase activity assays were performed using bacteria grown in two different media. Strains were grown either in Brucella broth, which is an iron-rich broth, or in MEMα, which is an iron-restricted broth. These two media were used to investigate changes in KatA expression under the different conditions since *katA* expression is repressed in presence of iron (van Vliet *et al.*, 1999). Iron is essential for bacterial growth because it is involved in metabolic processes and cell respiration (Cornelis *et al.*, 2011). However, an excess of iron is harmful to microorganisms due to the production of ROS via the Fenton reaction (Cornelis *et al.*, 2011). Therefore, a balance in iron metabolism is essential for bacterial survival. Iron metabolism plays an important role in *C. jejuni* metabolism and gene regulation (van Vliet *et al.*, 2002). Fur is the ferric uptake regulator responsible for maintaining the iron homeostasis (van Vliet *et al.*, 1998). Fur controls expression of ferritin, which controls intracellular homeostasis by storing and releasing iron (Ishikawa *et al.*, 2003). Dps binds to iron to prevent the generation of hydroxyl radicals under hydrogen peroxide stress via the Fenton reaction (Ishikawa *et al.*, 2003). Fur is a repressor and down-regulates the transcription of several genes when the intracellular concentration of Fe^{2+} is elevated (van Vliet *et al.*, 2002). Fe^{2+} is a co-factor to a Fur molecule that binds to the promoter region of iron-regulated genes blocking their transcription (Escolar *et al.*, 1999). Mutation of *fur* does not completely abolish the gene repression in *C. jejuni*, which indicates the presence of an iron-regulatory system independent of Fur (van Vliet *et al.*, 1998). Fur regulates the expression of some oxidative stress genes, such as *katA*, *fdxA* and *trxB* (Palyada et al., 2004). Iron may also regulate *C. jejuni* virulence, as protein glycosylation can be influenced by intracellular iron levels (Palyada *et al.*, 2004).

Both *katA* and *ahpC* expression are repressed by iron (van Vliet *et al.*, 1998). Furthermore, both *katA* and *ahpC* are constitutively repressed by PerR (van Vliet *et al.*, 1999). Mutation of *perR* de-repressed both *katA* and *ahpC* expression at high levels (van Vliet *et al.*, 1999). Growth of *C. jejuni* in iron limitation media was also shown

to increase *katA* and *ahpC* expression, although not at the same level as the de-repression caused by the *perR* mutation (van Vliet *et al.*, 1999). Iron also represses *tpx*, which has a role breaking down H_2O_2 (Palyada *et al.*, 2004, Atack *et al.*, 2008). It is also known that AhpC has a role in neutralising H_2O_2 in the cytoplasm, in addition to organic peroxides (Atack and Kelly, 2009).

The catalase activity levels from whole cell lysates indicated that both the 11168H *rrpA* and *rrpB* mutants had reduced catalase activity compared to the wild-type strain. The *rrpA* and *rrpB* complement strains exhibited wild-type level of catalase activity. The 11168H *rrpAB* double mutant also demonstrated catalase activity at a similar level compared to the wild-type strain, despite the increased resistance to H_2O_2 (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). This suggests that enzymes other than catalase may be playing a role in the breakdown of H_2O_2 in the 11168H *rrpAB* double mutant. Other genes expressing proteins with a role in the peroxide stress defence may be up-regulated to contribute to H_2O_2 breakdown, such as AhpC, Tpx or CCPs. As expected, the 11168H *perR* mutant demonstrated extremely high levels of catalase activity due to de-repression of *katA* expression (van Vliet *et al.*, 1999).

The 11168H *katA* mutant was used as a negative control in the catalase activity assay. When the 11168H *katA* mutant was grown in iron-rich media, no catalase activity was detected. However, when the 11168H *katA* mutant was grown in MEMα media, a low level of catalase activity was observed. This probably occurred because iron restricted media increases the expression of *ahpC* and *tpx*, both genes encoding enzymes with the ability to neutralise H_2O_2 (van Vliet *et al.*, 1998, Palyada *et al.*, 2004, Atack *et al.*, 2008, Atack and Kelly, 2009). A compensatory mechanism may also have occurred, up-regulating *ahpC* and *tpx.* A slight increase in catalase activity can be observed in the wild-type strain when grown in low iron media compared to high iron media. However, this was not statistically significant. This was an expected observation because *katA* and *ahpC* are up-regulated under iron restricted conditions (van Vliet *et al.*, 1999).

Electrophorectic Mobility Shift Assays (EMSA) have been performed to investigate whether RrpA and RrpB are auto-regulated as this feature is common to the MarR family of transcriptional regulators (Kumarevel, 2012). EMSA results showed that both RrpA and RrpB are auto-regulated. RrpA binds upstream of *rrpA,* and RrpB binds

upstream of *rrpB* (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). RrpA also binds upstream of *katA* indicating active regulation of *katA* expression (Gundogdu et al., 2015). However, RrpB does not appear to bind upstream of *katA* (Gundogdu et al., 2015). This indicates that RrpA directly regulates *katA* expression, whilst RrpB may affect *katA* expression indirectly. It was speculated that RrpA and RrpB may interact with each other to regulate *katA* expression. Following this observation, EMSA assays were performed to investigate if RrpA would bind upstream of the *rrpB* promoter and vice versa. RrpA was shown to bind upstream of *rrpB*. However, RrpB does not bind upstream of *rrpA* (Gundogdu *et al.*, unpublished data). This suggests that RrpA directly regulates *katA* and *rrpB* expression. RrpB does not appear to directly regulate either *katA* or *rrpA* expression, but RrpB may interact with RrpA to regulate *katA* expression. However, this is only true for strains that contain both genes (*rrpA* and *rrpB*). RrpA does not appear to regulate *katA* as efficiently in strains that only contain *rrpA*. Further studies are necessary to understand whether and how RrpB interacts with RrpA to regulate *katA* expression, and why RrpB does not seem to be important for all *C. jejuni* strains.

Gene expression based on RT-PCR was performed to further investigate if RrpA and RrpB affect *katA* expression. RT-PCR indicated that *katA* expression is reduced in the 11168H *rrpA* and *rrpB* mutants, whilst the 11168H *rrpAB* double mutant demonstrated a non-significant increase in *katA* expression. qPCR data also suggests that the 11168H *rrpA* and *rrpB* mutants have reduced *katA* expression and also indicate increased *katA* expression in the 11168H *rrpAB* double mutant. However, more qPCR replicates are required.

Preliminary RNA-seq data analysis demonstrated that a few genes related to the oxidative stress defence had a change of expression in the mutants compared to the wild-type strain. In agreement with the phenotypic assays, the 11168H *rrpA* mutant demonstrated reduced *katA* expression compared to the wild-type strain. However, the 11168H *rrpB* mutant and the 11168H *rrpAB* double mutant did not demonstrate any changes in *katA* expression. It was expected that the 11168H *rrpB* mutant would have reduced *katA* expression due to increased sensitivity to H_2O_2 and reduced catalase activity. It was also expected that the 11168H *rrpAB* double mutant would up-regulate oxidative stress genes with a role in neutralising H_2O_2. However, RNA was isolated from bacteria grown under microaerobic conditions. RNA should be isolated after

bacteria have been exposed to oxidative stress conditions. This will allow the investigation of changes in gene expression induced by the oxidative stress. Changes in *katA* expression may be observed comparing the wild-type strain and the different mutants after exposure to H_2O_2.

The 11168H *rrpB* mutant demonstrated up-regulation of *rrpB*. This was also observed in previous microarray data, which identified *rrpB* as the most up-regulated gene in the 11168H *rrpB* mutant (Gundogdu *et al.,* 2011). Further analysis demonstrated that *rrpB* expression is controlled by a negative autoregulation feedback mechanism and that the sequence upstream of the inserted kanamycin cassette was being transcribed in the 11168H *rrpB* mutant. This is probably why *rrpB* is also up-regulated in the 11168H *rrpAB* double mutant. The 11168H *rrpB* mutant also demonstrated up-regulation of *fur* and *trxA.* Thioredoxins have a role in the oxidative stress response by activating specific thiol-dependent antioxidant systems (Lu and Holmgren, 2014). When thioredoxin reduces a cytoplasmic enzyme, thioredoxin becomes oxidised, and will become reduced again by thioredoxin reductase (Ritz *et al.,* 2000). Thioredoxins can also directly reduce H_2O_2, quench single oxygen molecules and eliminate $OH^{\bullet}$ (Zeller and Klug, 2006). However, both TrxA and TrxB are necessary for a functioning Trx system. Fur and PerR co-regulate several genes involved in the oxidative stress defence mechanism in *C. jejuni* (Palyada *et al.,* 2009). *trxB* is one of the genes regulated by both Fur and PerR (Holmes *et al.,* 2005). However, which regulators control *trxA* expression is not known yet.

RT-PCR and RNA seq were performed on RNA isolated from bacteria grown under microaerobic conditions without any kind of oxidative stress exposure. These conditions were chosen to observe gene expression without external ROS stimulus. Further analyses are necessary to understand changes in gene expression during bacterial exposure to ROS. Future work will require that bacterial cultures be exposed to oxidative stress, such as sub-lethal levels of H_2O_2, or grown under aerobic conditions to identify important changes in gene expression. The changes in gene expression under ROS stress could then be compared to gene expression under microaerobic conditions.

The 11168H *rrpB perR* double mutant was highly resistant to H_2O_2, at the same level as the 11168H *perR* mutant. It is possible that mutation of *rrpB* is not dramatically

influencing the phenotype caused by the *perR* mutation. Mutation of *perR* results in extremely high expression of *katA* and *ahpC* (van Vliet et al., 1999). The 11168H *perR* mutant level of resistance to H_2O_2 stress and the levels of catalase activity observed were very high. The 11168H *rrpB* mutant demonstrated a significant but subtle phenotype compared to the 11168H wild-type strain. Therefore, it is probable that there will be no alteration in the H_2O_2 resistance phenotype of the 11168H *rrpB perR* mutant compared to the 11168H *perR* mutant.

Different wild-type strains and respective mutants were also investigated under H_2O_2 stress. The distribution of *rrpA* and *rrpB* amongst *C. jejuni* strains has been investigated (see Chapter 5). It was observed that not all *C. jejuni* strains contain *rrpB*. Further analyses demonstrated that 11168H and 81-176 contain both *rrpA* and *rrpB*, whilst 81116 and M1 contain only *rrpA*.

81-176 *rrpA* and *rrpB* mutants also exhibited increased sensitivity to H_2O_2 stress, demonstrating the same phenotype as the 11168H *rrpA* and *rrpB* mutants. However, *rrpA* mutants in the other two wild-type strains, 81116 and M1, were not more sensitive to H_2O_2 stress compared to the respective wild-type strains. The 81116 and M1 wild-type strains and the respective *rrpA* mutants were also more resistant to H_2O_2 stress compared to 11168H and 81-176 wild-type strains. This suggests that variation in the presence of *rrpB* may have an impact on the peroxide stress response.

Further studies supported the observation that variation in the distribution of *rrpA* and *rrpB* influences *C. jejuni* resistance to hydrogen peroxide stress. *C. jejuni* strains that contain only *rrpA* were demonstrated to have a natural tendency to be more resistant to H_2O_2 than *C. jejuni* strains that contain both *rrpA* and *rrpB* (Gundogdu *et al.*, unpublished data). When *rrpA* is mutated in the strains that only contain *rrpA*, no effect on H_2O_2 sensitivity is observed compared to the respective wild-type strain (Gundogdu *et al.*, unpublished data). Further investigations are necessary to understand the significance of the presence of RrpB, and whether RrpB interacts with RrpA, since not all *C. jejuni* strains contain *rrpB*.

Menadione was used as a generator of superoxide anions through redox cycling. Menadione is reduced by NADPH and oxidised by O_2 generating $O_2^{\bullet-}$ (Greenberg and Demple, 1989). This compound was used to investigate the activity of SodB in the different wild-type strains and mutants. SodB has an important role in *C. jejuni*

defence against oxidative stress and is the main enzyme that breaks down superoxides (Atack and Kelly, 2009).

11168H mutants lacking one of the three major oxidative stress scavengers were used to investigate the role of KatA, SodB and AhpC in the menadione stress response. The 11168H *katA* mutant did not show increased sensitivity compared to the wild-type strain. However, the 11168H *sodB* mutant was highly sensitive to menadione stress. This finding agrees with Stead and Park (2000) study where the *katA* mutant also did not show increased sensitivity to superoxide anions, whilst the *sodB* mutant demonstrated increased sensitivity. In this study, the *ahpC* mutant also demonstrated an increased sensitivity to menadione compared to the wild-type strain. Palyada *et al.* (2009) have shown that *C. jejuni* increases *ahpC* expression in presence of menadione. Both *sodB* and *ahpC* were up-regulated in the presence of menadione, but *sodB* expression was increased at a higher level compared to *ahpC* expression (Palyada et al., 2009). This indicates that besides SodB, AhpC also has a role in neutralising the toxic effects of superoxide anions and the 11168H *ahpC* mutant did indeed exhibit increased sensitivity to menadione in this study.

11168H *rrpA* and *rrpB* mutants were also exposed to menadione stress. Both mutants did not demonstrate any differences in sensitivity compared to the wild-type strain, suggesting that neither RrpA nor RrpB have a role in regulating *sodB* expression. However, the 11168H *rrpAB* double mutant demonstrated increased resistance to menadione stress, suggesting that absence of both RrpA and RrpB increases the expression of *sodB*. Microarray data demonstrated 1.24-fold increase of *sodB* expression in the 11168H *rrpB* mutant (Gundogdu *et al.*, 2011). In this study, RT-PCR did not demonstrate changes in *sodB* expression comparing the different mutants with the wild-type strain. *sodB* expression in the 11168H *rrpB* mutant seems to increase. However, this was not significant. RNA-seq data also did not show any changes in *sodB* expression.

Unlike the 11168H *rrpB* mutant, the 81-176 *rrpB* mutant demonstrated a small increase in sensitivity to menadione compared to the respective wild-type strains. This suggests that RrpB may have a more specific role in the superoxide stress response in this strain. No differences were observed in sensitivity to menadione stress with the 81-176, 81116 and M1 *rrpA* mutants compared to the respective wild-type strains.

Cumene hydroperoxide is an organic peroxide used to generate a different type of ROS involved in bacterial oxidative stress. In this study, cumene hydroperoxide was used to assess differences between mutants and wild-type strains. AhpC is the main enzyme that counters organic hydroperoxides in many different bacteria (Sherman *et al.*, 1996, Ellis and Poole, 1997, Lee *et al.*, 2014). Mutation of *C. jejuni ahpC* resulted in a strain with increased sensitivity to cumene hydroperoxide (Baillon *et al.*, 1999). In this study, the 11168H *ahpC* mutant also exhibited increased sensitivity to cumene hydroperoxide.

Both 11168H *katA* and *sodB* mutants demonstrated an increased resistance to cumene hydroperoxide compared to the wild-type strain. Palyada *et al.* (2009) demonstrated that both *katA* and *sodB* expression are up-regulated in the presence of cumene hydroperoxide. This suggests that KatA and SodB might also exhibit organic hydroperoxidase activity (Palyada *et al.*, 2009). The reason why 11168H *katA* and *sodB* mutants exhibit increased resistance is unclear. This may indicate that when *katA* is mutated, *sodB* is up-regulated as a compensatory mechanism to neutralise the toxic effects of cumene hydroperoxide, whilst *katA* may be up-regulated when *sodB* is mutated.

Both 11168H *rrpA* and *rrpB* mutants did not demonstrate any differences in sensitivity to cumene hydroperoxide compared to the wild-type strain. Therefore, neither RrpA nor RrpB appear to have a role in regulating AhpC. However, the 11168H *rrpAB* double mutant demonstrated increased resistance to cumene hydroperoxide compared to the wild-type strain, suggesting that the absence of both RrpA and RrpB may increase *ahpC* expression. Even though the 11168H *rrpB* mutant did not show increased sensitivity to cumene hydroperoxide, microarray data demonstrated that this mutant had 1.27-fold decreased *ahpC* expression (Gundogdu *et al.*, 2011). Furthermore, RT-PCR also demonstrated reduced *ahpC* expression in the 11168H *rrpB* mutant. However, RNA-seq did not show any changes in *ahpC* expression for any of the mutants investigated.

Similarly, the 11168H *perR* mutant also demonstrated an increased resistance to cumene hydroperoxide. PerR is a repressor of *katA* and *ahpC*. Therefore, a *perR* mutation leads to *katA* and *ahpC* overexpression (Palyada *et al.*, 2009), which explains the increased resistance of the 11168H *perR* mutant to cumene hydroperoxide. PerR

also regulates several other genes in the oxidative stress response and different regulators of *C. jejuni* oxidative stress responses have a tendency to overlap.

The 81-176 *rrpA* and *rrpB* mutants and the M1 *rrpA* mutant did not demonstrate differences in sensitivity compared to the respective wild-type strains. However, the 81116 *rrpA* mutant demonstrated an increased resistance to cumene hydroperoxide compared to the wild-type strain. This suggests that RrpA may play a different role in different wild-type strains. However, this phenotype could also be explained due to a point mutation in 81116 wild-type strain that affects *ahpC* expression. To test this hypothesis a new 81116 wild-type strain should be used to generate a new 81116 *rrpA* mutant, and then these strains should be exposed again to cumene hydroperoxide stress. Sequencing the 81116 wild-type strain and *rrpA* mutant could also indicate if any mutations had occurred to explain this phenotype.

Both 11168H *rrpA* and *rrpB* mutants displayed differences in KatA activity compared to the wild-type strain. However, when these mutants were assessed for SOD activity, no differences were found compared to the wild-type strain. This suggests that SodB is not responsible for the phenotypes observed in the 11168H *rrpAB* double mutant. 11168H *perR* and *katA* mutants had similar levels of SOD activity compared to the wild-type strain. However, the 11168H *ahpC* demonstrated increased levels, which is the opposite of what was expected. The 11168H *ahpC* mutant was more sensitive to menadione. Therefore, it was expected to have reduced SOD activity. However, Palyada *et al.* (2009) demonstrated that *sodB* expression is increased in presence of Menadione, which could explain the increased SOD activity in the 11168H *ahpC* mutant. Further investigation is necessary to understand this observation.

Both 11168H *rrpA* and *rrpB* mutants demonstrated to have increased sensitivity to aerobic stress compared to the wild-type strain (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). Both mutants showed reduced ability to survive when grown in broth under aerobic stress (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). These data suggest that RrpA and RrpB also have a role in the *C. jejuni* aerobic stress.

The assays performed demonstrated that RrpA and RrpB have a role in the *C. jejuni* oxidative stress defence mechanism. RrpA and RrpB have a role in the H_2O_2 defence mechanism through regulation of *katA* expression. RrpA has a direct role regulating *katA* expression. However, the exact role of RrpB is still not clear. RrpA and RrpB do

not seem to affect *sodB* or *ahpC* expression. The absence of both *rrpA* and *rrpB* in the 11168H *rrpAB* double mutant demonstrated increased resistance to H_2O_2, menadione and cumene hydroperoxide. However, RNA-seq analysis of this strain did not show any changes in the expression of *katA*, *sodB* and *ahpC*.

Further investigations are required to fully understand how these two regulators interact with other *C. jejuni* regulators of the oxidative stress and why the double mutation increased resistance to different oxidative stresses. Preliminary RNA-seq confirmed that RrpA is regulating *katA* expression. However, *katA* expression appears unaltered in the 11168H *rrpB* mutant and in the 11168H *rrpAB* double mutant. The RNA-seq data is currently being further analysed using different software to investigate more thoroughly differences in gene expression. Further analysis using ChIP-seq will also be performed to investigate RrpA and RrpB interaction with DNA and identification of the sequence binding sites. ChIP-seq will be an important tool to map all the binding sites throughout the *C. jejuni* genome, identifying exactly which genes are being regulated by RrpA and RrpB.

4 Further investigation into the role of RrpA and RrpB in the *C. jejuni*

4.1 Introduction

The 11168H *rrpB* mutant has previously been shown to exhibit a decreased ability for intracellular survival within Caco-2 human intestinal epithelial cells and J774A.1 mouse macrophages (Gundogdu *et al.*, 2011). Also has been demonstrated that RrpB was not involved in the nitrosative stress response (Gundogdu *et al.*, 2011). Both the 11168H *rrpA* and *rrpB* mutants demonstrated a reduction in virulence in the *Galleria mellonella* infection model (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). Electrophoretic mobility shift assays indicated that both RrpA and RrpB are DNA binding proteins. RrpA binds to the promoter region of *rrpA*, whilst RrpB binds to the promoter region of *rrpB* (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). Further analyses were performed to investigate other phenotypic differences caused by the mutation of *rrpA* or *rrpB* in the *C. jejuni* 11168H wild-type strain.

4.2 Results

4.2.1 Motility assays

C. jejuni is a highly motile bacterium (Hendrixson and DiRita, 2004). Flagella are important for biofilm formation (Kalmokoff *et al.*, 2006), secretion of virulence factors (Konkel *et al.*, 2004) and adhesion to and invasion of intestinal epithelial cells (Song *et al.*, 2004).

Motility assays were performed to investigate whether the mutation of *rrpA* or *rrpB* in the 11168H wild-type strain affected bacterial motility directly or if the insertion of the antibiotic resistance cassette resulted in any polar effects, which could affect downstream gene expression. Motility assays were performed as described in Section 2.4.1. Motility halos were measured after 24, 48 and 72 h incubation. No significant differences were observed in the diameter of the motility halo comparing the *rrpA* mutant, *rrpB* mutant or *rrpAB* double mutant with the 11168H wild-type strain (Figure 4.1 and Figure 4.2).

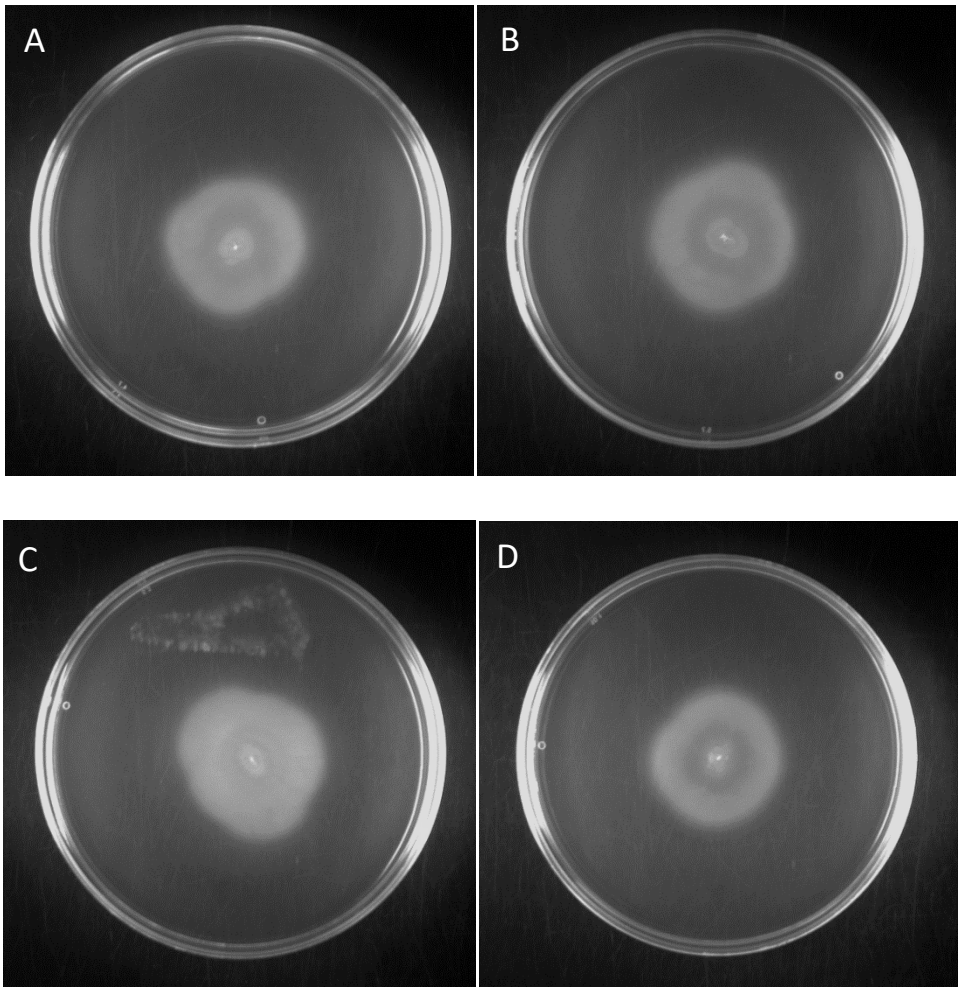

Figure 4.1 Motility assays for different *C. jejuni* mutants. A: 11168H wild-type strain; B: *rrpA* mutant; C: *rrpB* mutant, D: *rrpAB* double mutant. A bacterial suspension was prepared from a 24 h BA plate to an OD_{600} of 1.0 and 5 µl of this suspension was inoculated into the centre of a motility plate. Plates were incubated at 37ºC under microaerobic conditions. Halo diameter was measured after 24, 48 and 72 h. Images displayed were recorded after 72 h.

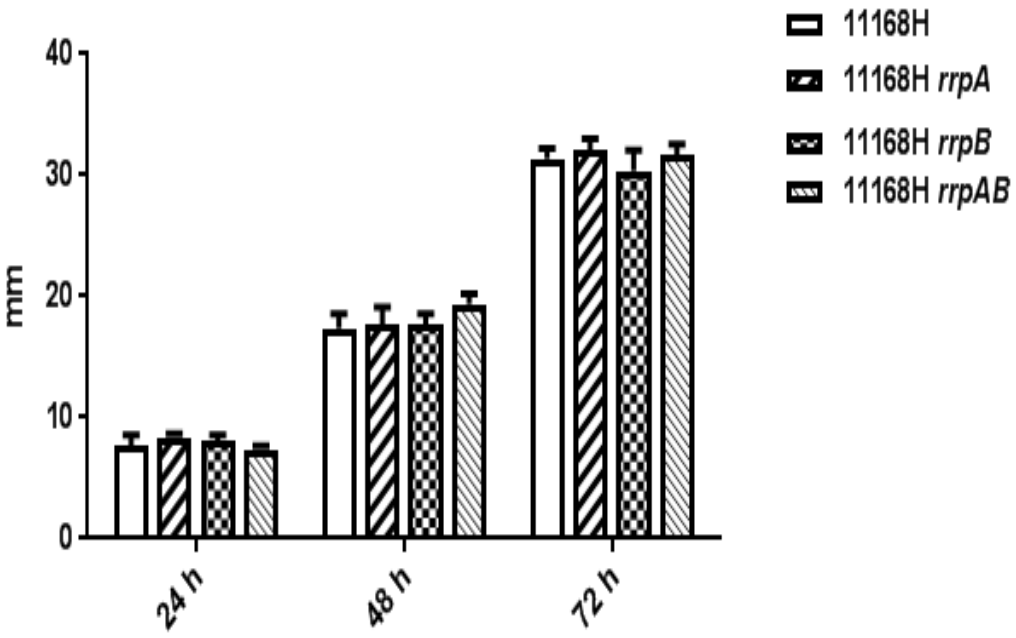

Figure 4.2 Motility assays for different *C. jejuni* mutants. A bacterial suspension was prepared from a 24 h BA plate to an OD_{600} of 1.0 and 5 µl of this suspension was inoculated into the centre of a motility plate. Plates were incubated at 37ºC under microaerobic conditions. Halo diameter was measured after 24, 48 and 72 h. Data represents at least three biological replicates.

No differences were also observed in the diameter of the motility halo of the *katA* mutant, *sodB* mutant, *perR* mutant or *rrpB perR* double mutant compared to the 11168H wild-type strain (Figure 4.3).

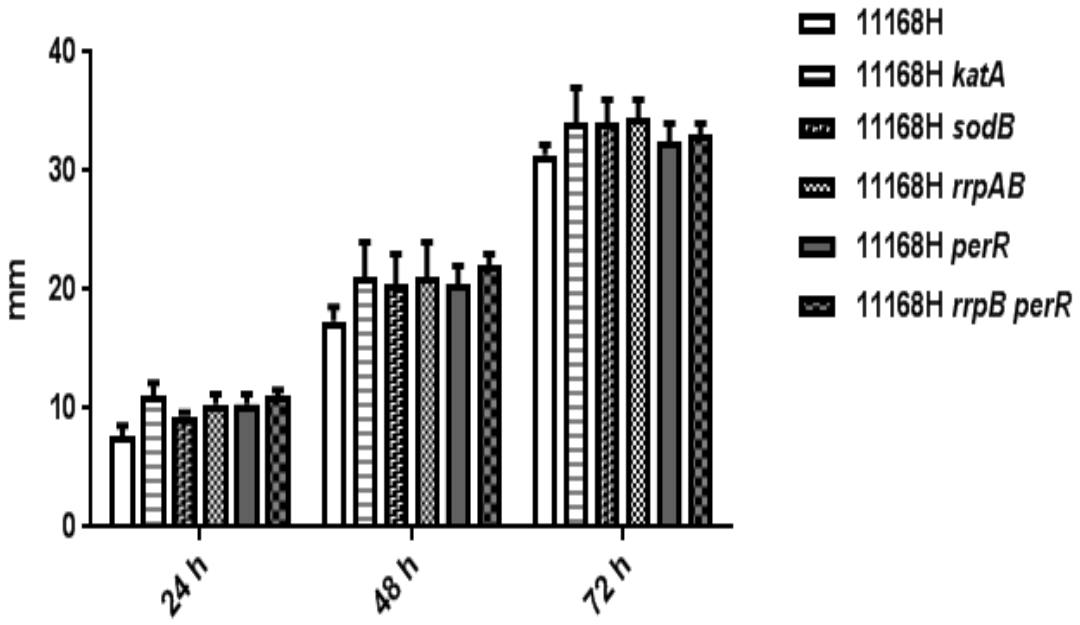

Figure 4.3 Motility assays for different *C. jejuni* mutants. A bacterial suspension was prepared from a 24 h BA plate to an OD_{600} of 1.0 and 5 µl of this suspension was inoculated into the centre of a motility plate. Plates were incubated at 37ºC under microaerobic conditions. Halo diameter was measured after 24, 48 and 72 h. Data represents at least three biological replicates.

4.2.2 *Galleria mellonella* haemolymph collection

G. mellonella larvae have been established as a good infection model to investigate the virulence of *Campylobacter* strains (Champion *et al.*, 2010). *G. mellonella* larvae were infected with the following: the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant and *rrpAB* double mutant to assess the effects of these mutations on virulence. *G. mellonella* were infected with a bacterial suspension containing 10^6 CFU for each strain. Haemolymph was collected after 16 h, serially diluted and pipetted onto BA plates, as described in Section 2.4.12. *C. jejuni* colonies were counted after 48 h. Reduced haemolymph CFUs were observed following infection with both the *rrpA* and *rrpB* mutants compared to infection with the 11168H wild-type strain. However,

these were not statistically significant (Figure 4.4). Infection with the *rrpAB* double mutant resulted in haemolymph CFUs similar to the wild-type strain.

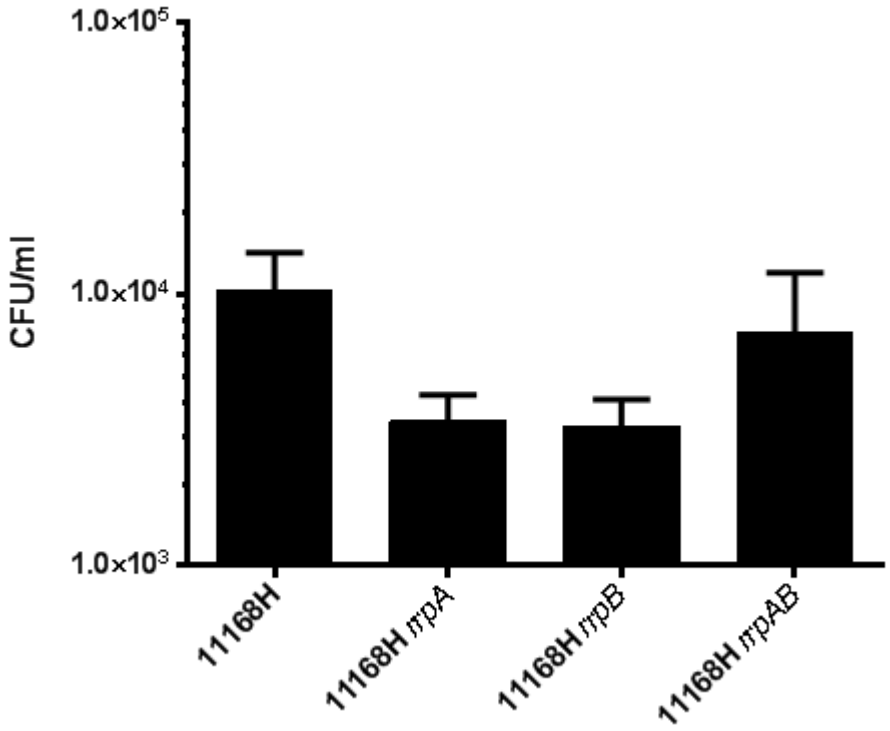

Figure 4.4 Survival of *C. jejuni* strains in *Galleria mellonella* larvae. 10 µl bacterial suspension (10^6 CFU) of the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant or *rrpAB* double mutant was injected into *G. mellonella* larvae. Larvae were incubated at 37ºC for 16 h. Haemolymph was drained from individual larvae, serially diluted (10^{-1} to 10^{-6}) and pipetted onto BA plates. CFUs were counted after 48 h incubation. The experiments were repeated 4 times. Data represents at least three biological replicates.

4.2.3 Biofilm formation

C. jejuni is capable of forming biofilms (Joshua *et al.*, 2006). Different environmental signals can trigger biofilm formation, for example the presence of environmental stresses are known to stimulate biofilm formation (O'Toole *et al.*, 2000). However, the mechanisms controlling biofilm formation in *C. jejuni* are still not yet fully understood (O'Toole *et al.*, 2000).

4.2.3.1 Biofilm formation in Mueller Hinton broth

Biofilm assays were performed to investigate whether mutation of *rrpA* or *rrpB* would affect the ability of *C. jejuni* to form biofilms. Biofilm formation assays were performed for the 11168H wild-type strain, *rrpA*, *rrpB*, *ahpC*, *katA*, *sodB* and *perR* mutants, and the *rrpAB* and *rrpB perR* double mutants as described in Section 2.4.9. Strains were inoculated in MH broth and incubated for 72 h under either aerobic or microaerobic conditions.

Both the *rrpA* and *rrpB* mutants exhibited a significant increase in biofilm formation under aerobic conditions compared to the wild-type strain. The *rrpAB* double mutant formed biofilms at a similar level as the wild-type strain. All the other mutants (*ahpC*, *katA*, *sodB*, *perR* and *rrpB perR*) also exhibited a significant increase in biofilm formation under aerobic conditions compared to the wild-type strain (Figure 4.5).

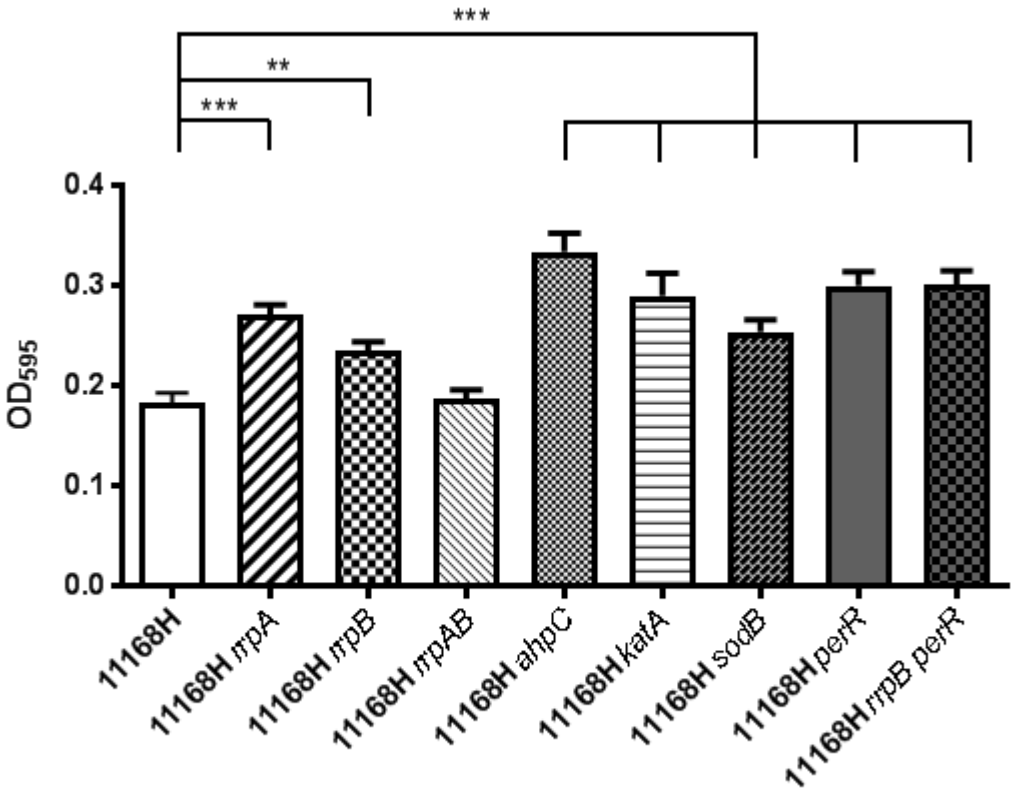

Figure 4.5 Capacity of the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant, *rrpAB* double mutant, *ahpC* mutant, *katA* mutant, *sodB* mutant, *perR* mutant and *rrpB perR* double mutant to form biofilms under aerobic conditions. Bacterial suspensions were prepared from 24 h BA plates and MH broth were inoculated to an OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then the OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Plates were incubated at 37ºC for 72 h under aerobic conditions. Biofilms were stained with 1% (w/v) crystal violet, destained and the OD_{595} recorded. Data represents at least three biological replicates. ** = $p < 0.01$; *** = $p < 0.001$.

Similar biofilm formation phenotypes were observed when the strains were incubated under microaerobic conditions. The *rrpA* mutant and *rrpB* mutant exhibited a significant increase in biofilm formation compared to the wild-type strain. The *rrpAB* double mutant formed biofilms at a similar level as the wild-type strain. All the other

mutants (*ahpC*, *katA*, *sodB*, *perR* and *rrpB perR*) also exhibited a significant increase in biofilm formation under microaerobic conditions compared to the wild-type strain (Figure 4.6).

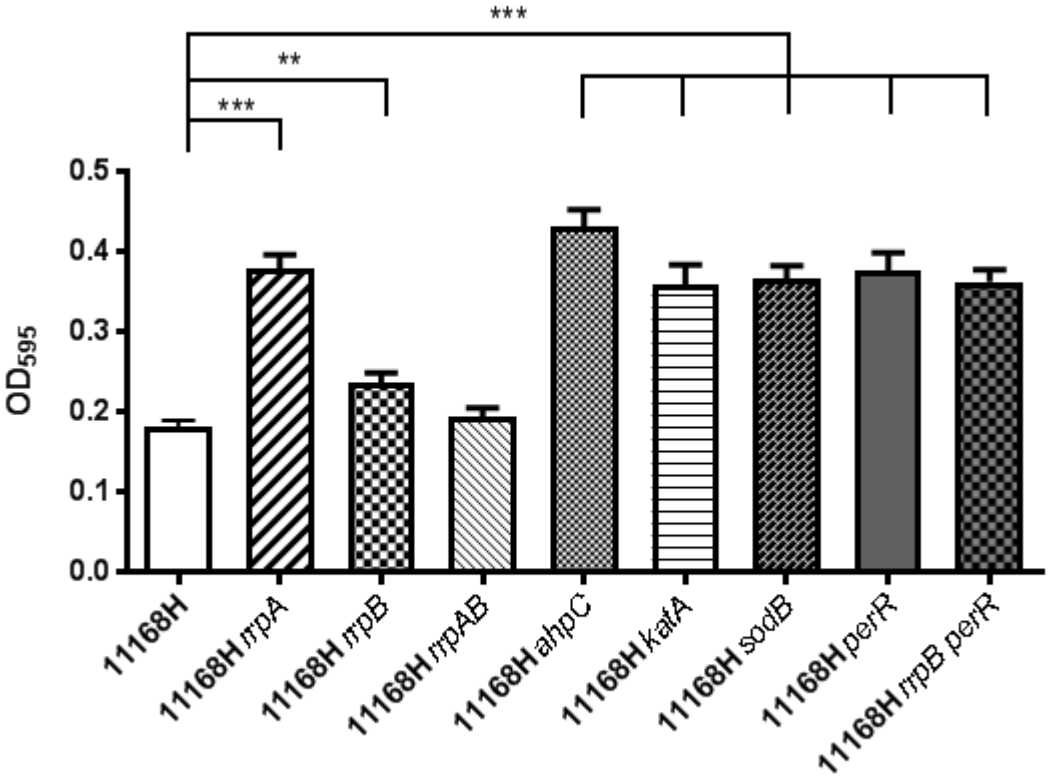

Figure 4.6 Capacity of the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant, *rrpAB* double mutant, *ahpC* mutant, *katA* mutant, *sodB* mutant, *perR* mutant and *rrpB perR* double mutant to form biofilms under microaerobic conditions. Bacterial suspensions were prepared from 24 h BA plates and MH broth were inoculated to an OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then the OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Plates were incubated at 37ºC for 72 h under microaerobic conditions. Biofilms were stained with 1% (w/v) crystal violet, destained and the OD_{595} recorded. Data represents at least three biological replicates. ** = $p < 0.01$; *** = $p < 0.001$.

4.2.3.2 Biofilm formation in Mueller Hinton supplemented with diluted chicken juice

Organic materials can play an important role in bacterial biofilm formation (Brown *et al.*, 2014). Compounds such as proteins, lipids, carbohydrates and sugars can promote a rich environment for bacterial development (Brown *et al.*, 2014). Chicken juice was diluted as described in Section 2.4.10.2 due to high levels of protein precipitation during the incubation period. This protein precipitation increased the staining with crystal violet, which was not related to bacterial biofilm formation. The dilution of the chicken juice prevented this precipitation.

Biofilm formation in presence of diluted chicken juice was performed on the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant and *rrpAB* double mutant. Strains were inoculated in MH broth + 5% (v/v) diluted chicken juice and incubated for 72 h under aerobic or microaerobic conditions. Biofilms were stained with crystal violet as described in Section 2.4.10.2. Diluted chicken juice did not affect biofilm formation under aerobic conditions, except with the *rrpA* mutant where decreased biofilm formation was observed compared to the *rrpA* mutant grown without chicken juice (Figure 4.7).

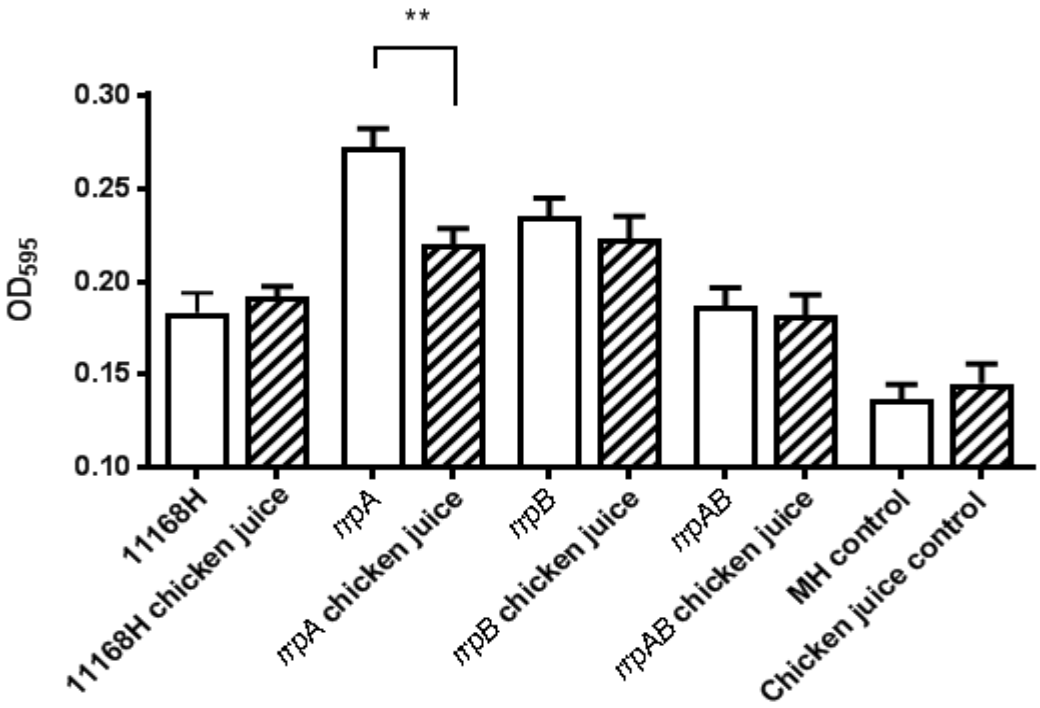

Figure 4.7 Capacity of the 11168H wild-type strain, *rrpA*, *rrpB* and *rrpAB* mutants to form biofilms in presence of diluted chicken juice under aerobic conditions. Bacterial suspensions were prepared from 24 h plates and MH broths were inoculated with OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Controls: MH and MH + 5% (v/v) diluted chicken juice. Plates were incubated at 37ºC for 72 h under aerobic conditions. Plates were stained with 1% (w/v) crystal violet and OD_{595} measured. Data represents at least three biological replicates. ** = $p < 0.01$.

Similar phenotypes were observed when the strains were incubated under microaerobic conditions in presence of undiluted chicken juice. Diluted chicken juice did not affect biofilm formation, except for the *rrpA* mutant that exhibited decreased biofilm formation compared to the *rrpA* mutant grown without chicken juice (Fig 4.8).

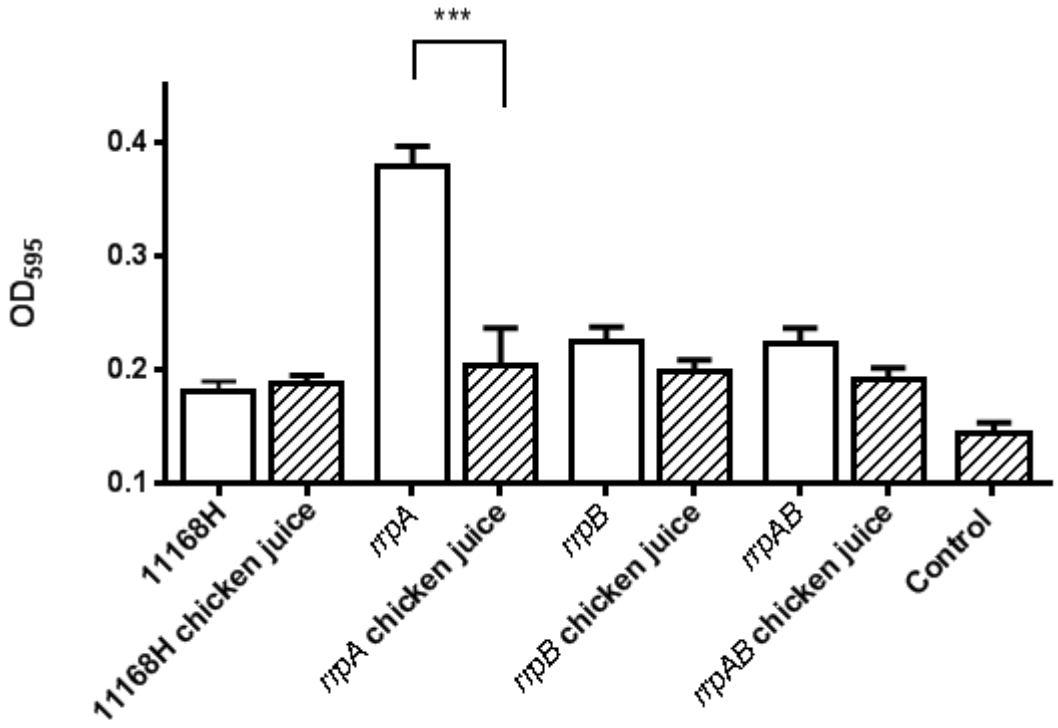

Figure 4.8 Capacity of the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant, and *rrpAB* double mutant to form biofilms in presence of diluted chicken juice under microaerobic conditions. Bacterial suspensions were prepared from 24 h plates and MH broths were inoculated with OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Controls: MH and MH + 5% (v/v) diluted chicken juice. Plates were incubated at 37ºC for 72 h under microaerobic conditions. Plates were stained with 1% (w/v) crystal violet and OD_{595} measured. Data represents at least three biological replicates. *** = $p < 0.001$.

4.2.3.3 Biofilm formation in Mueller Hinton supplemented with undiluted chicken juice

Based on previously published methodology (Brown *et al.*, 2014), undiluted chicken juice was added to media and stained with TTC. TTC was used to overcome the problems with protein precipitation and excess staining with crystal violet. TTC only stains the live bacteria within the biofilm because metabolically active cells reduce TTC to a red compound that can be measured colorimetrically. TTC was prepared as described in the Section 2.4.10.3.

Biofilm formation in presence of undiluted chicken juice was performed on the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant, and *rrpAB* double mutant. Strains were inoculated in MH broth + 5% (v/v) undiluted chicken juice and incubated for 72h under aerobic or microaerobic conditions. Biofilms were stained with TTC as discussed above. All strains incubated with undiluted chicken juice exhibited increased biofilm formation under aerobic conditions compared to biofilm formation in the absence of chicken juice. However, the wild-type strain exhibited a slightly larger increase in biofilm formation compared to *rrpA* mutant. The same phenotype was also observed for the *rrpB* mutant. The *rrpAB* double mutant exhibited increased biofilm formation at a similar level to the wild-type strain (Figure 4.9).

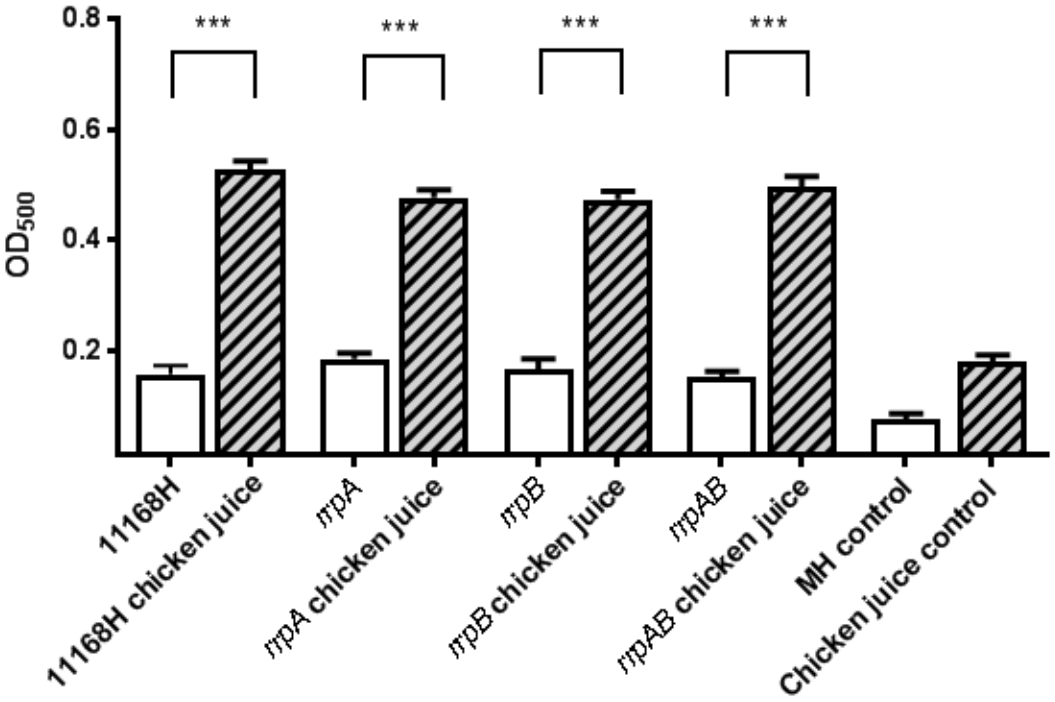

Figure 4.9 Capacity of the 11168H wild-type strain, *rrpA*, *rrpB* and *rrpAB* mutants to form biofilms in presence of undiluted chicken juice under aerobic conditions. Bacterial suspensions were prepared from 24 h plates and MH broths were inoculated with OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Controls: MH and MH + 5% (v/v) undiluted chicken juice. Plates were incubated at 37ºC for 72 h under aerobic conditions. Plates were stained with TTC and OD_{500} measured. Data represents at least three biological replicates. *** = $p < 0.001$.

A similar phenotype was observed when the strains were incubated under microaerobic conditions in presence of undiluted chicken juice. All strains increased biofilm formation under microaerobic conditions compared to biofilm formation in the absence of chicken juice (Figure 4.10). However, the *rrpA* mutant exhibited a

smaller increase in biofilm formation compared to the wild-type strain, as well as to the *rrpAB* double mutant.

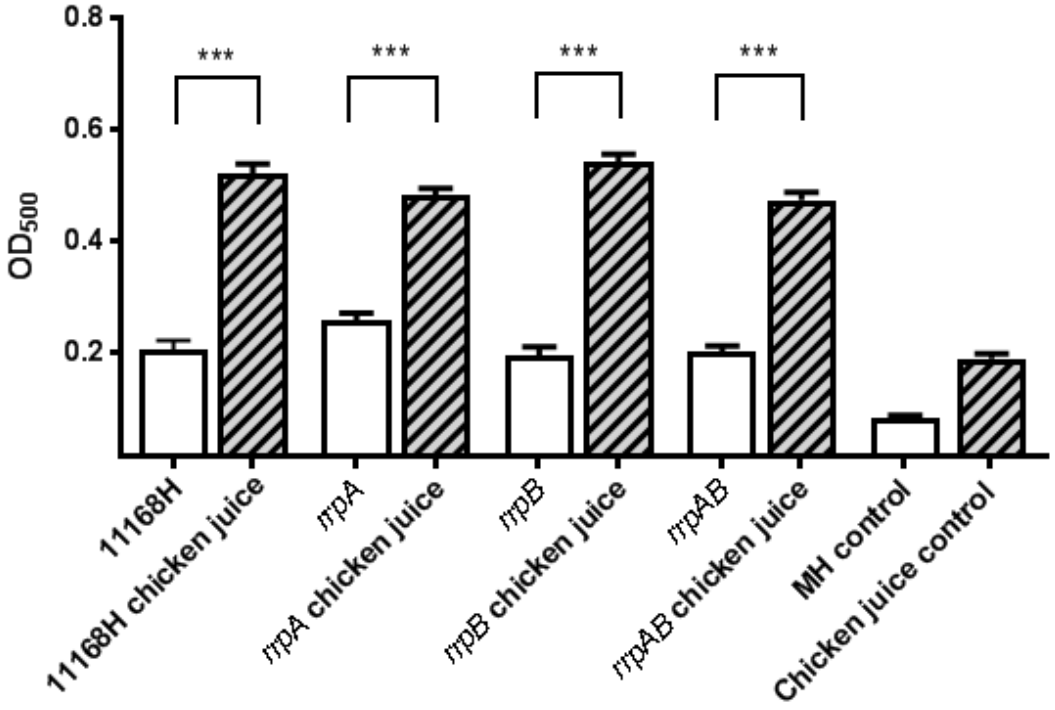

Figure 4.10 Capacity of the 11168H wild-type strain, *rrpA*, *rrpB* and *rrpAB* mutants to form biofilms in presence of undiluted chicken juice under microaerobic conditions. Bacterial suspensions were prepared from 24 h plates and MH broths were inoculated with OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Controls: MH and MH + 5% (v/v) undiluted chicken juice. Plates were incubated at 37ºC for 72 h under microaerobic conditions. Plates were stained with TTC and OD_{500} measured. Data represents at least three biological replicates. *** = $p < 0.001$.

4.2.4 Growth of the 11168H wild-type strain in the presence of sodium taurocholate

In order to investigate if the bile salt sodium taurocholate (ST) can influence the *C. jejuni* oxidative stress response, the 11168H wild-type strain was grown on BA plates containing two different concentrations of ST and then exposed to H_2O_2 stress. However, before performing the H_2O_2 assays, the 11168H wild-type strain was grown in Brucella broth containing the two different physiological concentrations of ST (0.1% (w/v) and 0.2% (w/v)) to assess any impact on bacterial growth or survival.

OD_{600} readings were recorded at different time points and compared to the 11168H wild-type strain grown in Brucella broth only. There were no differences in the readings performed at 3, 6 and 9 h. There was a difference in the readings performed at 16 h, where significantly lower OD_{600} readings were observed for the 11168H wild-type strain grown in the presence of both 0.1% ST (w/v) and 0.2% (w/v) ST. However, at 24 h, significantly lower OD_{600} readings were only observed for the 11168H wild-type strain grown in the presence of 0.2% ST (w/v) (Figure 4.11).

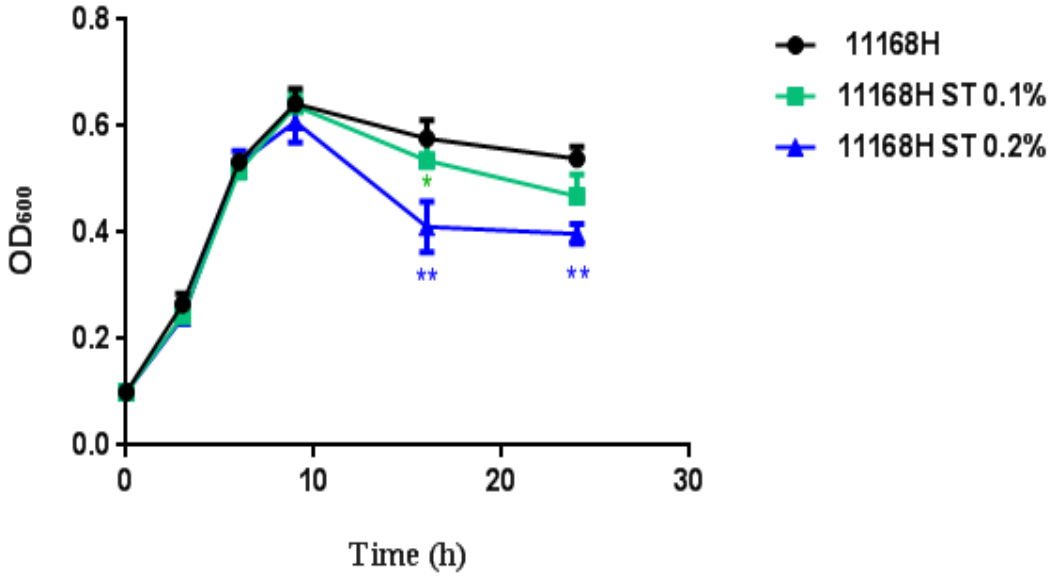

Figure 4.11 Growth curves for *C. jejuni* 11168H wild-type strain grown with and without sodium taurocholate. 11168H was grown in Brucella broth alone or in the presence of 0.1% (w/v) or 0.2% (w/v) ST at 37ºC under microaerobic conditions, shaking at 75 rpm. OD_{600} readings were recorded at 3, 6, 9, 16 and 24 h. Data represents at least three biological replicates. * = $p < 0.05$; ** = $p < 0.01$

To more accurately investigate the effect of ST on *C. jejuni* growth and survival, at each time point, one aliquot of the broth was collected, serial diluted (10^{-1} to 10^{-6}) and then plated onto BA plates for CFU counts. The presence of ST did not affect the growth or the survival of the 11168H wild-type strain at most time points analysed. The only exception was at 16 h in the presence of 0.2% (w/v) ST, where a statistically significant reduction in CFUs was observed compared to the CFUs in the absence of ST (Figure 4.12). However, there was no statistically significant reduction in CFUs after 24 h incubation in the presence of either 0.1% (w/v) ST or 0.2% (w/v) ST.

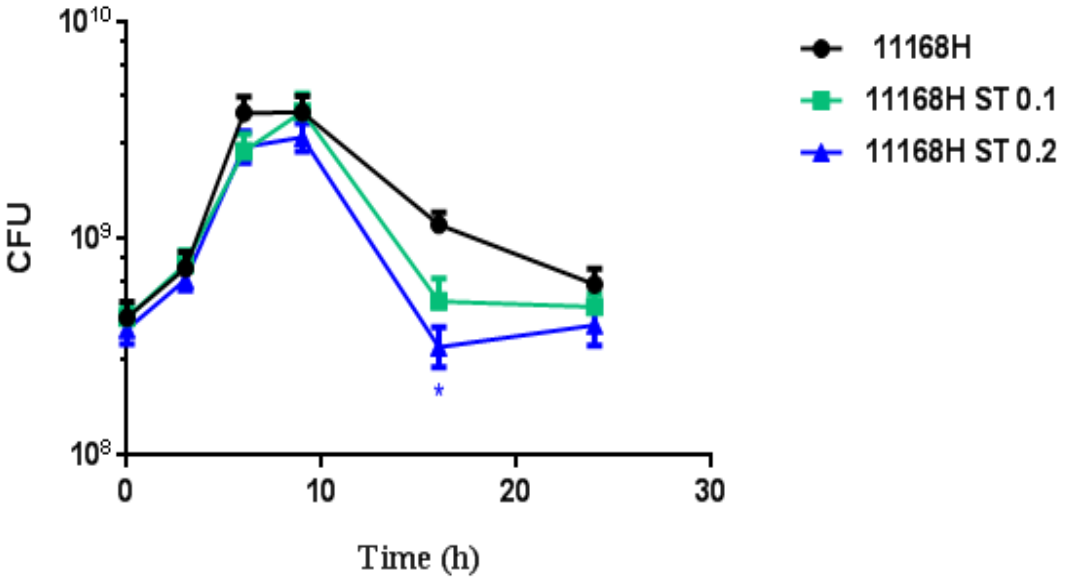

Figure 4.12 Growth curves for *C. jejuni* 11168H wild-type strain grown with and without sodium taurocholate. 11168H was grown in Brucella broth alone or in the presence of 0.1% (w/v) or 0.2% (w/v) ST at 37ºC under microaerobic conditions, shaking at 75 rpm. Serial dilutions were prepared at 3, 6, 9, 16 and 24 h and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = $p < 0.05$.

4.2.5 Sensitivity of the 11168H wild-type strain to hydrogen peroxide stress when grown in the presence of sodium taurocholate

Exposure to bile salts can alter bacterial gene expression and increase resistance to toxic compounds, such as diverse antimicrobials (Lin *et al.*, 2005). Therefore, the 11168H wild-type strain was grown in the presence or absence of ST and then exposed to different concentrations of H_2O_2 to investigate if exposure to this bile salt could alter bacterial sensitivity to H_2O_2 stress. 11168H grown in 0.1% (w/v) ST showed no difference in sensitivity to H_2O_2 compared to 11168H grown in the absence of ST. However, when grown in presence 0.2% (w/v) ST, 11168H exhibited a statistically

significant increase in sensitivity to H_2O_2 stress compared to growth in the absence of ST (Figure 4.13).

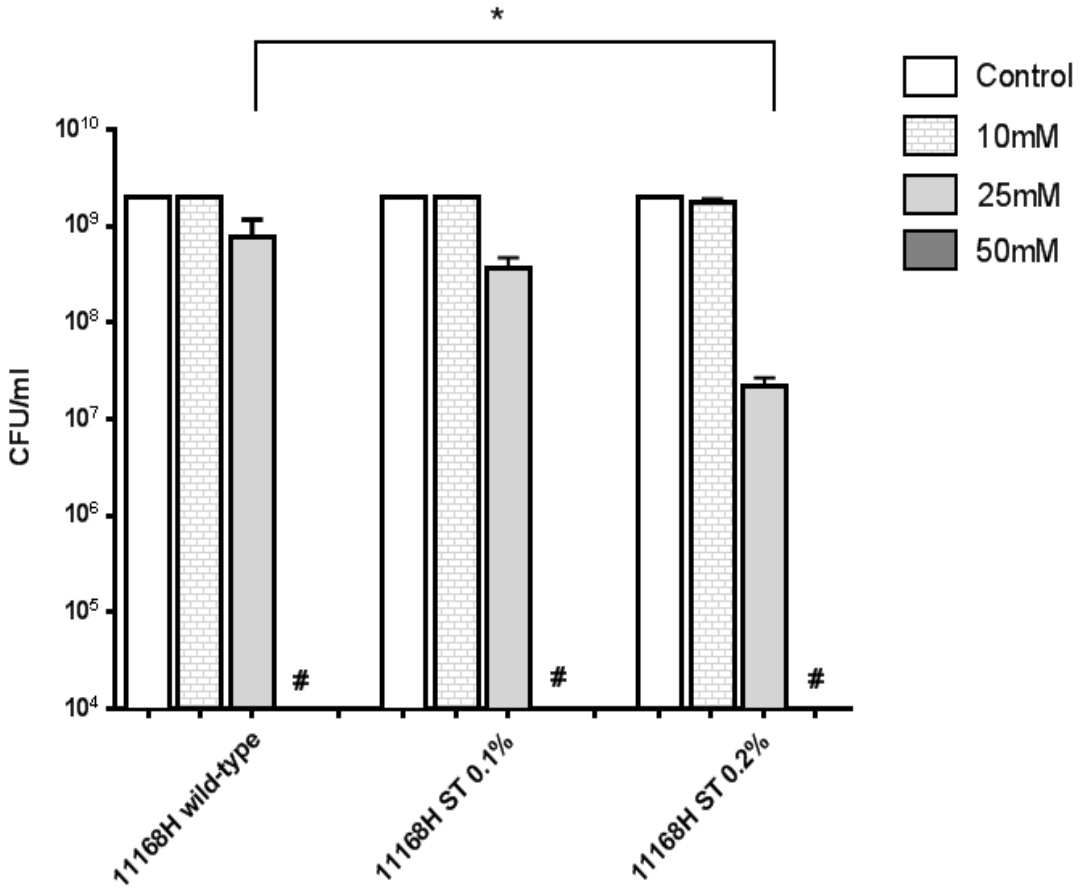

Figure 4.13 Sensitivity of the 11168H wild-type strain to hydrogen peroxide stress after exposure to sodium taurocholate. A bacterial suspension was prepared from a 24 h BA plate containing either no ST, 0.1% (w/v) ST or 0.2% (w/v) ST. The bacterial suspension (OD_{600} 1.0) was exposed to 10, 25 or 50 mM H_2O_2 for 15 min at 37ºC under microaerobic conditions. Serial dilutions were prepared from 10^{-1} to 10^{-6} and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = $p < 0.05$; # no growth.

4.2.6 Superoxide dismutase activity associated with *C. jejuni* OMVs

OMVs are a mechanism to deliver effector proteins extracellularly by *C. jejuni* (Kuehn and Kesty, 2005). *C. jejuni* 11168H OMVs have been shown to contain oxidative stress response enzymes (Elmi *et al.*, 2012, Jang *et al.*, 2014). OMVs were isolated from the 11168H wild-type strain and the *rrpA*, *rrpB*, *rrpAB*, *katA* and *perR* mutants as described in Section 2.4.3. The OMV samples were normalised to 100 ng/µl based on the protein concentration and SOD activity was investigated as described in Section 2.4.8. OMVs isolated from the *rrpA*, *rrpB* and *rrpAB* mutants all exhibited lower levels of SOD activity. However, no significant differences were observed compared to OMVs from the wild-type strain. SOD activity associated with OMVs isolated from the *perR* mutant was similar to that associated with OMVs from to the wild-type strain. However, OMVs isolated from the *katA* mutant exhibited a significant decrease in SOD activity compared to the wild-type strain (Figure 4.14).

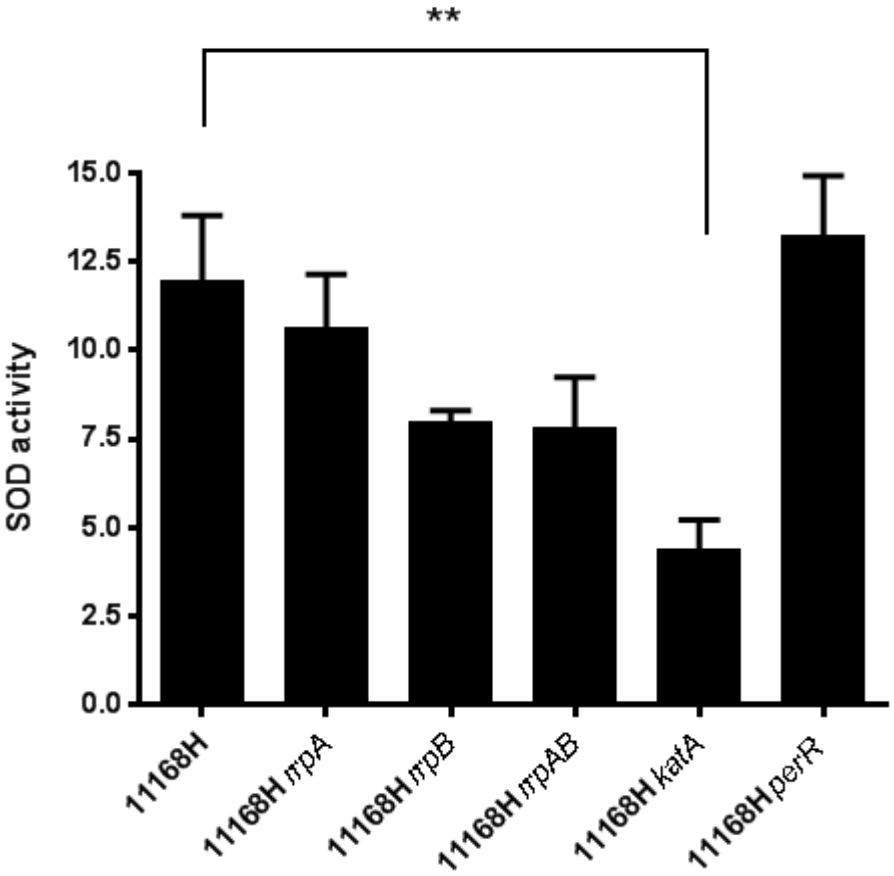

Figure 4.14 Superoxide dismutase activity assay associated with *C. jejuni* OMVs isolated from the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant, *rrpAB* double mutant, *katA* mutant and *perR* mutant. OMVs were isolated and protein concentrations were normalised to 100 ng/µl. Data represents at least three biological replicates. ** = $p < 0.01$.

When *C. jejuni* is grown in the presence of ST, OMV production is increased (Elmi *et al.*, unpublished data). Therefore, OMVs were isolated from the 11168H wild-type strain grown in the presence or absence of ST to investigate changes in SOD activity.

The 11168H wild-type strain was grown in presence of ST 0.1% (w/v) and 0.2% (w/v), and in absence of ST. OMVs isolated from the 11168H wild-type strain grown in the presence of 0.1% (w/v) ST exhibited no change in SOD activity compared to OMVs

isolated following growth in the absence of ST. However, OMVs isolated in presence of 0.2% (w/v) ST demonstrated a significant increase in SOD activity (Figure 4.15).

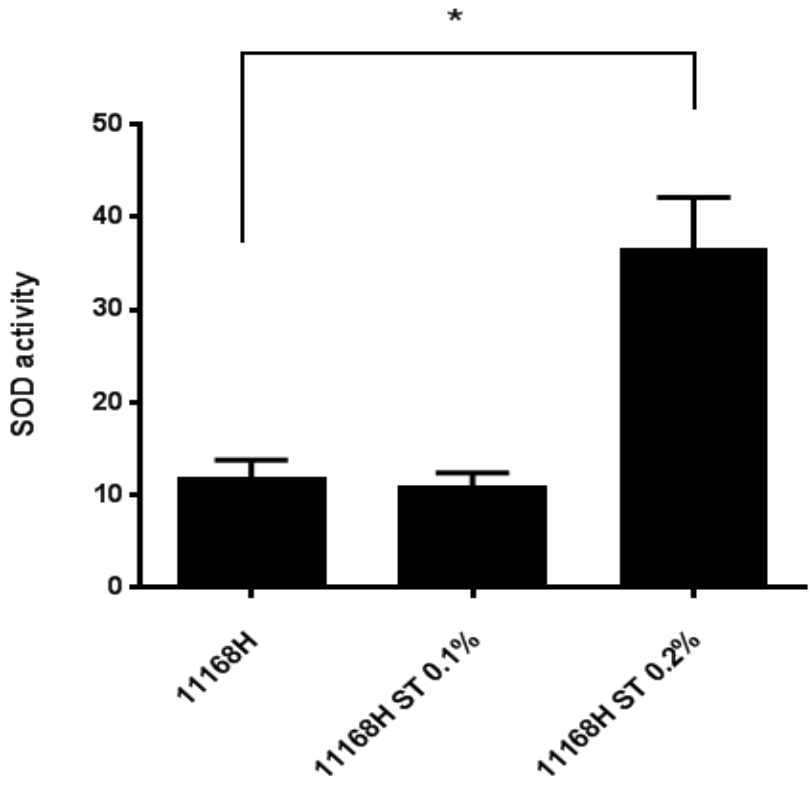

Figure 4.15 Superoxide dismutase activity assay associated with *C. jejuni* OMVs isolated from the 11168H wild-type strain grown in absence or in presence of sodium taurocholate (ST). OMVs were isolated from the 11168H wild-type strain grown in the absence of ST or in the presence of 0.1% (w/v) ST or 0.2% (w/v) ST. Protein concentrations were quantified and normalised to 100 ng/µl. Data represents at least three biological replicates. * = $p < 0.05$.

4.2.7 Catalase activity associated with *C. jejuni* OMVs

C. jejuni 11168H OMVs have been shown to contain oxidative stress enzymes (Elmi *et al.*, 2012, Jang *et al.*, 2014). Therefore, OMVs were isolated to investigate catalase activity. However, the catalase activity assay could not be performed on OMVs isolated from the *C. jejuni* 11168H wild-type strain or on OMVs isolated from the different mutants. The catalase activity kit contains chromogen reagent (3,5-dichloro-2-hydroxybenzenesulfonic acid) that oxidises 4-aminoantipyrine in the presence of H_2O_2 and horseradish peroxide producing a red quinoneimine dye. Some compounds, such as proteins and lipids, may interfere with the assay colour development.

The assay uses a blank control that consists of a chromogen reagent and an aliquot of 200 mM H_2O_2. H_2O_2 oxidises the chromogen reagent and the blank control will develop the highest amount of colour. When H_2O_2 is added to a test sample, the H_2O_2 will be neutralised by enzymes present in the sample. Therefore, the H_2O_2 that reacts with the chromogen reagent will produce less oxidation (colour) than the blank control.

When OMVs were used to investigate catalase activity, the colour development in the samples was higher than the blank control. It is possible that proteins associated with *C. jejuni* OMVs interfere with the assay reagent. However, it was possible to demonstrate visually that *C. jejuni* OMVs exhibit catalase activity by exposing a solution containing OMVs to H_2O_2. Catalase activity breaks down H_2O_2 to H_2O and O_2, and it was possible to observe the released O_2 molecules through the development of bubbles (Figure 4.16).

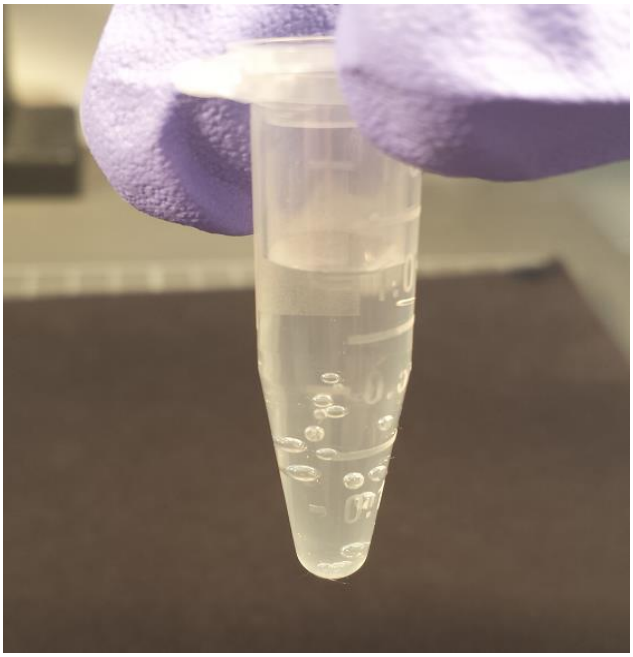

Figure 4.16 *C. jejuni* OMVs initiate the breakdown of H_2O_2 to water and oxygen. OMVs (100 ng/µl) were isolated from 11168H wild-type strain and added to a 200 mM H_2O_2 solution.

4.3 Discussion

To further investigate the roles of RrpA and RrpB, different assays were performed on the 11168H wild-type strain, *rrpA* mutant, *rrpB* mutant and *rrpAB* double mutant to investigate whether mutation of *rrpA* or *rrpB* resulted in other phenotypic changes. Motility assays, *Galleria mellonella* infection and biofilm formation assays were performed. This study also investigated whether the bile salt sodium taurocholate influences the 11168H wild-type strain resistance to H_2O_2 stress. The role of OMVs in the *C. jejuni* oxidative stress response was also investigated using catalase and SOD activity assays.

4.3.1 Motility

Motility is essential for *C. jejuni* colonisation. Wassenaar *et al.* (1991) demonstrated that a non-motile *C. jejuni* lost the capacity to adhere to and invade human intestinal epithelial cells. Yao *et al.* (1994) also demonstrated that different mutants with defects in motility were also unable to invade human cells. A different study also demonstrated that non-motile *C. jejuni* strains exhibited deficit in invasion *in vitro* (Russell and Blake, 1994). Non-motile *C. jejuni* also demonstrated no adhesion or invasion in mice (Yanagawa *et al.*, 1994).

Motility assays were performed to assess possible polar effects in the different mutants. Insertional mutations can cause polar effects by changing the transcriptional reading frame and by altering gene expression of genes located either upstream or downstream of the insertion site (Cheng *et al.*, 2015). It was important to investigate possible polar effects after mutating a gene to confirm if differences in the phenotype observed were a result of the mutation of the target gene or if they were a result of polar effects on adjacent genes (Roberts, 2000). Some *C. jejuni* mutations are known to affect motility via polar effects, such as the mutation of *luxS* that also affects *flaA* transcription and reduces motility (Jeon *et al.*, 2008). In the present study, none of the mutants demonstrated altered motility compared to wild-type strain. However, no further investigations were performed to confirm the absence of polar effects.

4.3.2 *Galleria mellonella* infection model

G. mellonella larvae have been used as a model of infection to investigate the virulence of various pathogenic bacteria, such as *Burkholderia cepacia* (Seed and Dennis, 2008), *Legionella pneumophila* (Harding *et al.*, 2012), *Pseudomonas aeruginosa* (Hendrickson *et al.*, 2001) and *Bacillus cereus* (Fedhila *et al.*, 2006). Insect immune systems have many characteristics in common with mammalian immune systems, including an innate non-adaptive immune response which is an efficient defence against microorganisms (Hoffmann, 1995). The immune response is divided in humoral and cellular responses (Lavine and Strand, 2002). Humoral defences comprise of antibacterial peptides, coagulation, melanisation, and production of ROS (Lavine and Strand, 2002). Cellular responses are responsible for phagocytosis and encapsulation (Lavine and Strand, 2002). Insect haemolymph detects bacterial LPS leading to a rapid coagulation response (Hoffmann, 1995). The recognition of foreign antigens occurs through haemocyte surface receptors or through opsonisation (Lavine and Strand, 2002). Inhibitors and antibacterial peptides are produced and released into the haemolymph to eliminate the bacteria (Hoffmann, 1995, Altincicek *et al.*, 2007). An important cellular defence reaction mechanism utilised by insects, including *G. mellonella,* is melanisation (Altincicek *et al.*, 2007). The melanisation process involves the production of cytotoxic molecules, such as ROS to eliminate infectious microorganisms sequestered in melanoic capsules (Hoffmann, 1995). Melanisation occurs around the invaded microorganisms, followed by phagocytosis by specialised cells (Hoffmann, 1995). Melanin can also release free radicals to help eliminate microorganisms (Hoffmann, 1995).

G. mellonella produce antioxidant enzymes as a protective response to oxidative stress to control endogenous levels of ROS (Buyukguzel *et al.*, 2010). Changes in the expression of enzymatic antioxidants occur during bacterial or viral infection (Wang *et al.*, 2001, Dubovskii *et al.*, 2005). The enzymes that demonstrated changes in expression were SOD, glutathione S-transferase (GT), and catalase (Dubovskii *et al.*, 2005). Catalase activity is decreased under bacterial infection (Dubovskii *et al.*, 2010). Infected *G. mellonella* increased oxidative stress and lipid peroxidation levels to eliminate the infectious pathogen (Dubovskii *et al.*, 2010). The increase of ROS by *G. mellonella* is a mechanism to eliminate the infection (Dubovskii *et al.*, 2010). As such,

G. mellonella is a useful model to study *C. jejnui* mutants deficient in oxidative stress responses.

Both the *rrpA* and *rrpB* mutants demonstrated reduced cytotoxicity in the *G. mellonella* model of infection compared to the wild-type strain (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). However, the *rrpAB* double mutant and *perR* mutant exhibited similar levels of cytotoxicity as the wild-type strain, whilst the *katA*, *sodB* and *ahpC* mutants all exhibited reduced levels of cytotoxicity compared to the wild-type strain (Gundogdu *et al.*, 2015). In the present study, *G. mellonella* larvae were injected with either the 11168H wild-type strain or different mutants. Haemolymph was drained from the larvae to investigate differences in bacterial survival. Reduced CFU counts were observed for both the *rrpA* and *rrpB* mutants, suggesting that *rrpA* and *rrpB* mutants were more sensitive to the *G. mellonella* innate immune response. This reinforces previous findings that *G. mellonella* larvae are less susceptible to killing when injected with the *rrpA* or *rrpB* mutants compared to the wild-type strain (Gundogdu *et al.*, 2011, Gundogdu *et al.*, 2015). However, this reduction in CFUs was not statistically significant. Infection with the *rrpAB* double mutant resulted in CFU counts similar to the wild-type strain. This indicates that the *rrpAB* double mutant has a similar capacity to resist to the *G. mellonella* innate immune system as the wild-type strain.

4.3.3 Biofilm formation

Biofilms are microbial communities formed by a polymeric matrix attached to a surface (Gambino and Cappitelli, 2016). Different environmental conditions can stimulate biofilm formation, such as limited nutrient availability, temperature variation and presence of environmental stresses (O'Toole *et al.*, 2000). *C. jejuni* biofilm formation is affected by environmental factors. In rich nutrient media, *C. jejuni* forms biofilm very poorly (Reeser *et al.*, 2007). However, less rich nutrient media, such as Muller Hinton broth, is more compatible with *C. jejuni* biofilm formation (Reeser *et al.*, 2007).

Biofilm formation is a bacterial survival strategy as biofilms confer higher resistance to adverse conditions (Gambino and Cappitelli, 2016). Bacterial cells have to form a

balance between the ROS produced by cytoplasmic metabolism and neutralisation of these ROS by scavenger enzymes to prevent damage to bacterial structures (Gambino and Cappitelli, 2016). Accumulated ROS activate the oxidative stress response, but also trigger adaptation to the hostile environment and biofilm formation (Gambino and Cappitelli, 2016). Environmental ROS are generated by UV radiation that leads to ROS formation intracellularly, as well as high or low temperatures which also increase the amount of free radicals within bacterial cells (Gambino and Cappitelli, 2016).

Low levels of ROS can induce bacterial adaptive responses and influence biofilm structure and morphology (Gambino and Cappitelli, 2016). Low concentrations of H_2O_2 have been shown to increase biofilm formation in *E. coli* (Jakubowsk and Walkowiak, 2015). The presence of sub-lethal doses of ROS lead to an increase in catalase and AhpC expression in *Azotobacter vinelandii*, but also increased biofilm formation through the increased production of polysaccharide extracellular matrix (Villa et al., 2012). Some bacterial regulators are involved in both of these processes, biofilm formation and ROS resistance mechanism. (Gambino and Cappitelli, 2016). In *C. jejuni*, CsrA plays an important role in biofilm formation and in the regulation of oxidative stress resistance (Fields and Thompson, 2008). However, it is still unclear how CsrA regulates genes involved in the oxidative stress response and in biofilm formation.

C. jejuni biofilm formation has been shown to be a direct response to oxidative stress. *C. jejuni* can enhance biofilm formation in response to oxidative stress (Asakura *et al.*, 2007a, Reuter *et al.*, 2010). It has been demonstrated that *C. jejuni* mutants which are more sensitive oxidative stress, such as *ahpC* and *katA* mutants, exhibit increased biofilm formation compared to the wild-type strain, with the *ahpC* mutant exhibiting the largest increase in biofilm formation (Oh and Jeon, 2014). Further investigation demonstrated that the increase in biofilm was associated with the accumulation of ROS within bacterial cells (Oh and Jeon, 2014).

In the present study, it was also demonstrated that different mutants with defects in the oxidative stress response increased biofilm formation compared to the wild-type strain. 11168H *ahpC, katA, perR, rrpA, rrpB* and *rrpB perR* mutants all demonstrated increase in biofilm formation. This suggests that these mutants are accumulating ROS compounds in the cytoplasm that triggers the increase in biofilm formation. Although

Oh and Jeon (2014) did not demonstrate increased biofilm formation for a 11168 *sodB* mutant, in the present study the 11168H *sodB* mutant demonstrated increased biofilm formation. However, there were significant differences in the techniques used. The period of incubation performed by Oh and Jeon (2014) was 48 h at 42ºC, whilst in this study the period of incubation was 72 h at 37ºC. There was no increase in biofilm formation demonstrated by the 11168H *rrpAB* double mutant compared to the wild-type strain.

Although *C. jejuni* can survive in different environments, the presence of organic material can increase the chances of bacteria survival under hostile conditions (Brown *et al.*, 2014). Proteins present in these organic liquids can increase *C. jejuni* biofilm formation (Brown *et al.*, 2014). Chicken juice is defined as the meat exudates obtained from defrosted chicken carcasses (Birk *et al.*, 2004, Brown *et al.*, 2014). The formation of ice crystals during freezing damages the meat cell structure, so the meat exudate is the substance obtained as a result of ruptured meat cells (Leygonie *et al.*, 2012). Meat exudates contain compounds such as nucleotides, amino acids, peptides, proteins and many soluble enzymes, forming a viscous liquid (Kima *et al.*, 2015) which provides a rich environment for bacterial growth (Birk *et al.*, 2004). Such compounds can prolong the viability of *Campylobacter* cells compared to incubation in BHI broth (Birk *et al.*, 2004). *C. jejuni* NCTC 11168 was shown to be viable for 8 weeks in chicken juice incubated at 5ºC under microaerobic conditions (Birk *et al.*, 2004). Milk is a different type of organic material with a rich protein content that can also provide an increase in bacterial attachment to surfaces (Barnes *et al.*, 1999). Pre-coating stainless steel with diluted milk solutions has been shown to increase bacterial attachment to the surface, such as with *Staphylococcus aureus, Listeria monocytogenes* and *Serratia marcescens* (Barnes *et al*., 1999).

When undiluted chicken juice was incubated at 37ºC, precipitation was observed, which increased crystal violet staining unrelated to biofilm formation. Brown *et al.* (2013) have also described this problem of interference with crystal violet staining due to chicken juice precipitation. As such, chicken juice was diluted to overcome the problem of interference with crystal violet staining. However, diluted chicken juice did not increase biofilm formation compared to strains grown in MH broth alone. The reason for this could be that the dilution reduced the amount of organic material available for the bacteria to grow and/or stimulate an increase in biofilm formation.

The only exception was with the 11168H *rrpA* mutant. The *rrpA* mutant grown in presence of diluted chicken juice showed reduced biofilm formation compared to MH broth alone. The reduction in biofilm formation was more pronounced under microaerobic conditions. The reason for this phenotype is not obvious. This suggests that the concentration of diluted chicken juice was enough to trigger a signal for the 11168H *rrpA* mutant to restore the phenotype observed in the wild-type strain. Further studies are necessary to investigate this phenotype.

As the chicken juice had to be highly diluted to avoid precipitation, it may no longer be representative of an organic liquid that could affect biofilm formation. Therefore, an alternative quantitative method using TTC was investigated. TTC is reduced by bacterial cell respiration and allows for quantification of viable cells in biofilms (Brown *et al.*, 2013). Brown *et al.* (2013) demonstrated that chicken juice compounds do not interfere with TTC and that TTC is a useful staining method as an alternative to crystal violet. When undiluted chicken juice was added to MH broth, an increase in biofilm formation was observed for all strains. The rich content of undiluted chicken juice allowed 11168H biofilm formation to be increased more than two-fold. Chicken juice has high concentration of particulates that form a conditioning layer in the abiotic surface facilitating bacterial attachment and increase in biofilm formation (Brown *et al.*, 2014). *C. jejuni* also attaches to chicken juice particulates (Brown *et al.*, 2014).

This study demonstrated that mutation of the genes encoding key oxidative stress enzymes increased biofilm formation. Oh and Jeon (2014) showed that this increased biofilm formation is due to the accumulation of ROS intracellularly. However, accumulation of ROS can also induce *C. jejuni* cells to enter a VBNC state (Ica *et al.*, 2012). Ica *et al.* (2012) demonstrated that *C. jejuni* biofilm cells enter a VBNC state, and that VBNC cells within the biofilms failed to grow in bacteriological media. VBNC cells are less active compared to planktonic cells (Tholozan *et al.*, 1999). VBNC cells produce a low level of AMP and no ATP or ADP compared to planktonic *C. jejuni* cells (Tholozan *et al.*, 1999). TTC is reduced by planktonic cells. However, VBNC cells do not reduce this compound as efficiently (Tholozan *et al.*, 1999).

The 11168H *rrpA* mutant demonstrated an increase in biofilm formation using crystal violet stain. However, the *rrpA* mutant did not show an increase in biofilm formation using TTC when grown in MH only compared to the wild-type strain. Crystal violet

stains both living and dead cells by binding to extracellular polysaccharide matrix formed by biofilms (Gomes *et al.*, 2014), whilst TTC is only reduced by metabolically active cells (Brown *et al.*, 2013). This could suggest that the *rrpA* mutant increases biofilm formation, but also forms VBNC cells more rapidly compared to the wild-type strain. Further studies are necessary to investigate if the other *C. jejuni* oxidative stress mutants would also display similar phenotypes using TTC.

4.3.4 Bile salts

Bile salts are present in the intestine and provide protection to the host against pathogenic bacteria through bactericidal activity (Meinersmann *et al.*, 2005). Primary bile salts (cholate and chenodeoxycholate) are typically conjugated to glycine or taurine (forming taurocholate and glycocholate) in the host liver (Sorg and Sonenshein, 2008). Most of the bile salts present in the human gut are conjugated. However, bacteria present in the small intestine can deconjugate bile salts, forming different compounds termed secondary bile salts, such as deoxycholate acid (Ridlon *et al.*, 2006). Pathogenic bacteria have evolved the ability to survive and grow in the presence of bile salts in the intestine and establish infection (Meinersmann *et al.*, 2005). Bile salts are known activators of multidrug efflux pumps in different bacteria (Piddock, 2006). In *C. jejuni*, bile salts activate expression of CmeABC, the major efflux pump mechanism, increasing resistance to several antimicrobials (Lin *et al.*, 2005).

Bile salts can change gene expression and increase *C. jejuni* resistance to antimicrobials (Kuehn and Kesty, 2005). It is suggested that bile salts can also activate different mechanisms of resistance, protection and damage repair (Meinersmann *et al.*, 2005). Bile salts activate stress responses in *E. coli* associated with membrane functions, oxidative stress and DNA damage (Berg *et al.*, 2002). In *C. jejuni*, bile salts induce the expression of Cia protein synthesis enhancing *C. jejuni* capacity to invade intestinal cells (Rivera-Amill *et al.*, 2001, Malik-Kale *et al.*, 2008).

The highest bile salt concentration in the human gut is 10 mM, with an average concentration of 3.4 mM (Northfield and McColl, 1973). The average human concentration of taurocholic acid in the gall bladder varies between 39 to 56 µmol/ml

(Fisher and Yousef, 1973). ST is a constituent of the bile salts present in the human caecum, with average concentration of 1.3% (Hamilton *et al.*, 2007). ST is an important bile salt found in the human gut. ST has been shown to induce the germination of *Clostridium difficile* spores (Sorg and Sonenshein, 2008).

This study demonstrated that *C. jejuni* grows well in the presence of either 0.1% w/v (2 mM) or 0.2% w/v (4 mM) ST. These concentrations are within the physiological range and similar to the concentration used by Malik-Kale *et al.* (2008), although this study used a different bile salt (sodium deoxycholate). Sodium deoxycholate increases the expression of *C. jejuni* virulence genes, such as *ciaB* (Rivera-Amill et al., 2001). CiaB increases bacterial ability to invade epithelial cells (Malik-Kale *et al.*, 2008). Sodium deoxycholate also increases the expression of *rrpB* and *katA* (Malik-Kale *et al.*, 2008). This suggests that sodium deoxycholate can increase *C. jejuni* resistance to oxidative stress. This study investigated the effects of ST in the *C. jejuni* H_2O_2 stress response.

The presence of 0.2% w/v ST was demonstrated to be a stressful condition for *C. jejuni*. When 11168H was grown in the presence of ST, the bacteria displayed a reduced OD_{600} and reduced CFU at the 16 h time point. However, the CFU count increased again at 24 h with no differences compared to growth in absence of ST. This demonstrates the ability of *C. jejuni* to survive in presence of bile salts. This ability is probably due to the activation of the CmeABC efflux pump that increases the resistance of *C. jejuni* to several antimicrobials (Lin et al., 2005). The 11168H wild-type strain was grown in presence of ST to investigate if exposure to this bile salt would affect the oxidative stress response. No differences in sensitivity to H_2O_2 were observed when the wild-type strain was grown in 0.1% w/v ST. However, an increase in sensitivity to H_2O_2 was observed when grown in 0.2% w/v ST.

The higher concentration of ST used was associated with an increase in the levels of stress for *C. jejuni in vitro.* However, this does not indicate that bile salts do not play a role in activating genes related to *C. jejuni* oxidative stress defence mechanism. As demonstrated previously, *C. jejuni* increases the expression of *rrpB* and *katA* (Malik-Kale *et al.*, 2008), which can increase the *C. jejuni* resistance to H_2O_2. When in the gut, *C. jejuni* is in contact with different bile salts that cause changes in gene expression, which can increase the bacteria resistance against hostile antimicrobial

compounds and ROS (Lin *et al.*, 2005, Malik-Kale *et al.*, 2008). A mixture of different bile salts may be more suitable for stimulating *C. jejuni* resistance to oxidative stress.

4.3.5 OMVs

Gram-negative bacteria secrete OMVs containing biologically active proteins extracellularly (Kulp and Kuehn, 2010). OMVs are a mechanism to deliver virulence factors to the environment as well as to host cells (Kuehn and Kesty, 2005). Therefore, bacteria can deliver a high concentration of effectors over greater distances (Kulp and Kuehn, 2010). OMVs contain bacterial lipids, membrane proteins and other insoluble compounds. Soluble compounds are protected within the OMVs from extracellular proteases (Kulp and Kuehn, 2010).

Like other secretion systems, OMV secretion can be regulated (Kulp and Kuehn, 2010). OMV production is influenced by environmental factors and bacterial stressors, which can increase vesiculation levels and change OMV content (Ellis and Kuehn, 2010, Kulp and Kuehn, 2010). OMVs provide protection for planktonic cells as a short-term defence against lethal doses of antibacterial agents (Kulp and Kuehn, 2010). Antibacterial agents increase OMV secretion, which then target these agents through binding and inactivation (Kulp and Kuehn, 2010). The increase in vesiculation provides the bacteria with better chances of survival (Ellis and Kuehn, 2010).

It has been shown that *P. aeruginosa* can increase OMVs production under oxidative stress conditions (Sabra *et al.*, 2003, Macdonald and Kuehn, 2013). van de Waterbeemd *et al.* (2013) demonstrated that oxidative stress acts as an intracellular signal to increase vesiculation in *Neisseria meningitides.* Proteomic analysis performed on *Acinetobacter baumannii* OMVs demonstrated the presence of catalase and superoxide dismutase (Kwon *et al.*, 2009). Therefore, oxidative stress conditions may also increase the OMVs production in *C. jejuni* strains.

OMV content from the *C. jejuni* 11168H wild-type strain was analysed by proteomic analysis indicating the presence of oxidative stress ezymes (Elmi *et al.*, 2012). The oxidative stress ezymes identified were TrxA, TrxB, AhpC and Tpx (Elmi *et al.*, 2012). Further proteomic analysis of NCTC 11168 OMVs showed that Dps, Tpx and AhpC were present in the OMVs (Jang *et al.*, 2014). However, 21 to 26% of the OMV

protein countent could not have been identified (Elmi *et al.*, 2012, Jang *et al.*, 2014). KatA and SodB were identified as associated with *C. jejuni* 11168H OMVs isolated in presence of ST (Elmi *et al.*, unpublished data). Therefore, *C. jejuni* OMVs have the potential to act against toxic oxygen compounds as a defence mechanism.

KatA or SodB have major roles in detoxifing ROS compounds and were identified as associated with *C. jejuni* OMVs (Elmi *et al.*, unpublished data). Therefore, to further investigate the OMVs role in oxidative stress response, catalase and superoxide dismutase activity assays were performed. *C. jejuni* OMVs were demonstrated to have catalase activity due to the release of oxygen molecules when OMVs were in presence of H_2O_2. However, the exact concentration of catalase activity present in the OMVs was not possible to measure due to OMV protein content interference with the assay reagent. OMVs isolated from the 11168H wild-type strain, *rrpA*, *rrpB*, *katA*, *perR* mutants and *rrpAB* double mutant were shown to possess SOD activity. Only OMVs isolated from the *katA* mutant possessed a reduced SOD activity. An increase in SOD activity associated with OMVs was observed when the 11168H wild-type strain was grown in the presence of ST. It has been observed that *C. jejuni* can increase OMV production and protein content when grown in presence of ST (Elmi *et al.*, unpublished data). ST also increased *C. jejuni* OMVs proteolytic activity (Elmi *et al.*, unpublished data). This demonstrates that ST changes gene expression in *C. jejuni* and increases the response to stressful conditions, such as oxidative stress. Presence of bile salts may be a natural trigger to stimulate *C. jejuni* to produce more enzymes and enhance its defence mechanism.

4.3.6 Conclusion

The mutation of *rrpA* or *rrpB* appears to affect other bacterial responses as well as the peroxide and aerobic stress responses. Mutation of either *rrpA* or *rrpB* reduces the bacterial ability to survive the *G. mellonella* innate immune response. This study also showed that accumulation of toxic compounds in *C. jejuni* cells triggers an increase in biofilm formation. The *rrpA* and *rrpB* mutations increased biofilm formation. However, the *rrpAB* double mutant did not exhibit increased biofilm formation. The different assays performed demonstrated that *C. jejuni* uses different mechanisms to cope with hostile environments and to increase the bacterium chances of survival. The

presence of organic liquids further increases the chances of *C. jejuni* survival through an increase in biofilm formation. OMVs play a role in the *C. jejuni* oxidative stress response to neutralise toxic ROS compounds present in the extracellular space. Bile salts affect *C. jejuni* gene expression increasing bacterial resistance to hostile compounds, such as antimicrobials and ROS. It has been demonstrated that bile salts directly increase KatA expression (Malik-Kale *et al.*, 2008). This study has demonstrated that bile salts increase SOD activity associated with OMVs. RrpA and RrpB appear to also play a role in both resistance to innate immune responses and in biofilm formation.

5 Analysis of the distribution of *rrpA* and *rrpB* in different *C. jejuni* wild-type strains

5.1 Introduction

Reanalysis of whole genome microarray data for 111 *C. jejuni* wild-type strains (Champion *et al.*, 2005) indicated a significant difference in the distribution of *rrpA* and *rrpB* amongst *C. jejuni* strains, with *rrpA* identified in over 95% of these strains, whilst *rrpB* was identified in only 50% of these strains (Gundogdu *et al.*, 2011). Sensitivity to peroxide and aerobic stress has also been shown to vary depending on whether a *C. jejuni* strain contains only *rrpA* or both *rrpA* and *rrpB* (Gundogdu *et al.*, unpublished data).

5.2 Results

5.2.1 Screening for *rrpA, rrpB* and *cosR* using PCR with degenerate primers

At the start of this study in April 2013, 20 *C. jejuni* strains from the Champion *et al.* (2005) study were screened for the presence of *rrpA*, *rrpB* and *cosR* using PCR with degenerate primers. This screen was performed in order to confirm the *in silico* reanalysis of the microarray data that indicated variation in the presence of *rrpB* amongst different *C. jejuni* wild-type strains. Degenerate primers allow screening to be performed for a specific gene even if there is variation in some nucleotide positions. Designed degenerate primers covered every possible nucleotide combination based on the published amino acid sequences.

The *C. jejuni* strains from the Champion *et al.* (2005) study were isolated from different sources and further analysed by Stabler *et al.* (2013) using both microarray and MLST analysis. MLST analysis identified the *C. jejuni* strains as being either livestock-associated or water & wild-life-associated clonal complexes (Appendix 1). Ten isolates were randomly selected from the livestock-associated clonal complexes and ten from the water & wild-life-associated clonal complexes (Table 5.1).

Table 5.1 *C. jejuni* wild-type strains selected from livestock-associated clonal complexes and water & wild-life-associated clonal complexes

Livestock		**Water & wild-life**	
11919	13040	M1	40917
11973	13249	12241	44119
12450	13713	31481	47693
12487	30280	33106	62914
12912	47886	34007	64555

PCR screening with degenerate *cosR* primers indicated that *cosR* is highly conserved amongst the *C. jejuni* wild-type strains screened (Figure 5.1). The reference strains also included in the PCR analysis were 11168H, 81-176, 81116 and *C. coli* 37. *cosR* was present in all these reference strains.

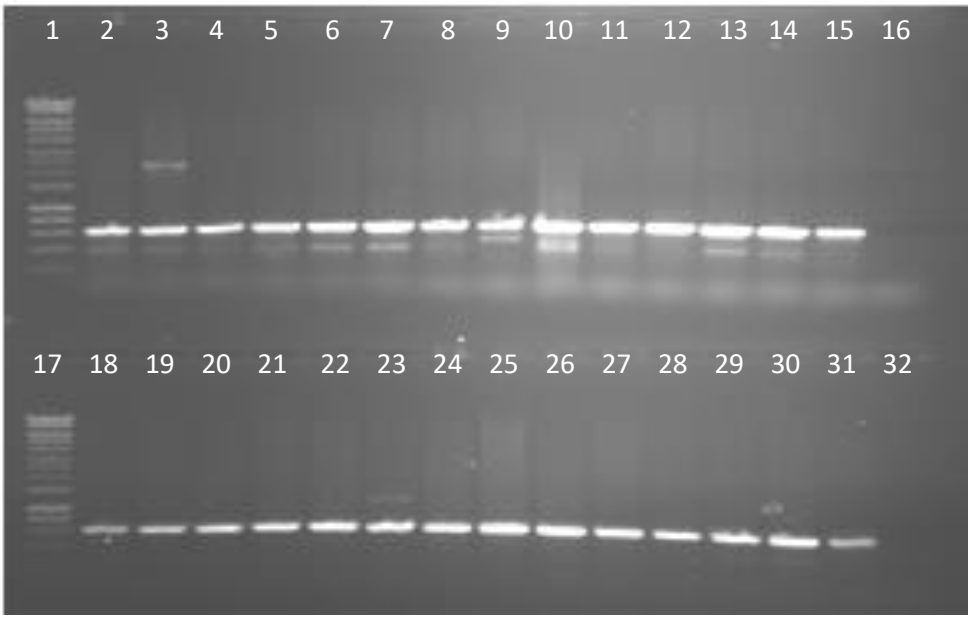

Figure 5.1 *cosR* degenerate primers were used to screen 20 isolates from livestock-associated or water & wild-life-associated clonal complexes. Lanes 1 and 17: ladder; Lane 2: M1; Lane 3: 12241; Lane 4: 31481; Lane 5: 33106; Lane 6: 34007; Lane 7: 40917; Lane 8: 44119; Lane 9: 47693; Lane 10: 62914; Lane 11: 64555; Lane 18: 11919; Lane 19: 11973; Lane 20: 12450; Lane 21: 12487; Lane 22: 12912; Lane 23: 13040; Lane 24: 13249; Lane 25: 13713; Lane 26: 30280; Lane 27: 47886; Lanes 12 and 28: 11168H; Lane 13 and 29: 81116; Lanes 14 and 30: 81-176; Lanes 15 and 31: *C. coli* 37; Lanes 16 and 32: negative control. The size of the amplified fragment is 0.654 kb.

PCR screening with degenerate *rrpA* or *rrpB* primers confirmed the variation in the distribution of *rrpB* amongst the different *C. jejuni* wild-type strains. Some wild-type strains contained only *rrpA,* whilst other strains contained both *rrpA* and *rrpB.* PCR screening with degenerate *rrpA* primers indicated that all strains from livestock-associated and water & wild-life-associated clonal complexes contained *rrpA. C. coli* 37 was the only strain that appeared not to contain *rrpA* (Figure 5.2).

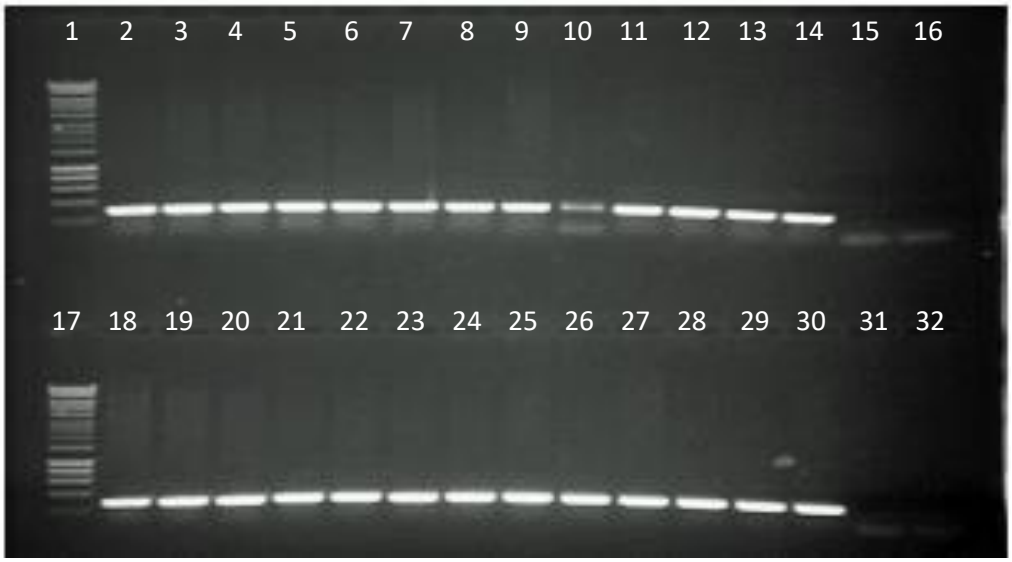

Figure 5.2 *rrpA* degenerate primers were used to screen 20 isolates from livestock-associated or water & wild-life-associated clonal complexes. Lanes 1 and 17: ladder; Lane 2: M1; Lane 3: 12241; Lane 4: 31481; Lane 5: 33106; Lane 6: 34007; Lane 7: 40917; Lane 8: 44119; Lane 9: 47693; Lane 10: 62914; Lane 11: 64555; Lane 18: 11919; Lane 19: 11973; Lane 20: 12450; Lane 21: 12487; Lane 22: 12912; Lane 23: 13040; Lane 24: 13249; Lane 25: 13713; Lane 26: 30280; Lane 27: 47886; Lanes 12 and 28: 11168H; Lane 13 and 29: 81116; Lanes 14 and 30: 81-176; Lanes 15 and 31: *C. coli* 37; Lanes 16 and 32: negative control. The size of the amplified fragment is 0.216 kb.

PCR screening with degenerate *rrpB* primers indicated variation in the presence of *rrpB* amongst the strains. Most water & wild-life-associated strains did not contain *rrpB*, except one (strain 12241). However, most livestock-associated strains contained *rrpB*, except one (strain 13040). As predicted, the positive controls 11168H and 81-176 contained *rrpB*, whilst the negative control 81116 and also *C. coli* 37 did not contain *rrpB* (Figure 5.3).

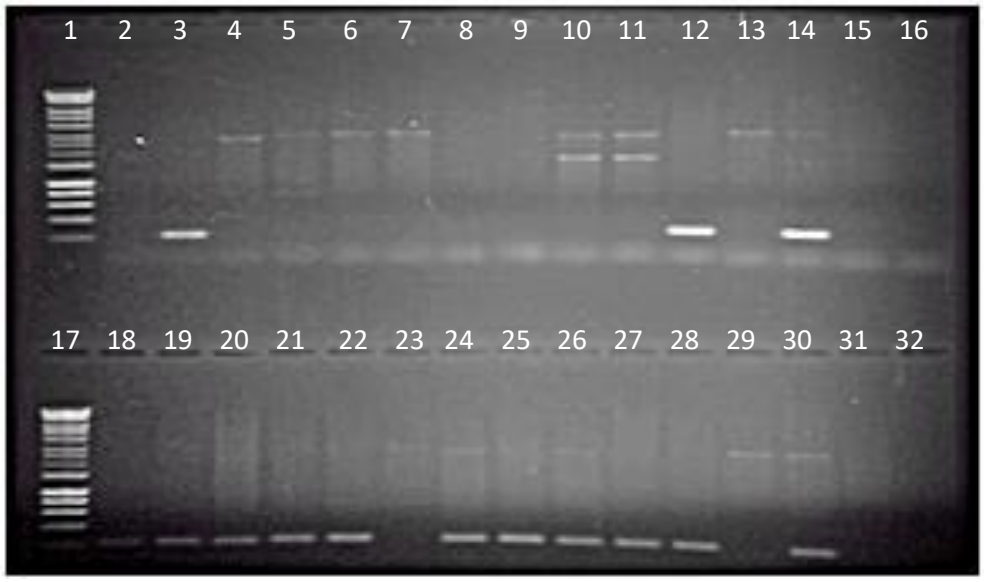

Figure 5.3 *rrpB* degenerate primers were used to screen 20 isolates from livestock-associated or water & wild-life-associated clonal complexes. Lanes 1 and 17: ladder; Lane 2: M1; Lane 3: 12241; Lane 4: 31481; Lane 5: 33106; Lane 6: 34007; Lane 7: 40917; Lane 8: 44119; Lane 9: 47693; Lane 10: 62914; Lane 11: 64555; Lane 18: 11919; Lane 19: 11973; Lane 20: 12450; Lane 21: 12487; Lane 22: 12912; Lane 23: 13040; Lane 24: 13249; Lane 25: 13713; Lane 26: 30280; Lane 27: 47886; Lanes 12 and 28: 11168H; Lane 13 and 29: 81116; Lanes 14 and 30: 81-176; Lanes 15 and 31: *C. coli* 37; Lanes 16 and 32: negative control. The size of the amplified fragment is 0.228 kb.

5.2.2 Prevalence of the *rrpA* and *rrpB* genes amongst 270 *C. jejuni* strains

The Stabler *et al.* (2013) study analysed a total of 270 *C. jejuni* strains based on whole genome microarray data and MLST analysis (Appendix 3). This study generated a phylogenetic tree that grouped *C. jejuni* isolates into nine subclades (C1 to C9). The subclades were divided into two categories based on source of isolation: livestock-associated and water & wild-life-associated clonal complexes. The livestock-associated strains dominated subclades C1 to C6, whilst the water & wild-life strains dominated subclades C7 to C9.

The *rrpA* and *rrpB* gene distribution was investigated using the whole genome microarray data from the Stabler *et al.* (2013) study. *rrpA* was identified in 129/133 strains within the C1-C6 subclades (97.0%) and in 130/137 strains within the C7-C9 subclades (94.9%). In total *rrpA* was identified in 259/270 strains, indicating that vast majority of *C. jejuni* strains contained *rrpA* (Table 5.2)

In contrast, *rrpB* was identified in 102/133 strains within the C1-C6 subclades (76.7%) and only in 19/137 strains in the C7-C9 subclades (13.9%). In total *rrpB* was identified in only 121/270 strains, indicating a more variable distribution according to subclade. Therefore, strains from livestock-associated subclades tend to contain both *rrpA* and *rrpB*, whilst strains from water & wild-life subclades tend to contain only *rrpA.*

It was also observed that within both the livestock and water & wild-life clades, there were anomalous subclades. Within the C1-C6 subclades (livestock-associated), most strains contained *rrpB*. However, most strains from the C6 subclade did not contain *rrpB*. In the C7-C9 subclades (water & wild-life-associated), most strains did not contain *rrpB*. However, 5/15 strains from the C9i subclade contained both *rrpA* and *rrpB*.

Table 5.2 *rrpA* and *rrpB* gene distribution according to subclades generated by MLST analysis

Livestock-associated			
Subclades	***rrpA***	***rrpA* & *rrpB***	**Neither**
C1	2 (10%)	27 (90%)	-
C2	-	27 (100%)	-
C3	-	21 (100%)	-
C4	1 (7.7%)	12 (92.3%)	-
C5	5 (26.3%)	14 (73.7%)	-
C6	20 (86.9%)	-	3 (13%)
Water & wild-life-associated			
Subclades	***rrpA***	***rrpA* & *rrpB***	**Neither**
C7	24 (96%)	1 (4%)	-
C8	35 (89.7%)	4 (10.3%)	-
C9i	7 (46.7%)	5 (33.3%)	3 (20%)
C9ii	45 (77.6%)	9 (15.5%)	4 (6.9%)

5.2.3 Prevalence of *rrpA* and *rrpB* in 3,746 *C. jejuni* and 486 *C. coli* genomes

A collaboration with the Institute of Food Research in Norwich allowed further investigation of the distribution of *rrpA* and *rrpB* using whole genome sequencing and MLST (Appendix 4). This analysis identified differences in *rrpA* and *rrpB* distribution amongst 4,232 *Campylobacter* genome sequences (3,746 *C. jejuni* and 486 *C. coli*) available in public databases (Cody *et al.*, 2013, Brown *et al.*, 2015). MLST identifies each strain to an ST due to a unique allelic profile of the seven housekeeping genes.

STs are grouped into clonal complexes (CC) by their similarity to a central allelic profile.

The vast majority (>96.4%) of the 3,746 *C. jejuni* genomes contained *rrpA*, whilst the presence of *rrpB* varied amongst these genomes, with 36.3% of *C. jejuni* genomes containing *rrpB*. The presence of *rrpB* appeared to be linked with MLST clonal complex. Some clonal complexes were strongly associated with strains that contained both *rrpA* and *rrpB*, such as CC-21 and CC-61. All genomes in the CC-61 contained both genes. In the CC-21, the vast majority of strains contained both genes, with just a few containing only *rrpA*. These two clonal complexes are usually associated with livestock (Kwan *et al.*, 2008, Rotariu *et al.*, 2009, Stabler *et al.*, 2013). However, strains in two other livestock-associated clonal complexes (CC-48 and CC-206) contained only *rrpA*.

Some water & wild-life-associated clonal complexes contained only *rrp*A, such as CC-45, CC-283, CC-354, CC-443, CC-574, CC-658, CC-257 and CC-460. However, some water & wild-life-associated clonal complexes demonstrated variation in the distribution of *rrpB*, such as CC-353, CC-607, CC-22, CC-42, CC-573 and CC-403. This suggests that some water & wild-life-associated clonal complexes contain a mixed distribution of strains, with some strains containing both genes whilst other strains contain only *rrpA*. Strains belonging to CC-464 also demonstrated variation in the presence of *rrpB*. This clonal complex has not been previously reported to be classified as either livestock-associated or water & wild-life-associated. The vast majority of *C. coli* genomes contained neither *rrpA* nor *rrpB*. Furthermore, most of the *C. coli* strains were assigned to CC-828. Only 12 of the 486 *C. coli* strains contained an *rrpA* orthologue and all but one of these genomes were assigned to a different lineage (clade 3) (Figure 5.4). Analysis of the amino acid sequences of RrpA and RrpB indicated a relationship between some clonal complexes and truncated versions of RrpA and RrpB. Strains belonging to the CC-607 were found to encode a shorter RrpA protein sequence that lacks the N-terminal 27 amino acids. Furthermore, a percentage of *C. jejuni* strains assigned to CC-353 were found to have a RrpB protein sequence either lacking the N-terminal 29 amino acids, the C-terminal 29 amino acids, or a combination of both 29 N-terminal and 9 C-terminal amino acids (Figure 5.5).

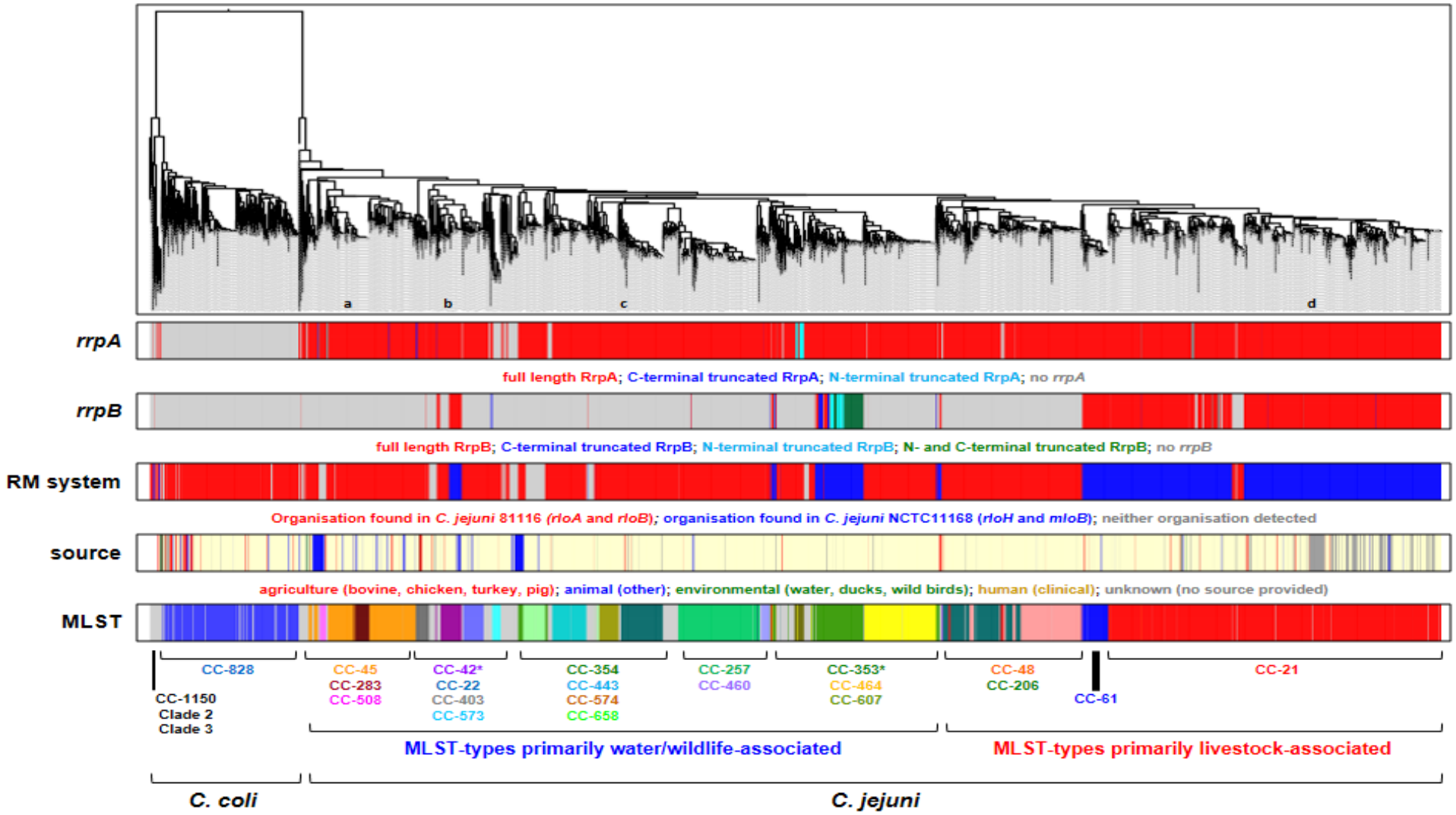
a
b
c
d
rrpA
full length RrpA; C-terminal truncated RrpA; N-terminal truncated RrpA; no rrpA
rrpB
full length RrpB; C-terminal truncated RrpB; N-terminal truncated RrpB; N- and C-terminal truncated RrpB; no rrpB
RM system
Organisation found in C. jejuni 81116 (rloA and rloB); organisation found in C. jejuni NCTC11168 (rloH and mloB); neither organisation detected
source
agriculture (bovine, chicken, turkey, pig); animal (other); environmental (water, ducks, wild birds); human (clinical); unknown (no source provided)
MLST
CC-828
CC-1150
Clade 2
Clade 3
CC-45
CC-283
CC-508
CC-42*
CC-22
CC-403
CC-573
CC-354
CC-443
CC-574
CC-658
CC-257
CC-460
CC-353*
CC-464
CC-607
CC-48
CC-206
CC-61
CC-21
MLST-types primarily water/wildlife-associated
MLST-types primarily livestock-associated
C. coli
C. jejuni

Figure 5.4 Prevalence of *rrpA* and *rrpB* amongst 3,746 *C. jejuni* and 486 *C. coli* genomes and MLST sequence types. Genomes were phylogenetically clustered as described in Section 2.5.9 (van Vliet and Kusters, 2015). The first two bars show the presence or absence of *rrpA* and *rrpB.* Red = presence, Grey = absence, Blue = C-terminal truncated, Light blue = N-terminal truncated, and Green = N- and C-terminal truncated RrpB. The third bar shows the RM system. Red indicates the organisation found in *C. jejuni* 81116 containing *rloA* and *rloB*, Blue indicates the organisation found in *C. jejuni* NCTC11168 containing *rloH* and *mloB*, and Grey indicates no organisation found. The fourth bar indicates the source of each strain. Red indicates isolation from animals (bovine, chicken, turkey and pig), Blue from other animals, Green from the environment (water, ducks and wild birds), Yellow from humans, and Grey from unknown sources. The fifth bar indicates the MLST clonal complexes where different colours represent different lineages. The asterisks at ST-42 and ST-353 indicate that these are mixed clonal complexes having a proportion of livestock-associated isolates. The lowercase letters indicate the approximate position of reference strains 81116 and M1 (a), 81-176 (b), RM1221 (c) and NCTC 11168 (d).

Further statistical analysis of the data present in Appendix 4 was performed to investigate the association of *rrpB* with the source of isolation. Table 5.3 indicates that most of the clinical isolates had *rrpA* only, and around 30% had both genes. Most of the livestock strains did not have *rrpA* or *rrpB*, and similar percentages were found for isolates containing both genes or only *rrpA.*

Table 5.3 *rrpA* and *rrpB* gene distribution related to the origin of the isolates

	rrpA* + *rrpB	***rrpA* only**	**Neither**	**Total**
Clinical	1111 (29.8%)	2128 (57%)	490 (13.2%)	3729
Livestock	25 (23.6%)	36 (34%)	45 (42.4%)	106
Other animals	21 (14.3%)	69 (46.9%)	57 (38.8%)	147
Environmental	3 (33.3%)	4 (44.5%)	2 (22.2%)	9
Unknown	197 (81.7%)	30 (12.5%)	14 (5.8%)	214

The correlation between the *C. jejuni* genomes and the RM systems can be seen in Table 5.4. The presence of *rrpB* in *C. jejuni* genomes strongly correlates with the RM type IF *hsd* system, which is represented by NCTC 11168. The RM type IAB *hsd* system does not correlate with *rrpB* and is represented by 81116.

Table 5.4 *rrpA* and *rrpB* gene distribution according to the RM system

	RM type IF 11168	**RM type IAB 81116**	**Neither**	**Total**
rrpA + *rrpB*	1339 (98.4%)	21 (1.5%)	1 (0.1%)	1361
rrpA only	34 (1.6%)	2046 (90.8%)	172 (7.6%)	2252
Neither	0	91 (68.4%)	42 (31.6%)	133

RrpA full-length and truncated versions

```
CjNCTC11168_RrpA      -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN
Cj81116_RrpA          -MTKENSQCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN
Cc76339_RrpA          VKNTKNSTCNYQECGFNYTLALISGKYKMSVLYCLYKDEIVRYNELNRILSPISFKTLTN
Cj414_RrpA            MKNTKNSTCNYQEYGFNYTLALISGKYKMSVLYCLYKDKIVRYNELKRILNPISFKTLTN
N-term_truncated      ----------------------------MSILYCLFRYEIVRYNELKRFLSSISFKTLTN
C-term_truncated      -MTKENSQCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN
                                                  **:****:: :*******:*:*..********

CjNCTC11168_RrpA      TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN-
Cj81116_RrpA          TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMCKWGEKDKKEKNA---
Cc76339_RrpA          VLRELESDGLITRKEYPQIPPKVEYSLSQKGQSFIPILQAMCDWGE-KNKRRIP---
Cj414_RrpA            VLRELENAGLIIRKEYPQIPPKVEYSLSKKGQSFIPILEAMCDWGRRKQKINILKIY
N-term_truncated      TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMCDWEEENKKLQGK---
C-term_truncated      TLRELENDGLIIRKEYA----------------------------------------
                      .*****. *** ****.
```

RrpB full-length and truncated versions

```
CjNCTC11168_RrpB       MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL
Cc2544_RrpB            MKKYHSPCPVETTLNLIGNKWKILIIRELLDGEKRFGELRKNISATKNQNISQNVLTQNL
N-term_truncated       ----------------------------MQGTKRFGELRKSISFTKNQNISQNVLTQNL
N+C-term_truncated     ----------------------------MQGTKRFGELRKSISFTKNQNISQNVLTQNL
C-term_truncated       MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL
                                                   ::* ********.** ***************

CjNCTC11168_RrpB        RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV
Cc2544_RrpB             RELEEAKLLKRKVYAEVPPRVEYSLTLLGSSLESVLKSLEIWGDKYKNMN
N-term_truncated        RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV
N+C-term_truncated      RELEEAKLIKRKVYAEVPPKVEYLLISLGNSLESILKSLEN---------
C-term_truncated        REIRRSKTDQTQSLCRSSSKG----------------------------
                        **:..:*  : :  .. ..:
```

Figure 5.5 Presence of N- and C-terminal truncations in *C. jejuni* and *C. coli* RrpA and RrpB proteins. Amino acid sequences were obtained from *C. jejuni* NCTC 11168, 81116 and 414, and *C. coli* 2544 and 76339. Other truncated versions were obtained from genome sequences listed in Appendix 4. Asterisks mean identical amino acids, colons and full stops show conservative substitutions. Alignments were made using ClustalX2. Yellow blocks indicate identical regions in all proteins, including regions with truncated proteins.

5.2.4 Plasticity region investigation amongst 3,746 *C. jejuni*

The great majority of *C. jejuni* strains contain *rrpA*, but the presence of *rrpB* is more variable. Based on this, further analysis was performed to investigate the genetic structure around the *rrpA* and *rrpB* genes. *rrpA* and *rrpB* are located near a type I Restriction-Modification (R-M) (*hsd*) system, which may suggest one possible explanation for the variation in the presence or absence of *rrpB* amongst different *C. jejuni* strains.

The analysis of the 3,746 *C. jejuni* genome sequences identified differences in the distribution of genes flanking *rrpA* and *rrpB.* Two conserved flanking regions were identified with a more variable central region. The upstream conserved region contains genes with unknown function and *rrpA*, whilst the downstream conserved region contains the arsenic resistance operon (*ars*), a methyl-accepting chemotaxis protein (*mcp*) gene and a paralysed flagellum gene *pflA*. In between these two conserved regions, there is a variable region that contains one of two versions of a "transferable plasticity region" including the type I R-M (*hsd*) system (Figure 5.6).

The NCTC 11168 version was used as a reference for strains with the variable region containing *rrpB.* The 81116 version was used as a reference for strains with the variable region lacking *rrpB*. The NCTC 11168 variable central region appears to be representative of all *rrpB*$^{+}$ strains, whilst the 81116 variable central region appears representative of all *rrpB*$^{-}$ strains.

C. coli genome sequences demonstrated a very similar structure to 81116. *C. coli* strains do not contain *rrpB*, and only a very few contain *rrpA.* This finding suggests that the transferable plasticity region does not occur in this region for *C. coli*.

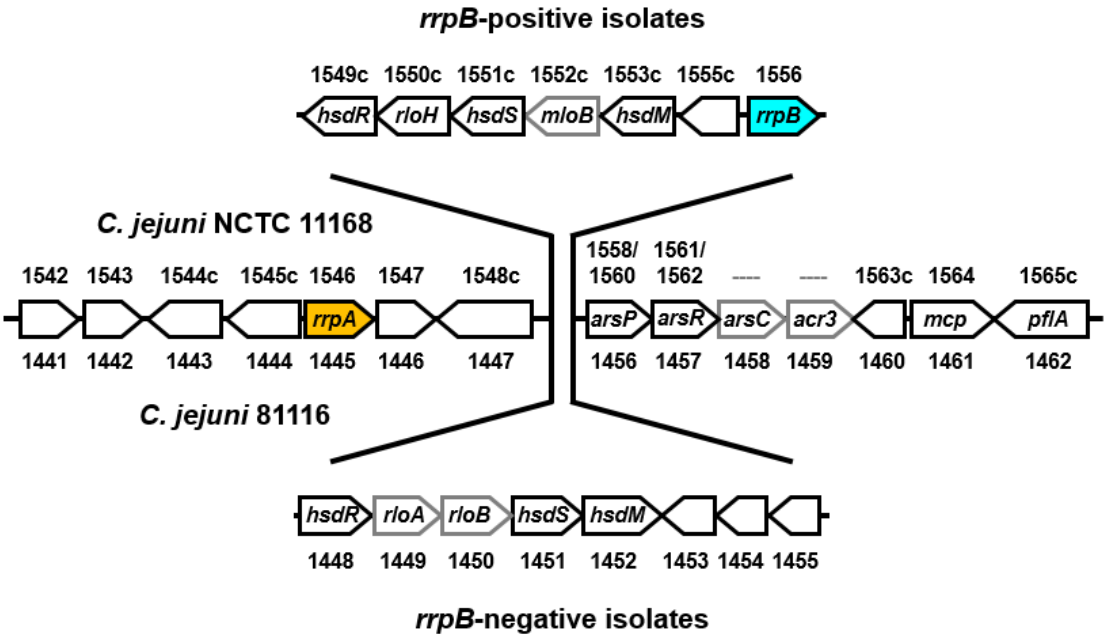

Figure 5.6 The genomes of NCTC 11168 and 81116 differ in a variable central region containing a type I Restriction-Modification (*hsd*) system. The transferrable plasticity region surrounded by conserved genes is linked with the type I Restriction-Modification (*hsd*) system, suggesting a mechanism for the variable distribution of *rrpB*. The figure shows the *C. jejuni* NCTC 11168 (*rrpB*$^{+}$) and 81116 (*rrpB*$^{-}$) genes and gene numbers. *C. jejuni* NCTC 11168 lacks the *arsC* and *acr3* genes. *arsP* and *arsR* are inactivated and annotated as *Cj1558*/*Cj1560* and *Cj1561*/*Cj1562* respectively.

5.3 Discussion

Presence of *rrpA* and *rrpB* in the *C. jejuni* genome appears to influence oxidative stress resistance. Therefore, a search for the presence or absence of *rrpA* and *rrpB* amongst different *C. jejuni* strains was performed. The first screening of different *C. jejuni* strains was performed using PCR with degenerate primers. Further investigations were performed using both whole genome microarray data and whole genome sequencing data with a greater number of *C. jejuni* strains. The variation in the presence of *rrpB* led to an investigation of the genetic structure surrounding *rrpA* and *rrpB.*

Screening for the *cosR, rrpA* and *rrpB* genes using PCR with degenerate primers was performed to determine the presence or absence of these genes in 20 different *C. jejuni* strains in order to confirm the reanalysis of the Champion *et al.* (2005) microarray data that indicated variation in the presence of *rrpB* amongst different wild-type strains (Gundogdu *et al.*, 2011). A degenerate primer has a mix of nucleotide sequences that covers all possible nucleotide combinations for a given protein sequence (Linhart and Shamir, 2005). Degenerate primers permit some flexibility in the specificity allowing the amplification of a gene sequence with some variations (Souvenir *et al.*, 2003). The more degenerate a primer, the more sequence combinations the primer mix contains (Linhart and Shamir, 2005). However, a few problems can arise from the use of degenerate primers, such as having to optimise the concentration of the degenerate primer pairs and the amplification of erroneous fragments (Souvenir *et al.*, 2003). A few erroneous amplifications were observed in the present study. However, as the size of the genes screened for were known and the correct fragments amplified as clearer and brighter bands than the erroneous fragments, this was not considered a major problem.

cosR was present in all strains screened using the degenerate primers. *cosR* is a highly conserved gene amongst different *Campylobacter* species, such as *C. jejuni, C. coli, C. lari, C. fetus, C. curvus* and *C. concisus* (Hwang *et al.*, 2011). CosR is a regulator of 480 genes involved in different cell functions, such as energy metabolism, cell respiration, heat shock response and regulation of oxidative stress responses (Hwang *et al.*, 2011, Hwang *et al.*, 2012). CosR is an important regulator of the oxidative stress defence, as CosR negatively regulates *sodB, dps*, *luxS* and *rrc* expression and

positively regulates *ahpC* and *katA* expression (Hwang *et al.*, 2011, Hwang *et al.*, 2012).

The study of Stabler *et al.* (2013) assigned *C. jejuni* strains as either livestock-associated or water & wild-life-associated clonal complexes based on the isolation source and MSLT analysis. PCR screening with degenerate primers for *rrpA* and *rrpB* demonstrated that all strains from livestock-associated subclades contained both *rrpA* and *rrpB*, except one strain that contained only *rrpA*. However, all strains from the water & wild-life subclades contained only *rrpA*, except one that contained both genes. This initial screening suggested that the *rrpA* and *rrpB* gene distribution could be related to the source of isolation and confirmed the reanalysis of the Champion *et al.* (2005) microarray data that indicated variation in the presence of *rrpB* amongst different wild-type strains.

Additional analysis of the *rrpA* and *rrpB* gene distribution was then performed on 270 *C. jejuni* strains using whole genome microarray data from the Stabler *et al.* (2013) study. This identified that the majority of the livestock-associated strains contained *rrpA* and *rrpB*, whilst the majority of the water & wild-life-associated strains contained only *rrpA*. These differences in the gene distribution suggest niche adaptation as the presence or absence of *rrpB* appears to correlate with the source of isolation (Stabler *et al.*, 2013).

A more in-depth analysis of the *rrpA* and *rrpB* gene distribution was performed using whole genome sequence data from 3,746 *C. jejuni* and 486 *C. coli* strains. The same pattern of *rrpA* and *rrpB* distribution was observed. The vast majority of *C. jejuni* strains contained *rrpA*, whilst only around 50% of the strains contained *rrpB*. With regard to *C. coli* strains, only very few contained *rrpA*, whilst most *C. coli* contained neither *rrpA* nor *rrpB*.

These strains were further analysed by MSLT. MLST is a powerful tool that can investigate different genotypes based on the genetic diversity of the housekeeping genes (Sheppard *et al.*, 2009). MLST can identify nucleotide differences in the housekeeping genes that allow the attribution of isolates to specific sources (Sheppard *et al.*, 2009). MLST can also identify host-restricted and host generalist lineages that can be found in both animal and clinical samples (Sheppard *et al.*, 2009, Sheppard *et al.*, 2014).

In the present study, MLST analysis identified strains belonging to different clonal complexes. It was observed that some clonal complexes were more associated with strains containing both *rrpA* and *rrpB* genes (*rrpB*$^{+}$), whilst others were more associated with strains containing only *rrpA* (*rrpB*$^{-}$). Clonal complexes were identified as livestock-associated or water & wild-life-associated based on different studies (Kwan *et al.*, 2008, Rotariu *et al.*, 2009, Stabler *et al.*, 2013). Other studies have also demonstrated the relationship between MLST clonal complex and source of isolation (Manning *et al.*, 2003, Zautner *et al.*, 2011).

Variation in gene distribution can indicate niche adaptation (Stabler *et al.*, 2013). Strains may have lost or gained *rrpB*, promoting some advantage in survival/resistance to a specific environment. In the present study, niche adaptation is suggested by the fact that strains with a specific profile (*rrpB*$^{+}$ or *rrpB*$^{-}$) were more related to a specific MLST clonal complex. Possibly *C. jejuni* has evolved genetically and adapted to survive under different conditions. Differences in gene distribution may affect different regulatory pathways that allow *C. jejuni* to survive under hostile oxygen conditions, such as in aerobic or anaerobic environments (Gaynor *et al.*, 2004).

Considering the 3,746 *C. jejuni* strains analysed by MSLT, a high percentage of strains identified in the CC-21 and CC-61 contained both *rrpA* and *rrpB*. Both CC-21 and CC-61 clonal complexes are defined as livestock-associated (Kwan *et al.*, 2008, Stabler *et al.*, 2013). This suggests that the presence of *rrpA* and *rrpB* may give an advantage to *C. jejuni* strains in the colonisation of livestock. In the Sheppard *et al.* (2009) study, most *C. jejuni* genotypes associated with human disease were genetically similar to isolates from chicken sources. Therefore, strains containing both *rrpA* and *rrpB* genes, which are related to livestock, might also be more likely to infect humans. Interestingly, the CC-21 and CC-61 are also two of the most associated with human infections (Manning *et al.*, 2003). Therefore, the presence of *rrpA* and *rrpB* in *C. jejuni* strains may also be important for human infection. However, in this study, most of the genomes analysed were clinical isolates and contained only *rrpA*.

Different truncated RrpA and RrpB amino acid sequences were identified in some *C. jejuni* isolates. However, no link was observed between the truncated versions and source of the isolates. The isolates with a truncated RrpA were related to the CC-607 and lacked the N-terminal 27 amino acids. A percentage of *C. jejuni* strains belonging

to CC-353 had a truncated RrpB lacking either the N-terminal 29 amino acids, the C-terminal 29 amino acids or lacked amino acids at both ends of the protein sequence. Gene truncation arises from a point mutation in the gene sequence, which can generate a stop codon resulting in interruption of the translation process (Clarke and Eriksson, 2000). Gene truncation can lead to a non-functional protein. However, some truncated proteins may still be active or partially active (Clarke and Eriksson, 2000). C-terminal residues play an important role in protein folding (Fedyukina and Cavagnero, 2011). Truncation of C-terminal residues can result in loss of native protein conformation and the protein can become inactive (Fedyukina and Cavagnero, 2011). The loss of just a few C-terminal residues is enough to generate a protein compact losing some of its secondary structure (Fedyukina and Cavagnero, 2011). This suggests that RrpA and RrpB proteins lacking the C-terminal amino acids may not be functional. Further studies are necessary to investigate if these proteins are still active.

Most strains belonging to CC-353 were isolated from humans and these strains contained both *rrpA* and *rrpB.* Dingle *et al.* (2002) demonstrated that CC-353 is highly associated with humans and chickens. This reinforces that livestock-associated clonal complexes tend to have both genes and are associated with human infection.

In this study the CC-403 is classified as water & wild-life-associated with the vast majority of strains isolated from humans and containing only *rrpA.* However, Morley *et al.* (2015) identified the CC-403 as livestock-associated, but not associated with any avian species. The strains were normally isolated from pigs, cattle and humans (Morley *et al.*, 2015). A great number of the CC-403 strains were isolated from humans, indicating that these strains have high capacity to cause diseases in humans (Morley *et al.*, 2015). This suggests niche adaptation for *C. jejuni* strains from CC-403, indicating that these strains have evolved and become less adapted to colonise avian hosts, but with greater capacity to infect humans (Morley *et al.*, 2015).

Further analysis of the genetic structure surrounding the *rrpA* and *rrpB* genes in the 3,746 *C. jejuni* strains identified a putative plasticity region between two conserved regions. Both genes are located near a type I R-M (*hsd*) system. Restriction enzymes are widely present in bacteria and are responsible for recognition of a specific nucleotide sequence in a DNA molecule and then cleavage of the DNA (Murray, 2000). R-M systems are a mechanism of bacterial defence that targets invading DNA

elements (Vasu and Nagaraja, 2013). R-M systems help to protect bacterial cells from foreign DNA thorough cleavage (Kobayashi, 2001). R-M systems are acquired by the bacterial genome from mobile elements through horizontal transfer (Kobayashi, 2001).

Normally, a restriction endonuclease is accompanied by a modification enzyme, with both enzymes comprising an R-M system (Murray, 2000). The modification enzyme, which is a DNA methyltransferase, protects the host DNA against the restriction endonuclease by methylation of specific nucleotides (Murray, 2000). Methylation activity ensures discrimination of bacterial self DNA, whilst DNA lacking methylation is recognised as non-self DNA (Vasu and Nagaraja, 2013). Unmethylated DNA will then be cleaved by the restriction endonuclease at specific sites. Cleavage occurs at phosphodiester bonds of endonucleotides (Vasu and Nagaraja, 2013).

R-M systems are acquired by horizontal gene transfer through mobile genetic elements or by other means, such as natural transformation, or small genomic integration hotspots (Oliveira *et al.*, 2014). Mobile genetic elements encode proteins that mediate their movement within the genome or can be inserted in a different genome, such as transposons, plasmids and bacteriophages (Frost *et al.*, 2005). R-M systems can also occasionally cause evolutionary changes in a genome (Kobayashi, 2001). Many R-M systems are linked with a recombinase homologue or a transposase homologue, which are responsible for genome rearrangements (insertion or deletion) (Kobayashi, 2001, Furuta *et al.*, 2010). R-M systems are essential for several bacterial DNA recombination processes. If the DNA cleaved is from a closely related bacterium, the DNA fragments can be used for homologous recombination (Vasu and Nagaraja, 2013).

R-M systems work at a selfish level, which means once incorporated into a bacterial genome, the R-M system cannot easily be removed (Kobayashi, 2001). This is demonstrated by the fact that an R-M operon on the bacterial chromosome cannot be easily replaced by homologous recombination (Kobayashi, 2001). If the R-M genes are lost, the unmethylated sites in the DNA can be digested by other R-M systems which can lead to cell death (Kobayashi, 2001). Therefore, an established R-M system becomes essential for the bacterial cell (Vasu and Nagaraja, 2013).

There are different types of R-M systems, such as type I, type II, type III and type IV systems (Murray, 2000). The classification is based on subunit composition, sequence recognition, cleavage position, co-factor requirements and substrate specificity (Vasu and Nagaraja, 2013). The *C. jejuni* NCTC 11168 genome sequence contains a putative type I R-M locus (Parkhill *et al.*, 2000). Type I R–M systems are common amongst prokaryotes and have been found in several different bacterial species (Murray, 2000). The type I R-M system is a bi-functional, multi-subunit complex containing *hsdR, hsdM* and *hsdS* genes, where *hsd* denotes "host specificity of DNA" (Murray, 2000, Miller *et al.*, 2005). The sequence recognised by a *hsd* system is not the cleavage site (Loenen *et al.*, 2014). Type I R-M enzymes translocate along DNA for considerable distances whilst remaining attached to their recognition sites. DNA will form large loops before being cleaved though breaking of phosphodiester bonds (Murray, 2000). DNA cleavage occurs randomly at asymmetric recognition site distances between 400 to 7,000 bp (Donahue and Peek, 2001).

The HsdS subunit has recognition domains that are responsible for recognising a specific DNA sequence for restriction and modification activities (Murray, 2000, Donahue and Peek, 2001). The HsdR subunit is responsible for the cleavage of DNA and the HsdM subunit is responsible for DNA methylation (Donahue and Peek, 2001). Normally, *hsdM* and *hsdS* are transcribed from the same promoter, whilst *hsdR* is transcribed from a separate promoter (Murray, 2000).

There are five type I R-M families, from A to E (Loenen *et al.*, 2014). These families are categorised based on complementation tests, antibody cross-reactivity and amino acid sequence (Loenen *et al.*, 2014). HsdR and HsdM proteins from different type I R-M families have regions of sequence homology. However, there is a low level of gene identity amongst the different type I families (Donahue and Peek, 2001).

The analysis of the *C. jejuni* type I R-M system performed by Miller *et al.* (2005) used 73 strains, including NCTC 11168, 81-176 and 81116, which are classical *C. jejuni* reference wild-types strains. This analysis was based on relatedness of nucleotide sequences and presence of characteristic spacer repeats within *hsdS.* Their results assigned some *hsd* systems to the classical type IC family, but also identified two new additional type I R-M families, termed type IAB and type IF (Miller *et al.*, 2005). Some *C. jejuni* strains had similarities to both IA and IB families, but not enough to

be assigned to either one. These strains demonstrated a sister affiliation to both families. Therefore, a new family designated IAB was proposed (Miller *et al.*, 2005). Similarly with the new IF family, the *hsd* locus demonstrated some similarities in the amino acid sequence to the ID family, but not enough to be assigned. Therefore, an additional IF *hsd* family was also proposed to be present in *C. jejuni* strains. 81116 has a type IAB *hsd* system, whilst both NCTC 11168 and 81-176 have a type IF *hsd* system (Miller *et al.*, 2005).

Further analysis of the genetic structure surrounding the *rrpA* and *rrpB* genes in the 3,746 *C. jejuni* strains identified a putative transferable plasticity region between two conserved regions. The variable central region contains one of two versions of a transferable plasticity region containing a type I R-M system. One version contains *rrpB* and the other version does not. Based on the Miller *et al.* (2005) study, different type I R-M families were observed amongst the different *C. jejuni* wild-type strains analysed. Further analysis demonstrated that *C. jejuni* strains belonging to the same R-M family have extensive gene rearrangements within the *hsd* loci.

However, it has been suggested that low sequence similarity between the IC, IAB and IF families would make inter-family recombination events unlikely. Morley *et al.* (2015) also identified specific R-M systems in *C. jejuni* strains from the ST-403 complex. These strains had specific intra-lineage recombination that regulated DNA acquisition and recombination. Both NCTC 11168 and 81-176 contain a type IF *hsd* system and both have the variable central region containing *rrpB*. This region is not mobile, but likely to undergo recombination within the IF family. The type IF *hsd* family represents the majority of isolates in the ST-21, ST-61, ST-42 and ST-353 clonal complexes. 81116 contains a type IAB *hsd* system and has the variable central region that does not contain *rrpB*. This version of the plasticity region represents the majority of *C. jejuni* strains in all other clonal complexes.

The Miller *et al.* (2005) study also described the presence of other ORFs in the *hsd* loci. Some ORFs were identified between *hsdR* and *hsdS* and designated *rloA* to *rloH* (R-linked ORF). Other ORFs were identified between *hsdS* and *hsdM* and designated *mloA* to *mloB* (M-linked ORF). The order of the *C. jejuni hsd* loci can vary amongst strains. The gene order in NCTC 11168 is *hsdM-hsdS-hsdR*, whilst the gene order in 81116 is *hsdR-hsdS-hsdM*.

The recombination process is facilitated by the *hsd* system, so it is possible that *rrpB* was either acquired or lost as part of the mobile plasticity region. The version containing *rrpB* is strongly associated with the IAB *hsd* family system. IAB *hsd* locus demonstrated higher degree of diversity particularly with respect to *rlo* genes (Miller *et al.*, 2005). The NCTC 11168 genome contains only one *rlo* gene (*rloH*). RloH has partial similarity to a class of ATP/GTP-binding proteins (Miller *et al.*, 2005). All other *rlo* and *mlo* genes encode putative proteins with as yet unidentified functions. *rlo* and *mlo* encode similar proteins with more than 70% amino acid identity between them (Miller *et al.*, 2005).

The upstream conserved region contains *rrpA* and other genes encoding proteins with unknown functions. The downstream conserved region contains the arsenic resistance operon (*ars*), a methyl-accepting chemotaxis protein (*mcp*) gene, a paralysed flagellum gene *pflA* and two other genes encoding proteins with unknown functions. Arsenic is ubiquitously found in the environment, such as in soil, water and air, in both inorganic and organic forms (Diorio *et al.*, 1995, Shen *et al.*, 2013). Arsenic is a toxic compound for bacteria, inhibiting phosphorylation and hindering energy generation systems (Shen *et al.*, 2013). Resistance mechanisms against arsenic compounds are essential for bacterial survival (Wang *et al.*, 2009). Arsenic resistance operons are found in both Gram-positive and Gram-negative bacteria (Diorio *et al.*, 1995). Arsenic resistance operons consist of three to five genes that are highly conserved (Diorio *et al.*, 1995). Various genes are responsible for arsenic resistance, including *arsR*, *arsA*, *arsB*, *arsC*, *arsD*, *arsH* and *arsM* (Wang *et al.*, 2009).

C. jejuni RM1221 strain contains a four gene arsenic resistance operon encoding a transcriptional repressor (ArsR), an arsenate reductase (ArsC) together with a putative membrane permease (ArsP) and an efflux protein (Acr3) (Wang et al., 2009). AsrR controls the expression of the other *ars* genes (Rosen, 2002). ArsR negatively regulates the expression of *ars* genes in the presence of arsenite substrates (Shen *et al.*, 2013). The role of ArsP in the arsenic resistance response remains to be clarified (Wang *et al.*, 2009). The organic form of arsenic is used to feed farm animals for the food chain, including both livestock and poultry (Shen *et al.*, 2013). *C. jejuni* is likely to be exposed to arsenic organic compounds that are present in the chicken gut (Sapkota *et al.*, 2006). When excreted by poultry, these degrade into metabolites such as arsenite and arsenate (Sapkota *et al.*, 2006).

NCTC 11168 and 81-176 are missing *arsC* and *acr3* in the *ars* locus (Wang *et al.*, 2009). Furthermore, NCTC 11168 *arsR* and *arsP* are degenerated as result of a single-nucleotide deletion in the ORF (Ahmed *et al.*, 2002, Wang *et al.*, 2009). Therefore, these two strains lack a functional *ars* operon. Strains that contain the *ars* operon are more resistant to arsenic (Wang *et al.*, 2009). Whilst NCTC 11168 lacks a functional *ars* operon, the genome contains homologs of *arsR*, *arsC* and *arsB* (Wang *et al.*, 2009, Shen *et al.*, 2013). These genes are located far apart on the chromosome (Wang *et al.*, 2009). ArsB and Acr3 are two unrelated arsenite transporters broadly present in different bacteria (Rosen, 2002). *arsB* and *acr3* are also present in *C. jejuni* strains (Wang *et al.*, 2009, Shen *et al.*, 2013). Some *C. jejuni* strains have either *arsB* or *acr3*, whilst some strains contain both genes (Shen *et al.*, 2013). ArsA is an ATPase family member present in various bacteria, but absent from *C jejuni* (Wang *et al.*, 2009, Shen *et al.*, 2013). The structure of ArsA and ArsB are related to the multiple drug resistance ATP-driven efflux pump (Diorio *et al.*, 1995). ArsA functions as an ATPase and ArsB contains transmembrane domains and has a role transporting arsenite out of the cell (Rosen, 2002). When ArsA is expressed in an operon together with ArsB, the detoxification of arsenite is more efficient (Dey and Rosen, 1995). However, ArsB can eliminate arsenite in the absence of ArsA (Dey and Rosen, 1995). When alone, ArsB uses the membrane potential in the efflux of arsenite (Wang *et al.*, 2009).

In *C. jejuni* NCTC 11168, ArsB is responsible for arsenite resistance, but not for arsenate resistance (Shen *et al.*, 2013). However, ArsC can convert arsenate to arsenite to then be eliminated by ArsB (Shen *et al.*, 2013). ArsB has a more important role in *C. jejuni* strains that lack the *ars* operon, such as NCTC 11168 (Shen *et al.*, 2013). However, the *ars* operon provides a higher level of resistance than ArsB alone (Shen *et al.*, 2013). *C. jejuni* requires either ArsB or Acr3 to resist arsenic in the environment (Shen *et al.*, 2013). ArsC is responsible for arsenic resistance in *C. jejuni* strains that contain an *ars* operon. However, in the NCTC 11168 strain, ArsC does not appear to play a role in arsenic resistance (Shen *et al.*, 2013).

The *pflA* (paralysed flagella) gene is also present in the downstream conserved region. PflA is important for motility as mutation of *pflA* results in a strain with a paralysed flagellum (Yao *et al.*, 1994). Strains where *pflA* has been mutated express a full length, but immobilised flagellum. Mutation of *pflA* also results in reduced invasion of epithelial cells (Yao *et al.*, 1994).

mcp has a methyl-accepting chemotaxis protein (MCP) signalling domain and is also present in the downstream conserved region as seen in the NCTC 11168 genome annotation. MCPs are trans-membrane proteins responsible for sensing environmental signals then transmitting to chemotactic proteins to promote the chemotactic orientation (Wadhams and Armitage, 2004). MCPs interact with a periplasmic binding protein and are responsible for detecting chemotactic signals extracellularly (Wadhams and Armitage, 2004). MCP activates CheA resulting in phosphorylation of CheY, which is a response regulator responsible for regulating the direction of the flagella rotation (Lertsethtakarn *et al.*, 2011). *C. jejuni* NCTC 11168 contains several putative MCPs that contain transmembrane motifs and periplasmic binding domains, such as Tlp1, Tlp2, Tlp3, DocB and DocC (Vegge et al., 2009). Mutation of any of these genes does not affect the chemotactic responses of the mutants compared to the wild-type strain (Vegge *et al.*, 2009). This suggests that mutation of only one *mcp* gene does not affect chemotaxis due to the overlapping sensing mechanisms of other MCPs (Vegge *et al.*, 2009). The chemotaxis mechanism is essential for *C. jejuni* colonisation (Marchant *et al.*, 2002).

This study has demonstrated that *rrpA* is conserved throughout *C. jejuni* strains, whilst the presence of *rrpB* is variable. *rrpB* is usually present in strains assigned to livestock-associated clonal complexes. One possible reason for the variation in the presence of *rrpB* is that this gene is located in a variable region of the genome, whilst *rrpA* is located in a conserved region. The variable region contains an R-M modification system that may be responsible for the genetic variation of different *C. jejuni* strains. Further studies are required to confirm this hypothesis.

6 Analysis of *C. jejuni* strains isolated in Brazil

6.1 Introduction

The laboratory *Coleção de Campylobacter* (Campylobacter Collection) at the *Fundação Oswaldo Cruz* (Oswaldo Cruz Foundation, known as Fiocruz) in Rio de Janeiro, Brazil kindly provided 43 *C. jejuni* strains to be included in this study. The Fiocruz is attached to the Brazilian Ministry of Health and promotes health, social development, scientific and technological knowledge. The strains were isolated from three different sources: humans, food and the environment. Twenty strains were isolated from human stools, twenty from chicken meat, two from creek water and one from aviary drinking water.

The epidemiology and symptomatology of Campylobacteriosis differs between developed and developing countries and the reasons behind this are not fully understood. *Campylobacter* infections in developed countries usually present with more severe symptoms compared to the disease presentation in developing countries. In developed countries, the disease occurs more frequently in children less than 4 years old, young adults and very elderly people (Padungton and Kaneene, 2003b, FSA, 2015). However, in developing countries the disease is more prevalent in children under 2 years old and it is not common in adults (Coker *et al.*, 2002, Padungton and Kaneene, 2003b). Asymptomatic older children with *Campylobacter* in their stools have been observed in developing countries, whilst this is not a common finding in developed countries (Coker *et al.*, 2002, Quetz *et al.*, 2010).

The aim of this study was to perform phenotypic and genomic comparisons between these Brazilian isolates and reference *C. jejuni* isolates used commonly in research to identify any important differences. Oxidative stress resistance and biofilm formation assays were used to investigate the capacity of these strains to survive in the environment.

6.2 Results

6.2.1 Sensitivity of Brazilian isolates to oxidative stress

C. jejuni possesses complex and not fully understood defence mechanisms against oxidative stress (Atack and Kelly, 2009). Microaerophilic bacteria are sensitive to high oxygen concentrations (Ludwig, 2004), yet *C. jejuni* is able to survive in different adverse oxygen environments (Kaakoush *et al.*, 2007, Hilbert *et al.*, 2010).

The sensitivity of the Brazilian isolates to *in vitro* oxidative stress was performed by exposure to H_2O_2 for 15 min under microaerobic conditions. Strains were exposed to four different concentrations of H_2O_2 (25, 50, 100 and 200 mM) as described in Section 2.4.6.1. Most of the chicken meat isolates were highly resistant to the different hydrogen peroxide concentrations used. All chicken meat isolates were resistant to 100 mM H_2O_2 and only three isolates (1019, 1022 and 1057) exhibited complete sensitivity to 200 mM H_2O_2 (Figure 6.1 and Figure 6.2). The remaining isolates were partially resistant to 200 mM H_2O_2.

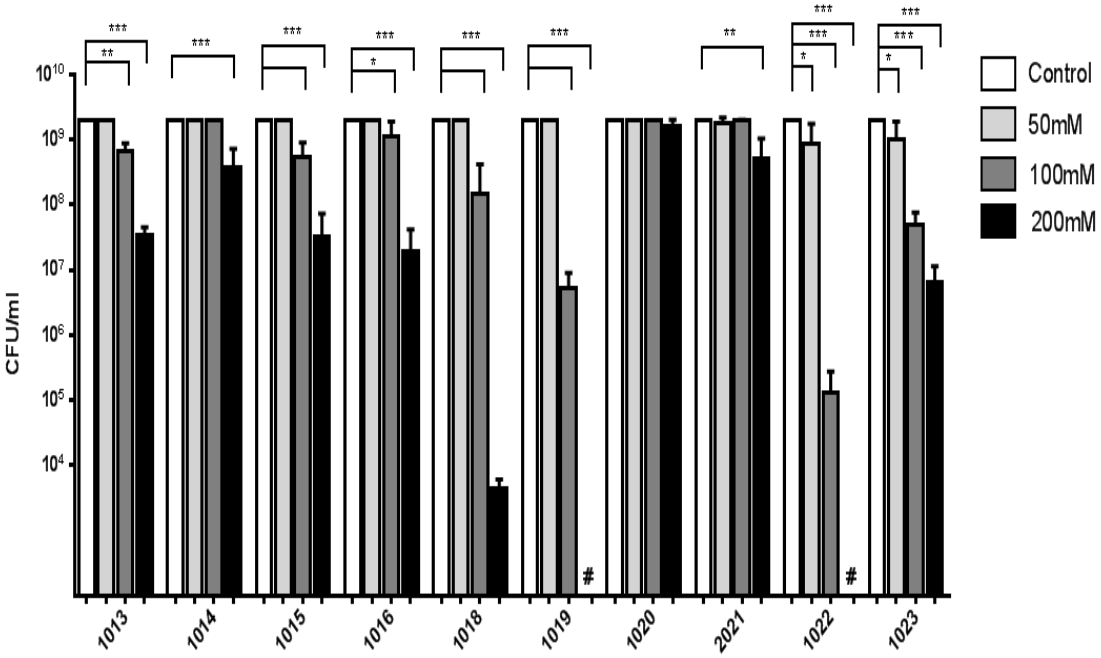

Figure 6.1 The sensitivity to hydrogen peroxide stress of Brazilian *C. jejuni* chicken meat isolates. Bacterial suspensions (OD_{600} 1.0) were exposed to 50, 100 or 200 mM H_2O_2 for 15 minutes at 37°C under microaerobic conditions. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = p <0.05; ** = p <0.01; *** = p <0.001; # no growth.

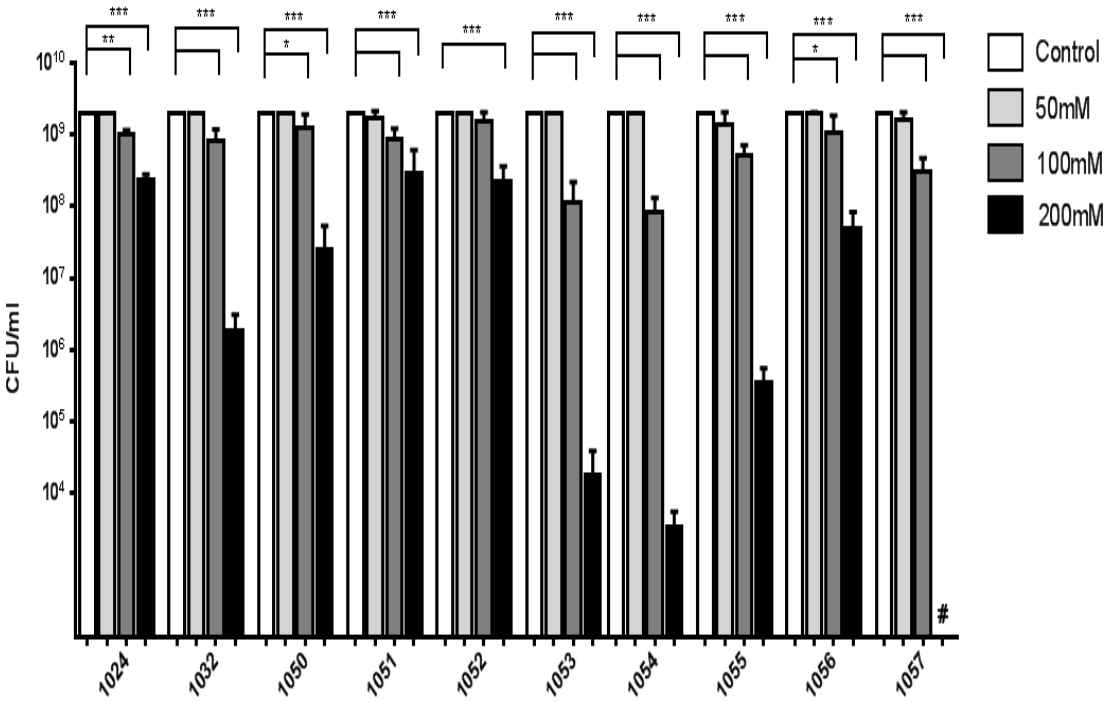

Figure 6.2 The sensitivity to hydrogen peroxide stress of Brazilian *C. jejuni* chicken meat isolates. Bacterial suspensions (OD_{600} 1.0) were exposed to 50, 100 or 200 mM H_2O_2 for 15 minutes at 37°C under microaerobic conditions. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = p <0.05; ** = p <0.01; *** = p <0.001; # no growth.

The sensitivity of the Brazilian *C. jejuni* strains isolated from human stools to H_2O_2 is shown in Figure 6.3 and Figure 6.4. The human isolates in Figure 6.3 demonstrated a higher resistance to H_2O_2 compared to the isolates in Figure 6.4.

Nine strains (478, 488, 492, 500, 507, 511, 593, 679 and 680) exhibited very high resistance to H_2O_2, surviving exposure to 200 mM H_2O_2. In comparison, the other eleven strains exhibited an increased sensitivity to H_2O_2. Seven of these strains (489, 491, 499, 505, 600, 611 and 677) exhibited resistance to only 25 mM H_2O_2. Two further isolates (607 and 608) were resistant to 50 mM H_2O_2 and two other isolates (1479 and 1490) were resistant to 100 mM H_2O_2.

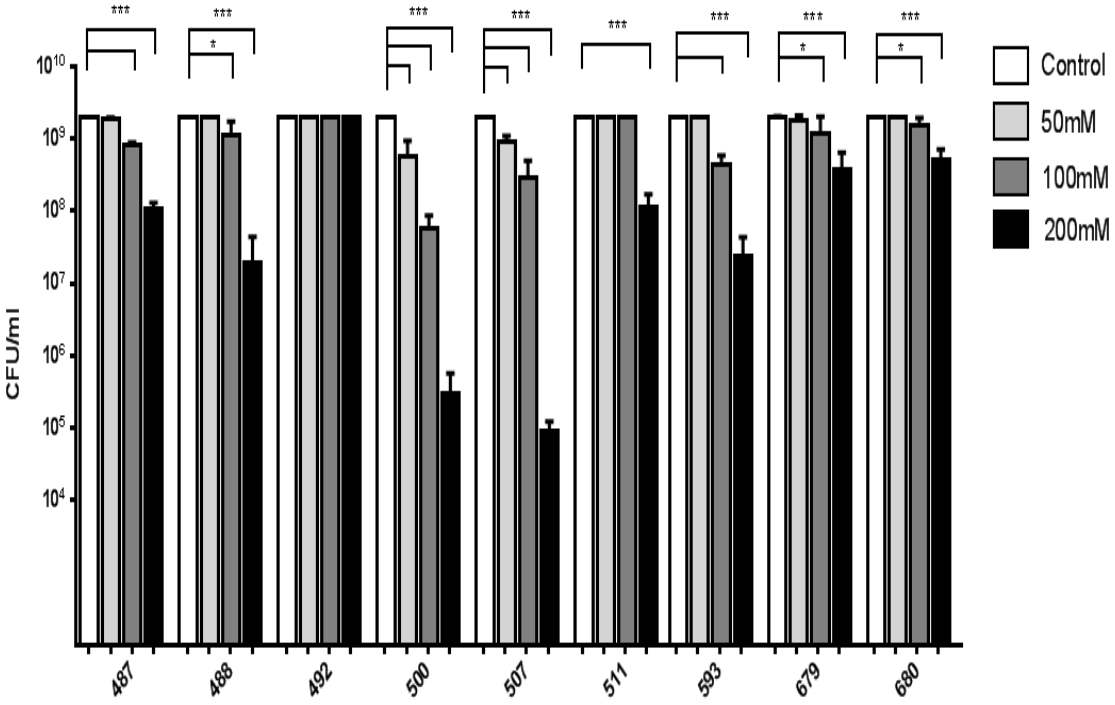

Figure 6.3 The sensitivity to hydrogen peroxide stress of Brazilian *C. jejuni* human isolates. Bacterial suspensions (OD_{600} 1.0) were exposed to 50, 100 or 200 mM H_2O_2 for 15 minutes at 37°C under microaerobic conditions. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = p <0.05; *** = p <0.001.

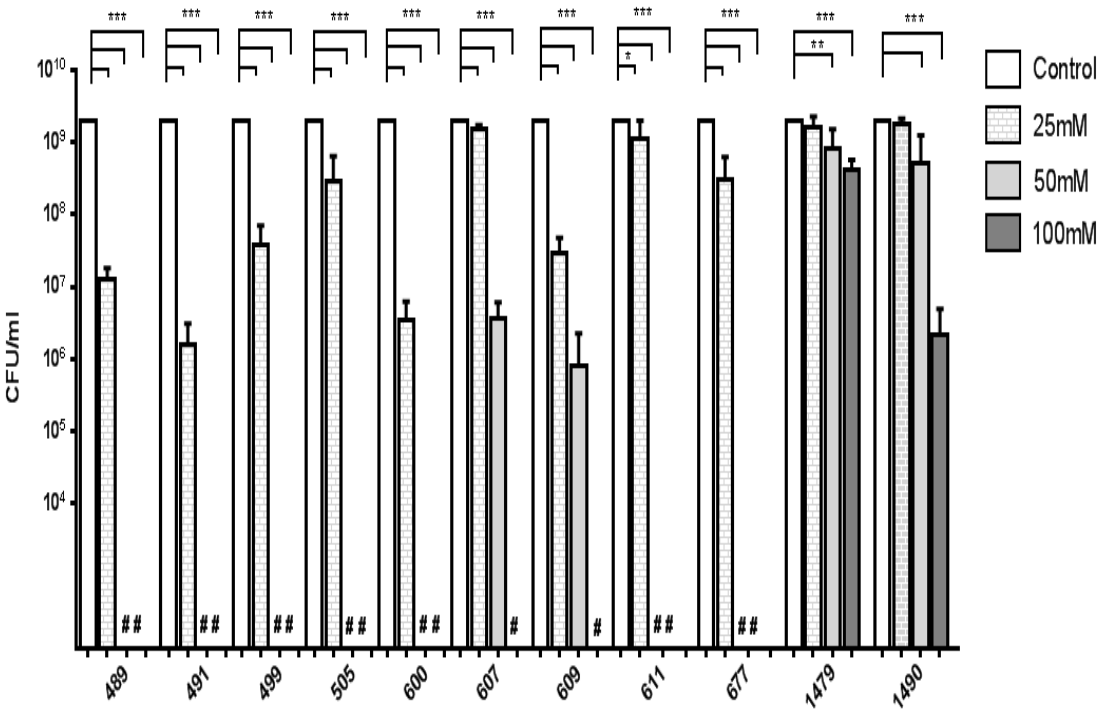

Figure 6.4 The sensitivity to hydrogen peroxide stress of Brazilian *C. jejuni* human isolates. Bacterial suspensions (OD_{600} 1.0) were exposed to 25, 50 or 100 mM H_2O_2 for 15 minutes at 37°C under microaerobic conditions. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. * = p <0.05; ** = p <0.01; *** = p <0.001; # no growth.

The sensitivity of the three Brazilian environmental isolates to H_2O_2 was also investigated. One strain was isolated from aviary drinking water (463), whilst the other two were isolated from creek water (830 and 831). Two of the environmental isolates (463 and 830) were resistant to 100 mM H_2O_2. The other environmental isolate (831) was only resistant to 25 mM H_2O_2 (Figure 6.5).

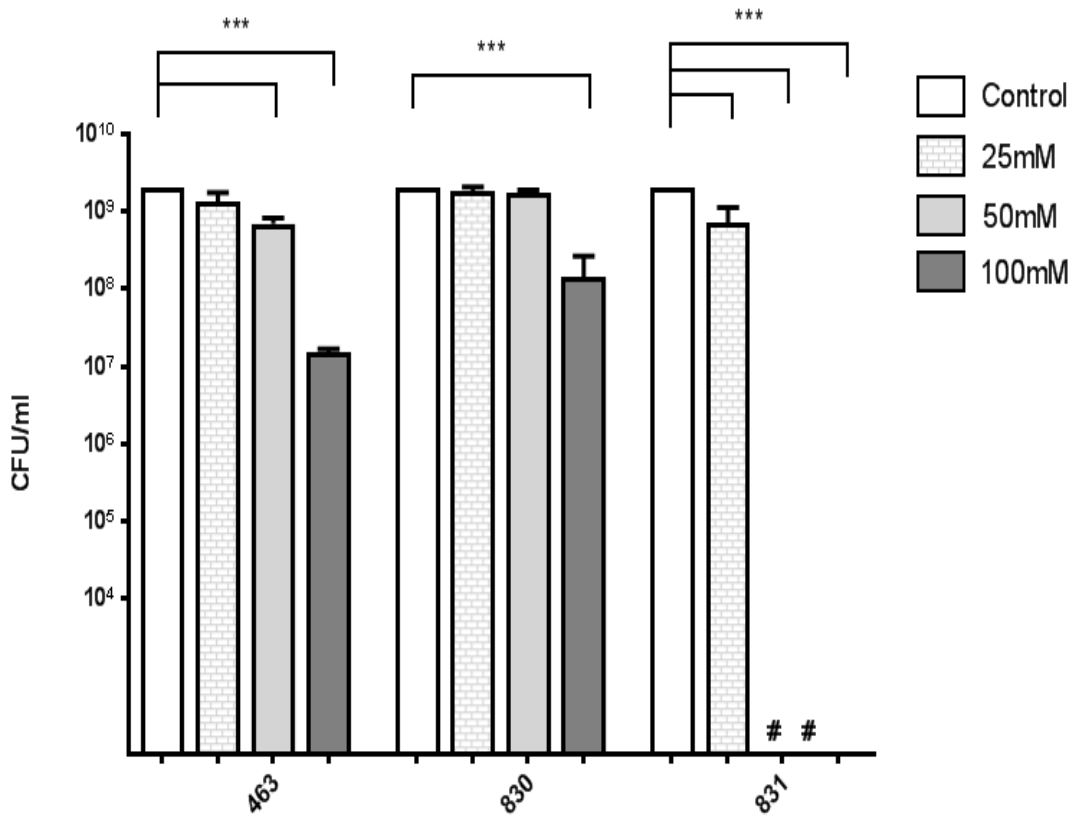

Figure 6.5 The sensitivity to hydrogen peroxide stress of Brazilian *C. jejuni* environmental isolates. Bacterial suspensions (OD_{600} 1.0) were exposed to 25, 50 or 100 mM H_2O_2 for 15 minutes at 37°C under microaerobic conditions. Serial dilutions were prepared (10^{-1} to 10^{-6}) and pipetted in duplicate onto BA plates. CFUs were counted after 48 h incubation. Data represents at least three biological replicates. *** = p <0.001; # no growth.

6.2.2 Ability of the Brazilian isolates to form biofilms

Biofilm formation is important for *C. jejuni* survival in hostile environments (O'Toole *et al.*, 2000). *C. jeuni* forms biofilm under different conditions (Reeser *et al.*, 2007). Environmental signals trigger biofilm formation, such as nutrient starvation, temperature, pH, osmolarity and changes in oxygen concentration (O'Toole *et al.*, 2000, Reeser *et al.*, 2007).

6.2.2.1 Biofilm formation under microaerobic conditions

The ability of the Brazilian isolates to form biofilms under microaerobic conditions was investigated. Biofilm formation in MH broth was investigated as described in Section 2.4.9.

All the chicken meat isolates demonstrated the capacity to form biofilms under microaerobic conditions. These strains demonstrated a mean absorbance of 0.18. Two strains (1013 and 1053) demonstrated a significant enhanced ability to form biofilm under microaerobic conditions compared to the mean of the other isolates (Figure 6.6).

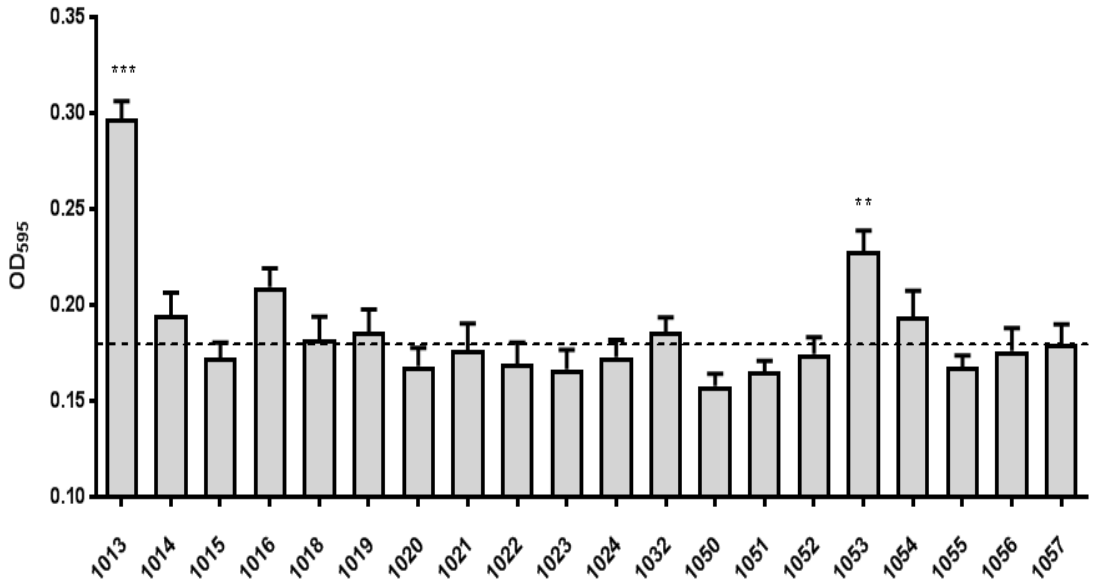

Figure 6.6 Capacity of the Brazilian *C. jejuni* chicken meat isolates to form biofilms under microaerobic conditions. Bacterial suspensions were prepared from 24 h BA plates and MH broth was inoculated to an OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then the OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Plates were incubated at 37ºC for 72 h under microaerobic conditions. Biofilms were stained with 1% (w/v) crystal violet, destained and the OD_{595} recorded. Data represents at least three biological replicates. ** = p <0.01; *** = p <0.001 compared to the mean (dashed line) OD_{595} of 0.18 for all 20 chicken meat isolates.

The Brazilian human isolates also demonstrated the capacity to form biofilms under microaerobic conditions. The strains demonstrated a mean absorbance of 0.17. One isolate (1479) demonstrated a significant greater ability to form biofilm compared to the mean of the other strains (Figure 6.7).

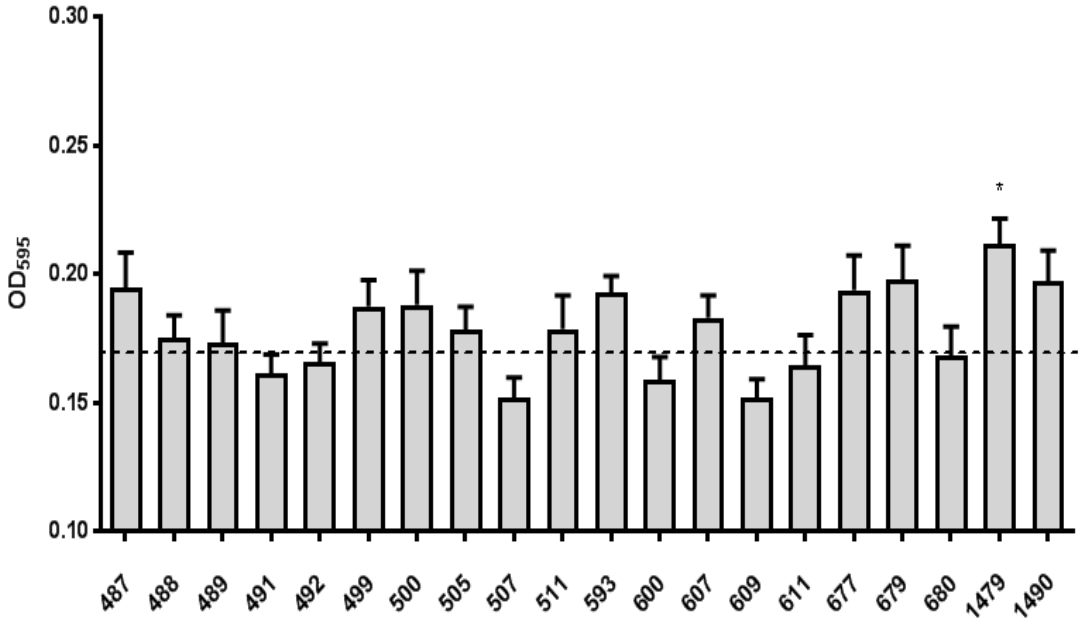

Figure 6.7 Capacity of the Brazilian *C. jejuni* human isolates to form biofilms under microaerobic conditions. Bacterial suspensions were prepared from 24 h BA plates and MH broth was inoculated to an OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then the OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Plates were incubated at 37ºC for 72 h under microaerobic conditions. Biofilms were stained with 1% (w/v) crystal violet, destained and the OD_{595} recorded. Data represents at least three biological replicates. * = p <0.05 compared to the mean (dashed line) OD_{595} of 0.17 for all 20 human isolates.

The Brazilian environmental isolates also demonstrated the ability to form biofilms under microaerobic conditions. These strains demonstrated a mean absorbance of 0.18. None of the environmental isolates had any significant difference in the ability to form biofilms compared to the mean of these isolates (Figure 6.8).

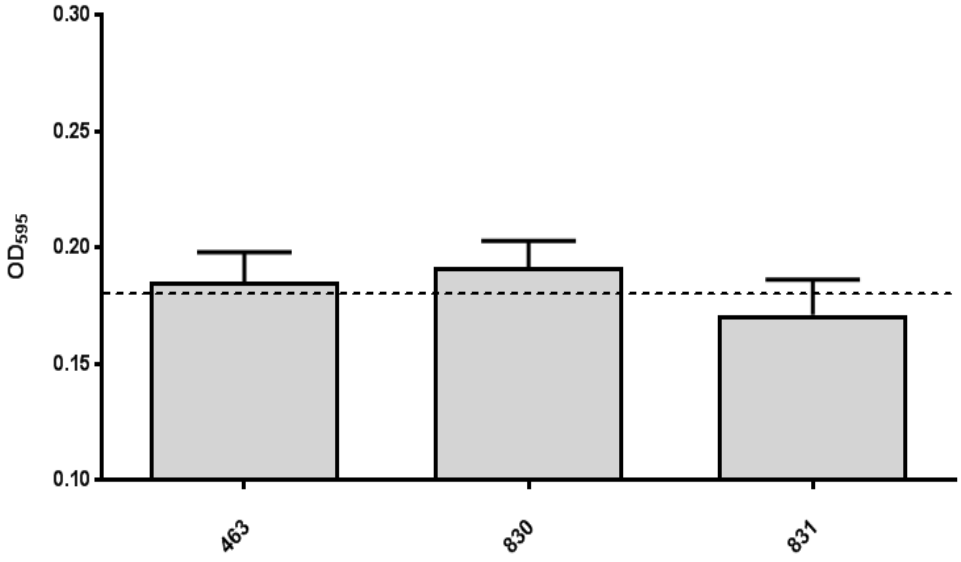

Figure 6.8 Capacity of the Brazilian *C. jejuni* environmental isolates to form biofilms under microaerobic conditions. Bacterial suspensions were prepared from 24 h BA plates and MH broth was inoculated to an OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then the OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Plates were incubated at 37ºC for 72 h under microaerobic conditions. Biofilms were stained with 1% (w/v) crystal violet, destained and the OD_{595} recorded. Data represents at least three biological replicates. Mean 0.18 (dashed line).

6.2.2.2 Biofilm formation under aerobic conditions

The ability of the Brazilian isolates to form biofilms under aerobic conditions was also investigated. Biofilm formation in MH broth was investigated as described in Section 2.4.9. The chicken meat strains demonstrated a mean absorbance of 0.17. Four chicken meat strains demonstrated significant enhanced ability to form biofilms under aerobic conditions (1013, 1014, 1016 and 1022). One strain (1052) demonstrated significant reduced biofilm formation compared to the mean of the other isolates (Figure 6.9).

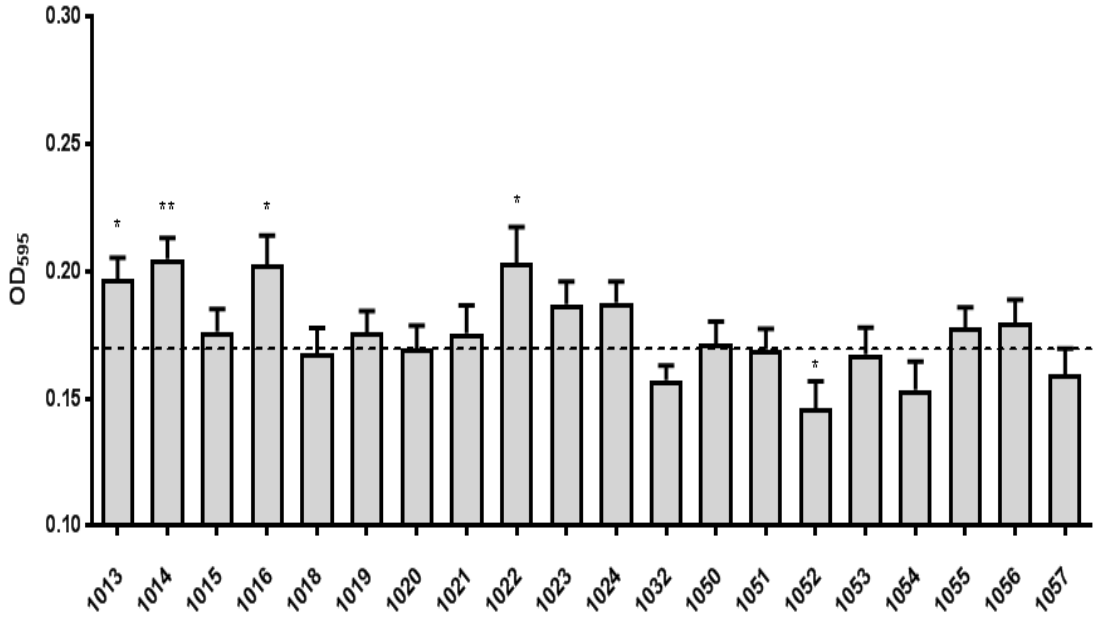

Figure 6.9 Capacity of the Brazilian *C. jejuni* chicken meat isolates to form biofilms under aerobic conditions. Bacterial suspensions were prepared from 24 h BA plates and MH broth was inoculated to an OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then the OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Plates were incubated at 37ºC for 72 h under microaerobic conditions. Biofilms were stained with 1% (w/v) crystal violet, destained and the OD_{595} recorded. Data represents at least three biological replicates. * = p <0.05; ** = p <0.01 compared to the mean (dashed line) OD_{595} of 0.17 for all 20 chicken meat isolates.

The human strains demonstrated a mean absorbance of 0.17. Three human strains demonstrated a significantly enhanced ability to form biofilms under aerobic conditions (505, 607 and 1479). One human strain (488) demonstrated a significantly reduced capacity to form biofilms compared to the mean of the other strains (Figure 6.10).

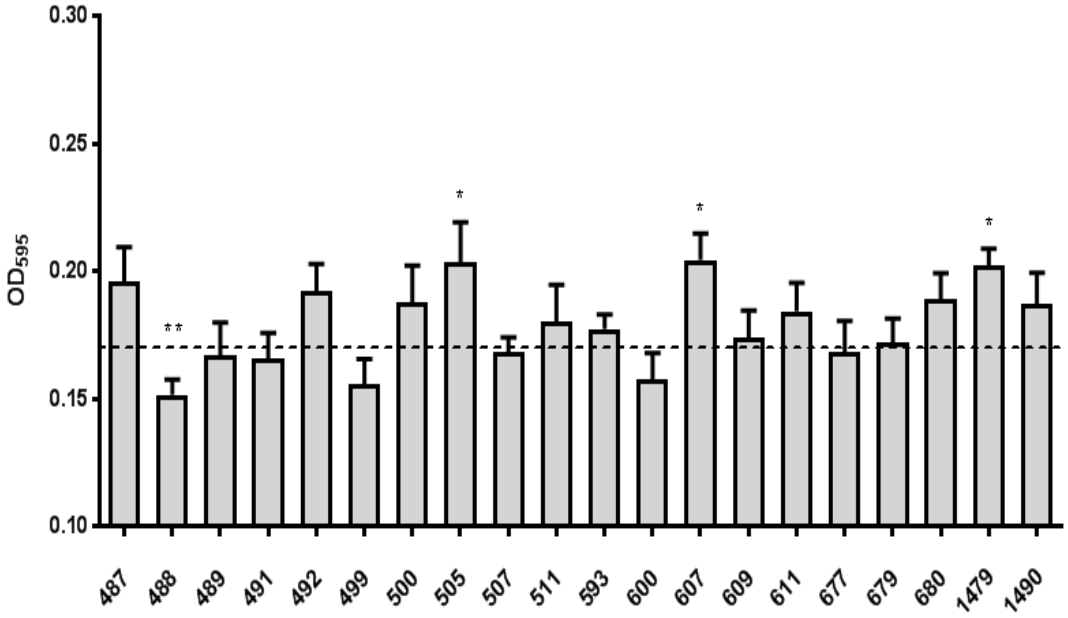

Figure 6.10 Capacity of the Brazilian *C. jejuni* human isolates to form biofilms under aerobic conditions. Bacterial suspensions were prepared from 24 h BA plates and MH broth was inoculated to an OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then the OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Plates were incubated at 37ºC for 72 h under microaerobic conditions. Biofilms were stained with 1% (w/v) crystal violet, destained and the OD_{595} recorded. Data represents at least three biological replicates. * = p <0.05; ** = p <0.01 compared to the mean (dashed line) OD_{595} of 0.17 for all 20 human isolates.

The Brazilian environmental isolates also demonstrated the ability to form biofilms under aerobic conditions. The environmental strains demonstrated a mean absorbance of 0.17. None of the environmental isolates had any significant difference in the ability to form biofilms compared to the mean of these isolates (Figure 6.11).

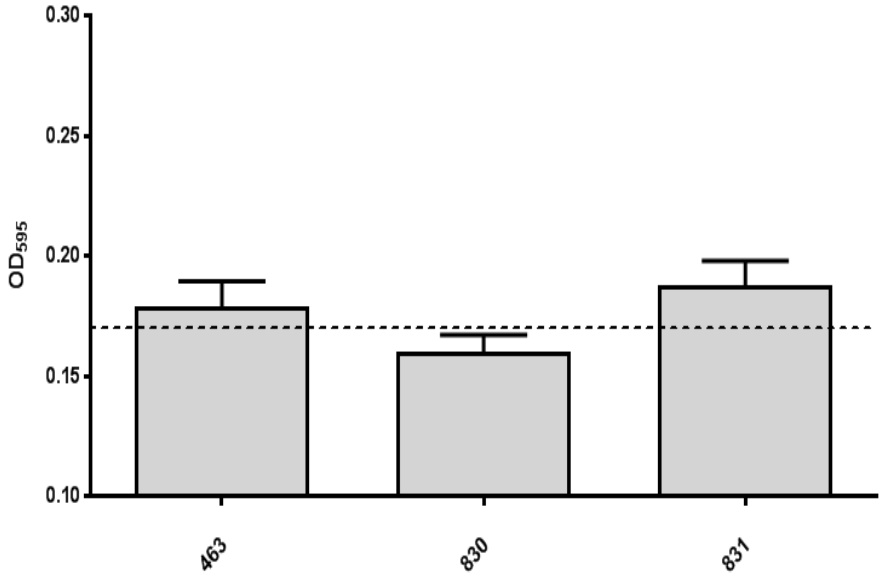

Figure 6.11 Capacity of the Brazilian *C. jejuni* environmental isolates to form biofilms under aerobic conditions. Bacterial suspensions were prepared from 24 h BA plates and MH broth was inoculated to an OD_{600} of 1.0. MH broths were pre-incubated for 5 h, then the OD_{600} was re-adjusted to 0.1 in 1 ml in 24 well-plates. Plates were incubated at 37ºC for 72 h under microaerobic conditions. Biofilms were stained with 1% (w/v) crystal violet, destained and the OD_{595} recorded. Data represents at least three biological replicates. Mean 0.17 (dashed line).

6.2.3 Whole genome sequencing

Whole genome sequencing was performed using Illumina MiSeq platform for all 43 Brazilian isolates as described in Section 2.5.16.6 (Appendix 6). This technique allows the investigation of the complete bacterial genome, allowing the identification of differences in gene content, mutations and gene distribution amongst the strains. Comparisons between different strains can be made and single nucleotide

polymorphism identified. Whole genome sequencing is also a useful tool for epidemiological investigations (Kao *et al.*, 2014).

6.2.4 Prevalence of *rrpA* and *rrpB* in Brazilian *C. jejuni* isolates

The distribution of *rrpA* and *rrpB* in the 43 Brazilian isolates was investigated using their whole genome sequences. BLAST searches were performed based on the RrpA and RrpB amino acid sequences of the NCTC 11168 wild-type genome, as described in Section 2.5.17. Blastx translates the whole genome sequences into amino acid sequences, and then compares the translated genomes to the target amino acid sequence.

The presence or absence of *rrpA* and *rrpB*, as well as the source of each *C. jejuni* strain is demonstrated in Table 6.1. Results are shown in percentage of amino acid similarity to NCTC 11168. A cut-off of 50% amino acid similarity was chosen for a gene to be considered as present.

Thirteen of the *C. jejuni* human isolates contained only *rrpA*, whilst only four strains (488, 492, 499 and 593) contained *rrpB.* However, three strains (489, 491 and 609) isolated from humans were identified as containing neither *rrpA* nor *rrpB.* The opposite was found in the *C. jejuni* chicken meat isolates, where most isolates contained both *rrpA* and *rrpB.* Seven chicken meat isolates contained only *rrpA* and 13 isolates contained both genes. For the environmental isolates, two strains (463 and 831) were identified as containing neither *rrpA* nor *rrpB*, whilst the other environmental strain (830) contained only *rrpA* (Table 6.1).

Table 6.1 Amino acid similarity of RrpA and RrpB from 43 Brazilian *C. jejuni* strains compared to NCTC 11168.

Strain	*rrpA*	*rrpB*	Sample source
487	68%	-	human stool
488	100%	89%	human stool
489	-	-	human stool
491	-	-	human stool
492	100%	100%	human stool
499	100%	94%	human stool
500	89%	-	human stool
505	96%	-	human stool
507	93%	-	human stool
511	89%	-	human stool
593	100%	100%	human stool
600	76%	-	human stool
607	96%	-	human stool
609	-	-	human stool
611	93%	-	human stool
677	96%	-	human stool
679	99%	-	human stool
680	100%	-	human stool
1479	99%	-	human stool
1490	99%	-	human stool
463	-	-	aviary water

830	93%	-	creek water
831	-	-	creek water
1013	100%	100%	poultry meat
1014	100%	100%	poultry meat
1015	99%	-	poultry meat
1016	100%	80%	poultry meat
1018	99%	-	poultry meat
1019	100%	-	poultry meat
1020	90%	-	poultry meat
1021	76%	-	poultry meat
1022	100%	80%	poultry meat
1023	100%	80%	poultry meat
1024	100%	80%	poultry meat
1032	100%	80%	poultry meat
1050	100%	100%	poultry meat
1051	100%	-	poultry meat
1052	100%	100%	poultry meat
1053	100%	-	poultry meat
1054	100%	80%	poultry meat
1055	100%	80%	poultry meat
1056	100%	100%	poultry meat
1057	100%	97%	poultry meat

Percentages indicate amino acid similarity to NCTC 11168 wild-type strain.

– indicates less than 50% amino acid similarity to the NCTC 11168 wild-type strain.

6.2.5 Presence of pathogenicity genes

Amino acid sequences were also used for pathogenicity gene searches in the different Brazilian strain genomes, as described in Section 2.5.17. Searches were performed to determine the presence or absence of pathogenicity genes based on the similarity to NCTC 11168 amino acid sequences. A cut-off of 50% amino acid similarity was chosen for a gene to be considered as present. The pathogenicity genes investigated are listed below (Table 6.2).

Most of the pathogenicity genes searched for in the genome sequences of the 43 Brazilian isolates were highly conserved. The genes present in all strains were *cadF*, *flaC* and *racR* (Table 6.3 and Table 6.4). Only one strain demonstrated absence of *flpA*, *dnaJ*, *hipO*, *peb1A* and *pldA*. Five strains had low similarity for one or two of the *cdtABC* genes. Strain 600 had low similarity for *cdtA*. Strain 463 had low similarity for *cdtB*. Strains 491 and 499 had low similarity for *cdtC*. Strain 831 had low similarity for *cdtB* and *cdtC*. Six strains had low similarity for *ciaB*. Seven strains had more than 50% similarity with *flaA*. However, 36 strains had very low similarity to *flaA*. Strain 491 had several genes identified as absent, including *cdtC*, *ciaB*, *flpA*, *dnaJ*, *hipO*, *jlpA*, *peb1A* and *pldA*, which all had low similarities to the respective NCTC 11168 amino acid sequences.

Table 6.2 List of pathogenicity genes searched for in the whole genome sequences of the 43 Brazilian isolates and the function of the encoded protein

Gene	Function
cdtA	Encodes CtdA which is a subunit of the Cytolethal Distending Toxin. CtdA might play a role in adhering to cell surface receptors (Lara-Tejero and Galan, 2001).
cdtB	Encodes CtdB which is the active subunit of the Cytolethal Distending Toxin. CdtB has DNase activity and causes cell cycle arrest (Elwell and Dreyfus, 2000).
cdtC	Encodes CtdC which is a subunit of the Cytolethal Distending Toxin. CtdC might play a role in adhering to cell surface receptors (Lara-Tejero and Galan, 2001).
ciaB	Encodes CiaB which is *Campylobacter* invasion antigen B. CiaB is required for invasion of epithelial cells (Konkel *et al.*, 2004).
cadF	Encodes CadF which is a *C. jejuni* adhesin that has fibronectin binding capacity (Konkel *et al.*, 1997).
dnaJ	Encodes DnaJ which is a heat shock protein responsible for regulating bacterial heat shock proteins. DnaJ is also required for chick colonisation (Konkel *et al.*, 1998).
flpA	Encodes FlpA which is a fibronectin binding protein, an important adhesin in *C. jejuni* (Flanagan *et al.*, 2009).
flaA	Encodes FlaA which is flagellin A, the major flagellin sub-unit and important for motility (Wassenaar *et al.*, 1991).

flaC	Encodes FlaC which is flagellin C. FlaC is secreted to the extracellular milieu and binds to the surface of epithelial cells (Song *et al.*, 2004). Important for innate immune response modulation (Faber *et al.*, 2016).
hipO	Encodes HipO which is a hippuricase enzyme. HipO is responsible for hydrolysing hippurate. Detection of *hipO* differentiates between *C. jejuni* and *C. coli* (Linton *et al.*, 1997).
jlpA	Encodes JlpA which is a surface-exposed lipoprotein. JlpA is an adhesin important for adherence to intestinal epithelial cells (Jin *et al.*, 2001).
peb1A	Encodes PEB1 which is surface-exposed lipoprotein. PEB1 is an important adhesin in *C. jejuni* (Pei and Blaser, 1993).
pldA	Encodes PldA which is an outer membrane phospholipase A with haemolytic activity (Grant *et al.*, 1997).
racR	Encodes RacR which is a response regulator important for chicken colonisation (Bras *et al.*, 1999).

Table 6.3 Presence of pathogenicity genes amongst Brazilian isolates based on gene similarity to 11168H wild-type strain

Strain	*cadF*	*cdtA*	*cdtB*	*cdtC*	*ciaB*	*flpA*	*dnaJ*	*flaA*	*flaC*	*hipO*	*jlpA*	*peb1A*	*pldA*	*racR*
487	99%	93%	100%	100%	37%	98%	99%	50%	100%	72%	98%	99%	48%	100%
488	98%	100%	100%	100%	82%	99%	99%	39%	99%	99%	98%	99%	56%	99%
489	87%	64%	78%	66%	79%	78%	86%	51%	95%	56%	70%	93%	80%	94%
491	54%	60%	59%	20%	21%	16%	24%	56%	56%	40%	12%	39%	28%	51%
492	98%	100%	100%	100%	99%	99%	99%	39%	100%	100%	88%	100%	99%	100%
499	78%	76%	100%	33%	44%	100%	99%	38%	95%	100%	50%	100%	56%	100%
500	100%	100%	100%	100%	30%	98%	99%	33%	99%	99%	43%	100%	93%	100%
505	98%	100%	100%	100%	99%	99%	99%	33%	99%	98%	98%	99%	99%	99%
507	97%	100%	100%	100%	95%	100%	99%	37%	100%	99%	98%	100%	100%	99%
511	100%	100%	99%	100%	95%	98%	100%	45%	98%	99%	98%	100%	99%	100%
593	98%	100%	100%	100%	62%	99%	99%	39%	100%	98%	52%	100%	68%	100%
600	66%	48%	84%	69%	26%	85%	53%	20%	91%	73%	98%	99%	67%	96%
607	99%	100%	100%	100%	99%	99%	99%	33%	99%	100%	98%	99%	99%	99%
609	87%	63%	78%	66%	91%	90%	93%	27%	94%	56%	70%	93%	84%	97%
611	100%	100%	100%	100%	99%	99%	98%	30%	98%	98%	100%	99%	97%	99%
677	99%	100%	100%	100%	99%	99%	99%	33%	99%	98%	98%	99%	99%	99%
679	99%	100%	100%	100%	99%	98%	99%	80%	95%	98%	92%	99%	88%	100%
680	100%	100%	100%	100%	99%	100%	100%	35%	100%	99%	98%	99%	98%	100%
1479	100%	100%	100%	100%	99%	99%	99%	41%	99%	98%	98%	100%	100%	100%
1490	100%	100%	100%	100%	70%	100%	100%	38%	100%	99%	98%	99%	98%	100%
463	87%	63%	45%	67%	92%	90%	93%	25%	94%	56%	70%	93%	85%	97%

Red indicates less than 50% gene similarity to *C. jejuni* NCTC 11168 strain

Table 6.4 Presence of pathogenicity genes amongst Brazilian isolates based on gene similarity to 11168H wild-type strain

Strain	*cadF*	*cdtA*	*cdtB*	*cdtC*	*ciaB*	*flpA*	*dnaJ*	*flaA*	*flaC*	*hipO*	*jlpA*	*peb1A*	*pldA*	*racR*
830	100%	99%	100%	100%	80%	100%	94%	34%	99%	98%	98%	99%	99%	87%
831	87%	63%	41%	46%	92%	90%	94%	36%	95%	56%	70%	93%	81%	94%
1013	99%	100%	100%	100%	81%	99%	99%	82%	99%	98%	98%	100%	98%	100%
1014	99%	75%	100%	100%	80%	99%	89%	52%	100%	51%	98%	69%	99%	100%
1015	100%	100%	100%	100%	81%	99%	98%	38%	100%	98%	98%	100%	100%	100%
1016	92%	100%	100%	100%	68%	99%	97%	35%	98%	98%	49%	100%	94%	100%
1018	100%	100%	100%	100%	68%	99%	99%	40%	99%	98%	98%	100%	100%	100%
1019	99%	100%	100%	100%	87%	100%	99%	34%	98%	60%	98%	100%	85%	100%
1020	99%	100%	100%	100%	99%	98%	99%	38%	98%	98%	98%	100%	99%	100%
1021	99%	100%	100%	100%	33%	98%	99%	81%	98%	74%	98%	100%	90%	100%
1022	99%	100%	100%	100%	99%	99%	99%	33%	99%	98%	98%	100%	98%	100%
1023	99%	100%	100%	100%	82%	99%	99%	33%	99%	98%	98%	100%	98%	100%
1024	98%	100%	100%	100%	99%	99%	99%	41%	99%	98%	98%	100%	98%	100%
1032	99%	100%	100%	100%	99%	99%	99%	33%	99%	98%	98%	100%	98%	100%
1050	98%	100%	100%	100%	99%	99%	99%	36%	100%	100%	99%	100%	99%	100%
1051	98%	100%	100%	100%	99%	99%	99%	36%	100%	100%	99%	100%	99%	100%
1052	98%	100%	99%	100%	81%	99%	99%	39%	100%	100%	51%	100%	99%	100%
1053	99%	100%	100%	100%	99%	99%	99%	33%	99%	98%	98%	100%	98%	100%
1054	99%	100%	100%	100%	99%	99%	99%	36%	99%	98%	98%	100%	98%	100%
1055	99%	100%	100%	100%	99%	99%	99%	33%	99%	98%	98%	100%	98%	100%
1056	98%	100%	100%	100%	59%	99%	88%	36%	97%	100%	99%	100%	99%	100%
1057	98%	89%	100%	100%	99%	99%	99%	39%	100%	100%	99%	100%	99%	100%

Red indicates less than 50% gene similarity to *C. jejuni* NCTC 11168 strain

6.2.6 Presence of the Type VI Secretion System

The T6SS has a cluster of 13 conserved genes responsible for the secretion system functionality (Basler *et al.*, 2012, Wang *et al.*, 2015). The T6SS delivers effectors to attack other bacteria and is required for full virulence (Tseng *et al.*, 2009, Wang *et al.*, 2015). Gene searches based on the amino acid sequences from the *C. jejuni* 414 wild-type genome were performed for all Brazilian isolates as described in Section 2.5.17. Thirty of the Brazilian isolates did not contain any of the T6SS genes. Eight isolates (487, 492, 511, 593, 679, 1014, 1051 and 1479) were identified as containing some *tss* genes, albeit not all of the 13 T6SS genes required for an active T6SS (Table 6.5). Only 5 isolates (488, 1050, 1052, 1056 and 1057) contained all the 13 T6SS genes and only one was a human isolate (488). However, strain 488 contained two genes (*tssG* and *tssM*) with a low similarity compared to the reference 414 strain. Four isolates from chicken meat (1050, 1052, 1056 and 1057) also contained all the 13 T6SS genes. Two further chicken meat isolates (1014 and 1051) contained 12 of the 13 T6SS genes, with only *tssA* identified as absent.

Table 6.5 Presence of Type 6 secretion system genes amongst Brazilian isolates based on gene similarity to 414 wild-type strain

Strain	*tssA*	*tssB*	*tssC*	*tssD*	*tssE*	*tssF*	*tssG*	*tssH*	*tssI*	*tssJ*	*tssK*	*tssL*	*tssM*
487	-	-	15.7%	70.2%	96.9%	-	44.0%	89.3%	64.0%	-	-	-	-
488	85.1%	95.7%	97.1%	70.2%	96.9%	96.2%	45.0%	57.0%	55.1%	81.8%	97.0%	91.0%	27.3%
492	-	-	90.3%	70.2%	-	39.6%	-	-	87.8%	-	-	-	-
511	-	95.7%	97.1%	-	96.9%	96.2%	92.4%	46.6%	88.7%	-	-	-	55.4%
593	-	-	76.7%	-	96.9%	-	91.7%	43.0%	88.7%	-	26.7%	94.1%	30.0%
679	-	-	97.3%	99.4%	96.2%	95.8%	92.4%	65.4%	54.4%	94.6%	96.8%	91.0%	89.6%
1479	48.0%	95.7%	97.5%	-	96.9%	96.2%	92.4%	-	58.0%	-	-	-	-
1014	-	95.7%	97.1%	99.4%	96.9%	96.2%	92.4%	90.9%	55.0%	94.6%	94.8%	93.2%	64.3%
1050	84.8%	95.7%	97.1%	99.4%	96.9%	96.2%	92.4%	90.9%	55.0%	94.6%	97.0%	91.9%	90.6%
1051	-	87.2%	97.1%	99.4%	96.9%	96.2%	92.4%	90.6%	55.0%	94.6%	97.0%	91.9%	90.6%
1052	84.8%	95.7%	97.1%	99.4%	96.9%	96.2%	92.4%	90.9%	55.0%	94.6%	97.0%	91.9%	90.6%
1056	84.8%	95.7%	97.1%	99.4%	96.9%	96.2%	92.4%	90.9%	55.0%	94.6%	97.0%	91.9%	90.6%
1057	74.2%	95.7%	97.1%	74.9%	96.9%	96.2%	92.4%	90.9%	55.0%	94.6%	97.0%	91.9%	90.6%

Red indicates less than 50% gene similarity to *C. jejuni* 414 strain

Thirty of the Brazilian isolates did not contain any of the T6SS genes

6.2.7 Brazilian isolates MLST analysis

MLST was used to further characterise the Brazilian isolates based on the nucleotide sequence of seven housekeeping genes as described in Section 2.5.18. MLST allows a comparison amongst different strains from different origins and laboratories. The PubMLST database was last updated on 18th May 2016. This data analysis was generated in August 2016. The 43 Brazilian isolates were assigned to 17 different STs. The *rrpA* and *rrpB* gene distribution was also investigated and compared to the identified STs, as performed previously in Section 5.2.3.

Fourteen strains were assigned to a unique ST, whilst the remaining strains were distributed amongst three STs (Table 6.6). Strains 680 and 1490 that only contain *rrpA* were assigned to ST-2782 and were both isolated from humans. Strains 1015, 1018 and 1479 also contained only *rrpA* and were assigned to ST-1359. Eight strains (488, 492, 593, 1050, 1051, 1052, 1056 and 1057) were assigned to ST-353. ST-353 was the ST with most strains assigned. All these strains contained both *rrpA* and *rrpB*, except strain 1051 that contained only *rrpA.* Isolates from both human and chicken meat were assigned to ST-353 and ST-1359.

Fifteen strains (34.8%) could not be assigned to any ST because the housekeeping gene allelic profiles did not match any of the defined STs present in the PubMLST database. The novel MLST types are going to be uploaded to the *C. jejuni* PubMLST database. Once uploaded, the novel profiles will be assigned to a new allele number and added to the publicly available database.

Twelve strains from chicken meat, 2 from human and 1 environmental strain were not assigned to any ST due to a unique allelic profile. These strains are not listed in Table 6.6.

Table 6.6 MLST analysis for 25 of the 43 Brazilian isolates

Strain	ST	Source	*rrpA/rrpB*
463	860	aviary water	-
487	*3852*	human	*rrpA*
489	*1166*	human	-
499	*21*	human	*rrpA rrpB*
500	*443*	human	*rrpA*
505	*41*	human	*rrpA*
507	*332*	human	*rrpA*
600	3630	human	*rrpA*
607	5913	human	*rrpA*
609	829	human	-
611	660	human	*rrpA*
679	5860	human	*rrpA*
831	830	creek water	-
680	2782	human	*rrpA*
1490	2782	human	*rrpA*
1479	1359	human	*rrpA*
1015	1359	chicken meat	*rrpA*
1018	1359	chicken meat	*rrpA*
1020	463	chicken meat	*rrpA*
488	*353*	human	*rrpA rrpB*
492	*353*	human	*rrpA rrpB*
593	353	human	*rrpA rrpB*

1050	353	chicken meat	*rrpA rrpB*
1051	353	chicken meat	*rrpA*
1052	353	chicken meat	*rrpA rrpB*
1056	353	chicken meat	*rrpA rrpB*
1057	353	chicken meat	*rrpA rrpB*

6.2.8 Plasticity region investigation amongst Brazilian isolates

The 43 Brazilian isolates were also investigated with respect to the genomic structure flanking the *rrpA* and *rrpB* genes. As seen in Section 5.2.4, analysis of other *C. jejuni* genomes revealed a genetic structure with two conserved flanking regions and a variable central region. There are two different central region versions, one in which *rrpB* is present and another in which *rrpB* is absent. All the Brazilian strains genome sequences were visualised using Artemis (Sanger Institute) software. The Brazilian strains that contained both *rrpA* and *rrpB* (*rrpB*$^+$) were compared to NCTC 11168 genome region (Table 6.6), whilst strains that contained only *rrpA* (*rrpB*$^-$) (Table 6.7) or contained neither gene (*rrpA*$^-$ *rrpB*$^-$) were compared to the 81116 genome region (Table 6.8).

The upstream conserved region was highly conserved amongst all *rrpB*$^+$ and *rrpB*$^-$ isolates. The Brazilian strains had all genes from the upstream region present, with only a few genes identified as probably fragmented. Only strain 1052 was identified as not containing the *Cj1544c* gene in the upstream conserved region. This upstream conserved region could not be identified amongst any of the *rrpA*$^-$ *rrpB*$^-$ strains.

All *rrpB*$^+$ strains contained *hsdR, hsdS* and *hsdM* from the R-M system. *rloH* was present in the variable central region between *hsdS* and *hsdM* in all *rrpB*$^+$ strains. However, two strains contained a fragmented version of *rloH*. Only strain 499 contained *mloB* in the variable central region between *hsdS* and *hsdM*. All other *rrpB*$^+$ isolates did not contain any ORF present between *hsdS* and *hsdM*. Nine isolates

contained a fragmented *rrpB* gene sequence, which comprises 45% of the strains that were identified as *rrpB*$^{+}$.

In the *rrpB*$^{+}$ downstream conserved region, only a few strains were identified as not containing the following genes: *arsR, arsC, Cj1561, Cj1562* and *Cj1563c.* A few strains were identified where *arsR* and *arsC* appear to be transcribed together with the absence of a stop codon between the genes. This was the case for strains 1013, 1022, 1023, 1024, 1032, 1050, 1054, 1055, 1056 and 1057. This was also observed for *Cj1561* and *Cj1562*, which also appear to be transcribed together in all these same strains, as well as strain 1016. Strain 593 contained four genes (*arsR, arsC, Cj1561* and *Cj1562*) that appear to be transcribed without any stop codons in between them. *mcp* and *pflA* were present in all the *rrpB*$^{+}$ strains. However, *mcp* was truncated in most strains. *mcp* and *pflA* were also fragmented in a few strains.

Considering the variable central region amongst the *rrpB*$^{-}$ isolates, only strains 507, 511, 1019, 1020, 1051 and 1053 contained all the *hsd* genes. Only one strain (507) contained *rloA* and *rloB*. Four strains (511, 679, 1479 and 1490) contained *rloC*, one (611) contained *rloF* and three (1019, 1051 and 1053) contained *rloH* instead of *rloA* or *rloB* downstream of *hsdR.*

Most *rrpB*$^{-}$ strains were identified with several genes absent from the central variable region and from the downstream conserved region. Four strains (487, 830, 1018 and 1021) were identified with all the genes absent from the central variable region. All the remaining *rrpB*$^{-}$ strains contained some of the genes belonging to the central variable region. Similar observations were made for the *rrpB*$^{-}$ strains in the downstream conserved region, where some genes were absent. *arsR, arsC, Cj1459* and *Cj1460c* were mostly absent amongst *rrpB*$^{-}$ strains. *mcp* was absent in four strains (500, 511, 679 and 1479). *mcp* was truncated or fragmented in all other strains. *pflA* was highly conserved. However, *pflA* was absent from strain 511. Furthermore, *pflA* was fragmented in three strains (600, 680 and 1021).

Few genes from the plasticity region could be identified in the *rrpA*$^{-}$ *rrpB*$^{-}$ strains. These strains appear to have a completely different genomic structure, with the absence of the R-M plasticity region containing the type I R-M (*hsd*) system. Some genes (*hsdR, hsdM, asrC, arsC* and *pflA*) were identified due to genomic annotation and were present in different regions of the genome.

Table 6.6 Plasticity region of Brazilian isolates containing both *rrpA* and *rrpB*

	11168H genes							*rrpB* positive												
					rrpA			*hsdR*	*rloH*	*hsdS*	*mloB*	*hsdM*		*rrpB*	*arsR*	*arsC*				*mcp*	*pflA*
Strain	*1542*	*1543*	*1544c*	*1545c*	*1546*	*1547*	*1548c*	*1549c*	*1550c*	*1551c*	*1552c*	*1553c*	*1555c*	*1556*	*1558*	*1560*	*1561*	*1562*	*1563c*	*1564*	*1565c*
488	+	+	+	+	+	+	+	+	+	+	-	+	+	+	-	+	+	-	+	+	+
492	+	+	+	+	+	+	+	+	+	+	-	+	2	+	+	+	-	-	+	+	+
499	+	+	+	+	+	+	+	+	2	+	+	+	+	+	-	-	-	-	+	+	2
593	+	+	+	+	+	+	+	+	+	+	-	+	2	+	+				+	+	+
1013	+	+	+	+	+	+	+	+	+	+	-	+	+	2	+		+		+	+	+
1014	+	+	+	+	+	+	+	+	+	+	-	+	2	+	-	+	-	-	-	+	2
1016	+	2	2	+	+	+	+	2	+	+	-	+	+	2	-	+	+		+	+	+
1022	2	2	+	+	+	+	+	+	+	+	-	+	+	2	+		+		+	+	+
1023	+	+	+	+	+	+	+	+	+	+	-	+	+	2	+		+		+	+	+
1024	+	+	+	+	+	+	+	+	+	+	-	+	+	2	+		+		+	+	+
1032	+	+	+	+	+	+	+	+	+	+	-	+	+	2	+		+		+	+	+
1050	+	+	+	+	+	+	+	+	+	+	-	+	+	2	+		+		+	+	+
1052	+	+	-	+	+	2	2	2	2	+	-	+	2	+	-	+	-	-	-	2	2
1054	+	+	+	+	+	+	+	+	+	+	-	+	+	2	+		+		+	+	+
1055	+	+	+	+	+	+	+	+	+	+	-	+	+	2	+		+		+	+	+
1056	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+ 2		+		+	+	+
1057	+	+	+	+	+	+	+	+	+	+	-	+	2	+	+		+		+	+	+

+ gene presence / + shorter gene sequence compared to NCTC 11168 strain

- gene absence / 2 truncated gene

Absence of | between genes indicates lack of stop codon

Table 6.7 Plasticity region of Brazilian isolates containing only *rrpA* gene

	81116 genes							*rrpB* negative														
					rrpA			*hsdR*	*rloA*	*rloB*	*hsdS*	*hsdM*					*arsR*	*arsC*			*mcp*	*pflA*
Strain	*1441*	*1442*	*1443c*	*1444c*	*1445*	*1446*	*1447*	*1448*	*1449*	*1450*	*1451*	*1452*	*1453c*	*1454c*	*1455c*	*1456*	*1457*	*1458*	*1459*	*1460c*	*1461*	*1462c*
487	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
500	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
505	+	+	+	+	+	2	+	-	-	-	-	+	+	+	+	-	+	-	-	+	2	+
507	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	+	+
511	+	+	+	+	+	+	+	2	*rloC*	-	+	+	-	2	+	-	-	-	-	-	-	-
600	2	+	2	+	+	+	+	+	-	-	-	-	-	-	-	-	+	-	-	+	+	2
607	+	+	+	+	+	2	+	-	-	-	-	+	+	+	+	-	+	-	-	+	+	+
611	+	+	+	+	+	+	+	+	*rloF*	-	-	-	-	-	-	-	-	-	-	-	2	+
677	+	+	+	+	+	2	+	-	-	-	-	+	+	+	+	-	+	-	-	+	+	+
679	+	+	+	+	+	+	+	+	*rloC*	-	-	-	-	-	-	-	-	-	-	-	-	+
680	+	+	+	+	+	2	+	+	-	-	-	+	+	+	+	+	+	-	-	+	2	2
1479	+	+	+	+	+	+	+	+	*rloC*	-	-	-	-	-	-	-	-	-	-	-	-	+
1490	+	+	+	+	+	2	+	+	*rloC*	-	-	-	-	-	-	-	-	-	-	-	+	+
830	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	2	-	-	-	2	+
1015	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	2	+
1018	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
1019	+	+	+	+	+	+	+	+	*rloH*	-	+	+	-	-	-	-	-	-	-	+	+	+
1020	+	+	+	+	+	+	+	+	-	-	+	+	+	2	2	-	+	+	+	+	+	+
1021	2	2	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	2
1051	+	+	+	+	+	+	+	+	*rloH*	-	+	+	-	-	-	-	+	-	-	-	+	+
1053	+	+	+	+	+	+	+	+	*rloH*	-	+	+	-	-	-	-	+	-	-	+	+	+

+ gene presence / + shorter gene sequence compared to NCTC 11168 strain

- gene absence / 2 truncated gene

Table 6.8 Plasticity region of Brazilian isolates containing neither *rrpA* or *rrpB*

	81116 genes							*rrpB* negative														
					rrpA			*hsdR*	*rloA*	*rloB*	*hsdS*	*hsdM*					*arsR*	*arsC*			*mcp*	*pflA*
Strain	*1441*	*1442*	*1443c*	*1444c*	*1445*	*1446*	*1447*	*1448*	*1449*	*1450*	*1451*	*1452*	*1453c*	*1454c*	*1455c*	*1456*	*1457*	*1458*	*1459*	*1460c*	*1461*	*1462c*
489	-	-	-	-	-	-	-	2	-	-	-	+	-	-	-	-	+	+	-	-	-	+
491	-	-	-	-	-	-	-	2	-	-	-	+	-	-	-	-	+	+	-	-	-	2
609	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	+	-	-	-	+
463	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	+	+	-	-	-	+
831	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	-	-	-	+

+ gene presence compared to NCTC 11168 strain

- gene absence

2 truncated gene

6.3 Discussion

For phenotypic characterisations, the 43 Brazilian strains were investigated for oxidative stress resistance and the capacity to form biofilms. Whole genomic sequencing was performed for all 43 strains and the assembled genomes searched for pathogenicity genes. MLST analysis was also performed to further characterise the Brazilian strains. This information could contribute to the field of *C. jejuni* studies in Brazil.

6.3.1 Oxidative stress

C. jejuni is the leading cause of bacterial gastrointestinal infections in humans worldwide. Bacterial metabolism produces low levels of ROS that are normally neutralised by constitutive antioxidant defences (Paiva and Bozza, 2014). However, *C. jejuni* can experience higher ROS concentrations and associated stresses when the bacterium is exposed to the host immune defence or environmental oxygen levels (Murphy *et al.*, 2006). *C. jejuni* is commonly found in chicken meat products (Berndtson *et al.*, 1996, Golz *et al.*, 2014), demonstrating that the bacterium is well adapted to survive in these types of food products and under environmental oxygen levels.

The Brazilian chicken meat isolates were shown to be highly resistant to H_2O_2, with most of these isolates able to resist a high level (> 100mM) of H_2O_2 exposure. In the present study, different strains exhibited different sensitivities to H_2O_2. This was also observed by Kaakoush *et al.* (2007), where different *C. jejuni* strains demonstrated differences in oxygen tolerance. *C. jejuni* can survive in chicken meat at 4ºC for 14 days (Sampers *et al.*, 2010). *C. jejuni* can also survive in other types of meat, such as beef and pork for long periods at 4ºC (Balamurugan *et al.*, 2011). *C. jejuni* was also shown to be able to survive in co-culture with *Pseudomonas* species for 48 h at 35ºC under atmospheric oxygen concentrations (Hilbert *et al.*, 2010). In the Hilbert *et al.* (2010) study, *C. jejuni* was co-incubated with *Pseudomonas* species because the Pseudomonadaceae are a well-adapted food-spoiling family commonly found in meats. These studies demonstrated the *C. jejuni* ability to survive in meat products under high oxygen concentrations and in presence of other bacteria. In agreement, the

Brazilian chicken meat isolates were shown to be highly capable of resisting H_2O_2 stress and probably are also highly capable of surviving environmental oxygen concentrations.

The Brazilian human isolates were also investigated for sensitivity to H_2O_2 stress and demonstrated variable levels of sensitivity to H_2O_2, with some strains being highly resistant and others exhibiting increased sensitivity. *C. jejuni* is a complex bacterium and the fundamental requirements for host cell colonisation have not yet been fully identified. However, the capacity to resist to H_2O_2 and other ROS are an important factor in order to survive the host immune responses to bacterial infection (Knight, 2000). Immune cells, such as neutrophils and macrophages, recognise and engulf invading bacteria (Knight, 2000). Bacteria then trigger the respiratory burst, which makes phagocytes elevate their oxygen consumption (Paiva and Bozza, 2014). The respiratory burst increases the oxygen uptake, culminating with ROS formation by NADPH oxidase (NOX2) to eliminate invading microorganisms (Knight, 2000, Paiva and Bozza, 2014). In order for pathogenic bacteria to survive, either bacterial enzymes must neutralise the toxic ROS compounds or a mechanism to evade phagocytosis is required. The host immune response will vary depending on the surface antigens displayed by different microorganisms (Paiva and Bozza, 2014). The increase in ROS levels will then cause direct damage to the microorganisms, such as lipid peroxidation, cleavage of DNA strands, nucleotide oxidation and deamination, as well as oxidation of methionine residues (Paiva and Bozza, 2014). The respiratory burst generates O_2^- which will be rapidly dismutated to H_2O_2 by SOD that causes direct oxidative damage to many pathogens (Paiva and Bozza, 2014). The variable resistance to H_2O_2 of the human isolates demonstrates that resistance to oxidative stress is important for surviving ROS generated by immune system. However, other mechanisms may have a more important role in gut colonisation.

The Brazilian environmental isolates also demonstrated variable resistance to H_2O_2 stress. Two of the environmental isolates demonstrated higher resistance to H_2O_2 stress, whilst the other isolate was shown to be more sensitive to H_2O_2 stress. The presence of *C. jejuni* in river water is a common finding, and this can be attributed to wild bird faecal contamination (Savill *et al.*, 2001, Carter *et al.*, 2009, Van Dyke *et al.*, 2010). Cools *et al.* (2003) demonstrated that *C. jejuni* from different sources are capable of surviving in drinking water for several days. Cools *et al.* (2003) also

demonstrated that poultry isolates survived for longer periods (over 30 days) compared to human and water isolates at 4ºC (Cools *et al.*, 2003). In this study, all chicken meat isolates were highly resistant to H_2O_2, whilst human and environmental isolates had variable resistance to H_2O_2. This suggests that chicken isolates have greater capacity of survival under hostile conditions.

Most of the *C. jejuni* strains in the Talibart *et al.* (2000) study became non-culturable (VBNC) after 30 days in water at 4ºC. This suggests that water provides a stressful environment for *C. jejuni* cells, with the bacteria exposed to different stresses, such as oxidative, oligotrophic, hypothermal and hypo-osmotic stress (Talibart *et al.*, 2000). This reinforces the fact that *C. jejuni* strains are highly capable of surviving in many different hostile environments.

6.3.2 Biofilm formation

The ability of *C. jejuni* to form biofilms is important for bacterial survival. However, according to Reeser *et al.* (2007), biofilm formation does not correlate with pathogenicity. Different factors, such as temperature, nutritional compounds and oxygen concentration, affect biofilm formation (Reeser *et al.*, 2007). *C. jejuni* ability to form biofilms is an important feature for resisting in hostile environments (Brown *et al.*, 2015). *C. jejuni* can form biofilms on various abiotic surfaces (Reeser *et al.*, 2007).

In this study, the Brazilian *C. jejuni* isolates demonstrated the capacity for biofilm formation. The mean OD_{595} reading for the Brazilian isolates biofilm formation was similar to the OD_{595} reading for 11168H biofilm formation under microaerobic and aerobic conditions (see Chapter 4). This is an important feature that demonstrates a capacity to persist in the environment. Biofilm formation depends on environmental factors. However, variation in individual cell surface structures and molecules are also important in determining bacterial attachment to surfaces and biofilm formation properties (Van Houdt and Michiels, 2010).

Nutrient availability can affect *C. jejuni* capacity to form biofilms. It has been demonstrated that low nutrient media supports biofilm formation better than rich

nutrient media (Reeser *et al.*, 2007). Therefore, strains were grown in Mueller Hinton broth instead of Brucella broth. Mueller Hinton broth contains beef infusion and casein hydrolysate that provides nitrogen and vitamin sources. Brucella broth is a more nutrient rich media compared to Mueller Hinton, containing casein, digested animal tissue, yeast extract, dextrose and sodium chloride. Mueller Hinton broth has been demonstrated to be more compatible with *C. jejuni* biofilm formation (Reeser *et al.*, 2007).

In the Reeser *et al.* (2007) study, biofilm formation was increased under favourable *C. jejuni* conditions, such as a microaerobic atmosphere. In the Reuter *et al.* (2010) study, *C. jejuni* strains showed a greater ability to form biofilms under aerobic conditions compared to microaerobic conditions. However, this difference in biofilm formation was only observed after 48 h incubation, whilst after 72 h, the level of biofilm formation was the same under aerobic and microaerobic conditions (Reuter *et al.*, 2010). These two studies contained significant differences in methodology, which could explain the different results observed. The Reeser *et al.* (2007) study investigated M129 wild-type strain biofilm formation using Mueller Hinton broth in polystyrene plates at 37ºC. The Reuter *et al.* (2010) study investigated 11168 wild-type strain biofilm formation using Brucella broth in glass tubes at 42ºC. Differences in the temperature and abiotic surface, as well as variation between strains, probably influenced the differences in *C. jejuni* biofilm formation observed in these two studies.

Bacterial biofilm formation has major implications within the food industry, creating a persistent source of food contamination (Van Houdt and Michiels, 2010). Biofilms are believed to contribute to *C. jejuni* survival in the food chain (Brown *et al.*, 2015). Biofilms in the food industry are a high risk for food contamination. Furthermore, biofilms are highly associated with persistent human bacterial infections (Srey *et al.*, 2013). Biofilm formation in food processing plants is common and can occur rather rapidly (Van Houdt and Michiels, 2010). Organic residues on surfaces of food processing materials facilitate the attachment of bacteria initiating biofilm formation (Srey *et al.*, 2013). The food industry requires efficient strategies to decontaminate food processing equipment and to eliminate bacterial biofilms (Simoes *et al.*, 2010). Antimicrobial disinfectants widely used in the food industry are known to be more efficient against planktonic bacteria and less efficient against bacteria within biofilms

(Srey *et al.*, 2013). Biofilms create a protection for bacterial cells from exposure to disinfectant products (Van Houdt and Michiels, 2010).

Pre-existing biofilms facilitate *C. jejuni* attachment and biofilm formation (Trachoo *et al.*, 2002). Trachoo *et al.* (2002) co-cultured *C. jejuni* with pre-existent biofilms, such as a *Pseudomonas* biofilms, demonstrating that co-cultured biofilms enhanced the survival of *C. jejuni.* Trachoo and Frank (2002) tested different sanitisers on *C. jejuni* cells and on co-cultured biofilms. The results showed that the susceptibility of *C. jejuni* was decreased in biofilms, reducing the effectiveness of the sanitisers (Trachoo and Frank, 2002).

6.3.3 Presence of pathogenicity genes amongst the Brazilian isolates

Blastx searches based on amino acid sequence were performed for pathogenicity genes on all the Brazilian isolates. Gene sequence searches are routinely used by researchers to investigate sequence homology amongst different strains. Sequences that share significant similarity are considered homologs, indicating that the two sequences have similar structure and a common ancestor (Pearson, 2013). Amino acid similarity searches are much more sensitive than nucleotide similarity searches (Pearson, 2013). DNA alignments tend to have less accurate statistical analysis and miss the detection of homologs (Pearson, 2013). Brazilian isolates whole genomes were mapped to NCTC 11168 as reference strain. The use of a reference strain is important for a correct assembly as this method provides better output than the *de novo* assembly. A few of the Brazilian strains contained neither *rrpA* nor *rrpB.* Since the vast majority of *C. jejuni* strains in Chapter 5 contain *rrpA*, the presence of *hipO* was included in the gene searches to confirm if the Brazilian isolates were all identified correctly as *C. jejuni* strains. *hipO* encodes a hippuricase enzyme which converts hippuric acid to benzoic acid and glycine (Hani and Chan, 1995). This gene differentiates between *C. jejuni* and *C. coli* because *hipO* is only present in *C. jejuni* (Hani and Chan, 1995). The hippuricase biochemical test is routinely used to differentiate *C. jejuni* from *C. coli.* However, some *C. jejuni* strains produce only low levels of hippuricase leading to false-negative results (Totten *et al.*, 1987, Denis *et al.*, 1999). False-positive results for *C. coli* in the biochemical test have also been observed (Denis *et al.*, 1999).

cadF is a highly conserved gene and CadF is important for bacterial adhesion to intestinal epithelial cells (Konkel *et al.*, 2005). The *cadF* identity level amongst *C. jejuni* strains is high at above 90%, as well as for *C. coli* strains. However, the identity level between the two species is about 88% (Shams *et al.*, 2016). Therefore, *cadF* can also be used to differentiate between the two species (Shams *et al.*, 2016). Identity indicates the percentage of residues that are in the exact same position comparing two different sequences (Fassler and Cooper, 2011). Similarity indicates that residues have similar properties, but are not identical when comparing two different sequences (Garner, 2012). Similarity searches are widely used and reliable. A high similarity infers similar protein structure and homology (Pearson, 2013).

Strains 463, 489, 491, 609 and 831 contained neither *rrpA* nor *rrpB.* These strains also demonstrated similarity above the cut-off, but at not a very high level (around 50%) compared to NCTC 11168 HipO. These strains also demonstrated CadF similarity below 90%, and as a consequence, they also had less than 90% identity compared to NCTC 11168. These observations suggest that these strains were incorrectly identified as *C. jejuni.* Further investigation using PCR primer sequences was performed to correctly identify the *Campylobacter* species (Wang *et al.*, 2002). PCR primers of species-specific genes were Blasted against the genome of each one of the unidentified Brazilian strains. All these isolates were identified as *C. coli* based on Blast searchers using *C. coli glyA* primers. Three other strains also showed low similarity to either CadF or HipO. Strain 1014 had low HipO similarity, but high CadF similarity. Further genome analysis demonstrated assembly error for *hipO,* which explains the low similarity to NCTC 11168 *hipO.* Strains 499 and 600 had less than 90% CadF similarity, but high HipO similarity. Further Blast analysis demonstrated 100% *cadF* similarity to different *C. jejuni* strains, which confirmed that these isolates are *C. jejuni*. Strain 491 was identified as *C. coli.* This explains the strain low similarity for several of the genes investigated. Whole genome sequencing can also be used to identify species. This can be done by Blast seachers of species-specific genes against the whole genome of the strains that need to be identified.

In this study, the *cdtABC* operon was highly prevalent amongst the *C. jejuni* strains. However, a few strains had one of the three genes missing. *cdt* genes are highly conserved and prevalent in *C. jejuni* and *C. coli* strains isolated from chicken (Eyigor

et al., 1999, Bang *et al.*, 2001). However, *cdt* genes are more associated with *C. jejuni* strains (Bang *et al.*, 2001, Martinez *et al.*, 2006, Jain *et al.*, 2008). Furthermore, *C. coli* strains produce much lower levels of the CDT toxin (Eyigor *et al.*, 1999, Bang *et al.*, 2001). Mutation of *cdt* genes can generate a strain unable to produce CDT toxin (Abuoun *et al.*, 2005). *C. jejuni* strains containing *cdtB* are more adherent and invasive to HeLa cells as compared to strains without *cdtB* (Jain *et al.*, 2008). *cdt* genes are highly prevalent in strains isolated from humans (Fernandes *et al.*, 2010, Mortensen *et al.*, 2011). However, the presence of these genes does not correlate with severity of the infection (Mortensen *et al.*, 2011). *cdtB* is a DNase I homolog that damages DNA (Jinadasa *et al.*, 2011). CDT causes cell death of several mammalian cell lines. However, the mechanism of apoptosis is still not clear (Jinadasa *et al.*, 2011). CdtB induces Interleukin-8 from human intestinal cells (Borrmann *et al.*, 2007, Van Deun *et al.*, 2007). Intestinal inflammation and necrosis are observed in intestines of experimentally infected mice (Jain *et al.*, 2008).

Most Brazilian isolates had *flaA* absent, only three strains demonstrated a high degree of similarity to NCTC 11168 FlaA, whilst four strains had around 50% similarity. FlaA is the major flagellin in *C. jejuni* (Wassenaar *et al.*, 1991). FlaA is required for a fully functioning flagella and for invasion of epithelial cells (Joslin and Hendrixson, 2009). Absence of *flaA* reduced motility and colonisation of chickens (Wassenaar *et al.*, 1993). A *C. jejuni flaA* mutant produces a truncated flagella filament, but was still able to secrete Cia proteins (Yao *et al.*, 1994, Konkel *et al.*, 2004). However, a minimum flagella structure is required to secrete Cia proteins (Konkel *et al.*, 2004). Further investigation into the Brazilian isolates motility should be performed. However, it is unlikely that these isolates have non-functional flagella. The Brazilian strains *flaA* may have a less conserved sequence compared to NCTC 11168.

Six Brazilian isolates demonstrated the absence of *ciaB.* CiaB is important for invasion of epithelial cells (Rivera-Amill *et al.*, 2001, Konkel *et al.*, 2004). This suggests that these strains may have an invasion defect. Further studies about invasion capacity should be performed on these strains to confirm any invasion deficiency. Different studies have demonstrated that 5% to 9% of *C. jejuni* strains lack *ciaB* (Zheng *et al.*, 2006, Talukder *et al.*, 2008).

Except for strain 491, all the Brazilian isolates had *flpA, dnaJ, flaC, peb1A, pldA* and *racR.* Flanagan *et al.* (2009) also showed that *cadF, jlpA, peb1A, and flpA* are conserved amongst *C. jejuni* strains. *cadF, flpA, jlpA* and *peb1A* are genes that code for important *C. jejuni* adhesins responsible for binding to host intestinal epithelial cells. Absence of CadF, FlpA or Peb1 has been shown to reduce the ability of *C. jejuni* to adhere to and invade intestinal epithelium cells (Pei and Blaser, 1993, Monteville *et al.*, 2003, Flanagan *et al.*, 2009). Whilst JlpA is a lipoprotein important for adhesion, the lack of JlpA has been shown to have no influence on invasion (Jin *et al.*, 2001). It is suggested that JlpA triggers the host immune response and is important for cell signalling (Jin *et al.*, 2003). Three Brazilian strains did not contain *jlpA* in this study. Further studies are necessary to better understand the role of JlpA. PldA is a phospholipase A located in the outer membrane that has haemolytic activity important for *C. jejuni* virulence (Grant *et al.*, 1997). *pldA* was present in all the Brazilian strains. In *C. concisus,* PldA demonstrated haemolytic and cytolytic activities (Istivan and Coloe, 2006). Further investigation is necessary to better understand the role of PldA during *C. jejuni* infection.

All Brazilian strains contain *flaC* and *racR. dnaJ* was absent from one strain. FlaC is a conserved protein that plays an important role in cell invasion (Song *et al.*, 2004). The absence of *flaC* leads to an invasion defect. FlaC depends on a functional flagellum to be exported extracellularly (Song *et al.*, 2004). *racR* was highly prevalent amongst the Brazilian isolates. This is in agreement with the Talukder *et al.* (2008) study that showed *racR* present in 100% of the *C. jejuni* strains analysed. The RacR response regulator and DnaJ play a role in the colonisation of chick intestinal cells (Konkel *et al.*, 1997, Bras *et al.*, 1999) and may be important for human infection.

6.3.4 *rrpA* and *rrpB* gene distribution and hydrogen peroxide stress

The *rrpA* and *rrpB* gene distribution were investigated in the Brazilian isolates. Most of the Brazilian strains isolated from humans contained only *rrpA,* whilst most of the chicken meat isolates contained both genes.

The NCTC 11168 genome was the selected as the template that all other genomes were mapped against. 39.5% of the Brazilian isolates contained both *rrpA* and *rrpB.*

Most of these strains were isolated from poultry meat, whilst only a few were isolated from humans. All poultry meat isolates demonstrated high resistance to H_2O_2 stress. These strains contained either only *rrpA* or both genes, which suggests that presence of *rrpA* or *rrpB* did not affect the resistance to H_2O_2 stress.

With regards to the human isolates, five isolates demonstrated increased sensitivity to H_2O_2 and these contained only *rrpA*. However, all other human isolates containing only *rrpA* were highly resistant to H_2O_2. The human isolates containing both genes were also highly resistant to H_2O_2 stress. However, only one human isolate demonstrated increased sensitivity to H_2O_2 stress compared to all other isolates containing both *rrpA* and *rrpB.*

Strains with neither *rrpA* nor *rrpB,* which are probably not *C. jejuni,* demonstrated variable sensitivity to H_2O_2. Three strains were isolated from humans. Two demonstrated increased sensitivity to H_2O_2 and the other was resistant to 50 mM H_2O_2. The other two strains lacking both *rrpA* and *rrpB* were from environmental sources. One environmental strain was highly sensitive whilst the other had increased resistance. Further analysis is required to identify the species of these isolates.

It has been suggested that strains containing only *rrpA* exhibit increased resistance to H_2O_2 stress. In Chapter 3, it was shown that the 81116 and M1 strains (which contain only *rrpA*) were more resistant to H_2O_2 compared to 11168H and 81-176 (which contain both genes). Analysis with a greater number of *C. jejuni* strains has also showed that strains containing only *rrpA* were more resistant to H_2O_2 stress compared to strains containing *rrpA* and *rrpB* (Gundogdu et al., unpublished data). This does not seem to apply to the Brazilian isolates. The Brazilian isolates had a more variable profile of sensitivity to H_2O_2 stress based on the *rrpA* and *rrpB* gene distribution. *rrpA* and *rrpB* genes may be playing only a minor role regarding the resistance to H_2O_2. However, a greater number of Brazilian strains should be investigated.

6.3.5 *rrpA* and *rrpB* gene distribution and MLST

The Brazilian isolates were analysed by MLST and assigned to STs. MSLT allows the investigation of different bacterial populations within the same species based on

variation of housekeeping genes (Dingle *et al.*, 2002). Housekeeping genes evolve slowly because they are under stabilising selection to maintain metabolic functions (Urwin and Maiden, 2003). This means that nucleotide variation in the housekeeping genes are favoured by synonymous substitution, which will not alter the protein amino acid sequence (Urwin and Maiden, 2003).

It was observed that 34.8% of the strains had completely new genetic characteristics in the housekeeping genes, so they could not be assigned to a specific ST. These strains exhibited new allelic sequences not previously identified in the *C. jejuni* PubMLST database. The identification of these new profiles could be useful for Brazilian researchers. Future Brazilian researchers will be able to identify other strains that have the same ST profiles identified in this study. These new profiles could indicate evolution due to local environmental pressure. This information can also be useful for other South American countries that may also identify different *Campylobacter* isolates with the same profile. Future research will also reveal if these novel ST profiles are related only to Brazil or if they are also found in other countries.

As observed in Section 5.2.3, *rrpB* gene distribution is related with MLST clonal complexes. Some clonal complexes were more associated with strains containing only *rrpA*, or containing both *rrpA* and *rrpB*. This is in agreement with a different study that demonstrated that some *C. jejuni* metabolism-related features are more associated with specific STs (de Haan *et al.*, 2012). However, it was suggested that this may be a consequence of host ST association (de Haan *et al.*, 2012) or niche adaptation (Manning *et al.*, 2003).

Eight Brazilian isolates were assigned to ST-353. All these strains contained *rrpA* and *rrpB*, except one, which contained only *rrpA*. Strains assigned to this ST were isolated from both human and chicken meat sources. These findings are in agreement with the Dingle *et al.* (2002) study that demonstrated that ST-353 is associated with human disease, but also with live chickens and chicken meat. In Section 5.2.3, strains assigned to ST-353 were mostly isolated from humans and contained both genes.

Most of the Brazilian human isolates only contained *rrpA* (*rrpB*$^{-}$) and were assigned to several different clonal complexes, such as ST-2782. Only one human isolate was identified as belonging to ST-21 and contained both genes. ST-21 belongs to the clonal complex ST-21, which is highly related to human infections (Manning *et al.*, 2003).

This clonal complex also contains a high percentage of *rrpB*$^{+}$ strains, as seen in Section 5.2.3. ST-21 is also defined as livestock-associated (Kwan *et al.*, 2008, Stabler *et al.*, 2013). ST-2782 also belongs to clonal complex 21 and is associated with human infection based on analysis performed in Section 5.2.3 (Appendix 4). The association of an ST with a source may indicate that the isolates have adapted to a specific niche. Niche adaptation may create a selective pressure to maintain the genetic structure (Manning *et al.*, 2003).

As seen in Section 5.2.3, it is suggested that the presence of *rrpA* and *rrpB* in *C. jejuni* strains may be important for human infection and colonisation. Some strains that affect humans seem to be related to livestock-associated clonal complexes and, therefore, are *rrpB*$^{+}$. However, this does not seem to apply to the Brazilian isolates, as most human isolates were *rrpB*$^{-}$. To determine whether this is a trend in Brazil, further investigations should be performed with a larger number of strains.

Loss or gain of genes can be an evolutionary process due to selection pressures (Morley *et al.*, 2015). Some STs, such as ST-403 are associated with gene decay and multiple independent deletions and mutations, which indicates evolution and niche adaptation (Morley *et al.*, 2015).

6.3.6 Plasticity region investigation amongst Brazilian isolates

The Brazilian isolates were investigated for genomic structure of the plasticity region surrounding *rrpA* and *rrpB* based on whole genome sequence data. Next generation genomic sequencing is a short-read sequencing platform widely used in research, which, however, has some limitations (Nowrousian, 2010). The short sequence read system is prone to make errors on genomic assembly (Nowrousian, 2010). Highly fragmented genomes will have a decreased genome assembly quality (Klassen and Currie, 2012). Sequencing fragmented ORFs can generate biased information and issues with annotation (Klassen and Currie, 2012). Small gene sequences can have a false-negative annotation, whilst a fragmented ORF can have a false-positive annotation to each of the ORF fragments separately (Klassen and Currie, 2012). The Brazilian isolate genomes contained several fragmented genes, which might have occurred due to assembly problems.

The genetic structure of the two conserved regions and the variable central region of 11168H (*rrpB*$^+$) and 81116 (*rrpB*$^-$) were used as templates to compare with the Brazilian isolates. *rrpB*$^+$ isolates demonstrated a highly conserved genomic structure including both flanking regions and the central plasticity region. All *rrpB*$^+$ isolates contained the R-M *hsd* system. All strains contained *rloH* in the ORF between *hsdR* and *hsdS*. However, there was one gene absent in all *rrpB*$^+$ isolates, except one, which was *mloB*. This indicates that highly frequently no gene was inserted in the ORF between *hsdS* and *hsdM*.

Some genes present in the Brazilian isolates were identified as truncated. Truncation could have happened due to a frameshift mutation as reported by Miller *et al.* (2005). They also identified truncated genes in the *hsdR* gene region due to a frameshift mutation. These genes are presumably non-functional. Frameshift mutations are probably caused by slipped-strand mispairing within an upstream coding repeat (Miller *et al.*, 2005).

rrpB$^-$ isolates had several genes absent. Most of the absent genes were from either the central variable region or the downstream conserved region. No gene was missing from the upstream conserved region. This demonstrated a higher genetic variability for the Brazilian *rrpB*$^-$ strains and that the downstream region was less conserved. However, the gene absence could have risen from assembly problems.

6.3.7 Conclusion

Brazilian chicken meat isolates were shown to be highly resistant to H_2O_2. This highlights the risk of meat contamination and the high capability of *C. jejuni* to survive under hostile conditions. Brazilian human isolates had variable resistance to H_2O_2. All Brazilian strains demonstrated the ability to form biofilms, which is also an important feature for surviving in the environment and important for food contamination in the food industry. RrpA and RrpB did not seem to correlate with the strains ability to resist H_2O_2. However, this should be confirmed using a greater number of strains.

7 Final Discussion

7.1 Role of RrpA and RrpB in regulation *C. jejuni* oxidative stress

The investigations in this study into the role of RrpA and RrpB in the regulation of *C. jejuni* oxidative stress responses have shown that these two regulators play a role in both the peroxide and aerobic stress responses.

RrpA and RrpB were identified as members of the MarR-type family of transcriptional regulators which have a winged helix-turn-helix DNA binding motif (Kumarevel *et al.*, 2009). This domain is present in different bacteria, such as *S. typhimurium* and *E. coli* (Kumarevel *et al.*, 2009). MarR regulators are involved in important biological processes, such as antibiotic resistance, oxidative stress and pathogenesis (Saridakis *et al.*, 2008, Kumarevel, 2012). However, the exact mechanisms of regulation are not well understood (Saridakis *et al.*, 2008).

Different regulatory proteins belonging to the MarR family have been shown to have a dimer structure based on crystal structure analysis (Saridakis *et al.*, 2008, Kumarevel *et al.*, 2009). The regulators form homodimer structures and each monomer has a winged helix-turn-helix DNA binding motif (Kumarevel, 2012). The homodimers bind to double-stranded DNA (Kumarevel, 2012). As RrpA and RrpB belong to the MarR family, these regulators may also form homodimers. However, further investigation is necessary to confirm the RrpA and RrpB structures.

The mechanism of regulation by MarR transcriptional regulators involves changes of conformation in the DNA binding domain upon effector binding (Saridakis *et al.*, 2008). The effector modulates the DNA binding capacity through changes in the protein conformation (Saridakis *et al.*, 2008). The exact effector of RrpA and RrpB is still not known. However, ROS may be a potential effector of these regulators. RrpA and RrpB are self-regulators that repress their own transcription. RrpA and RrpB may be sensitive to ROS, which could change the protein conformation preventing the binding to DNA. This would then up-regulate *rrpA* and *rrpB* expression, allowing RrpA and RrpB to regulate the expression of other genes. RrpA regulates *katA* and *rrpB* expression. How RrpB regulates KatA is still unknown. Other regulators of *C. jejuni* oxidative stress defence, such as PerR, Fur and CosR may also have a role regulating the expression of RrpA and/or RrpB. As suggested by RNA seq data, Fur

might regulate *rrpB* expression. We could speculate that regulators such as PerR, Fur and CosR could regulate *rrpA* negatively, and thus, RrpA would regulate *katA* positively. Analysis of gene expression demonstrated that RrpA directly regulates *katA* expression and also regulates *rrpB* expression (Figure 7.1). Preliminary analysis did not suggest RrpB regulation of *katA* expression, even though the *rrpB* mutant demonstrated to affect *katA* expression.

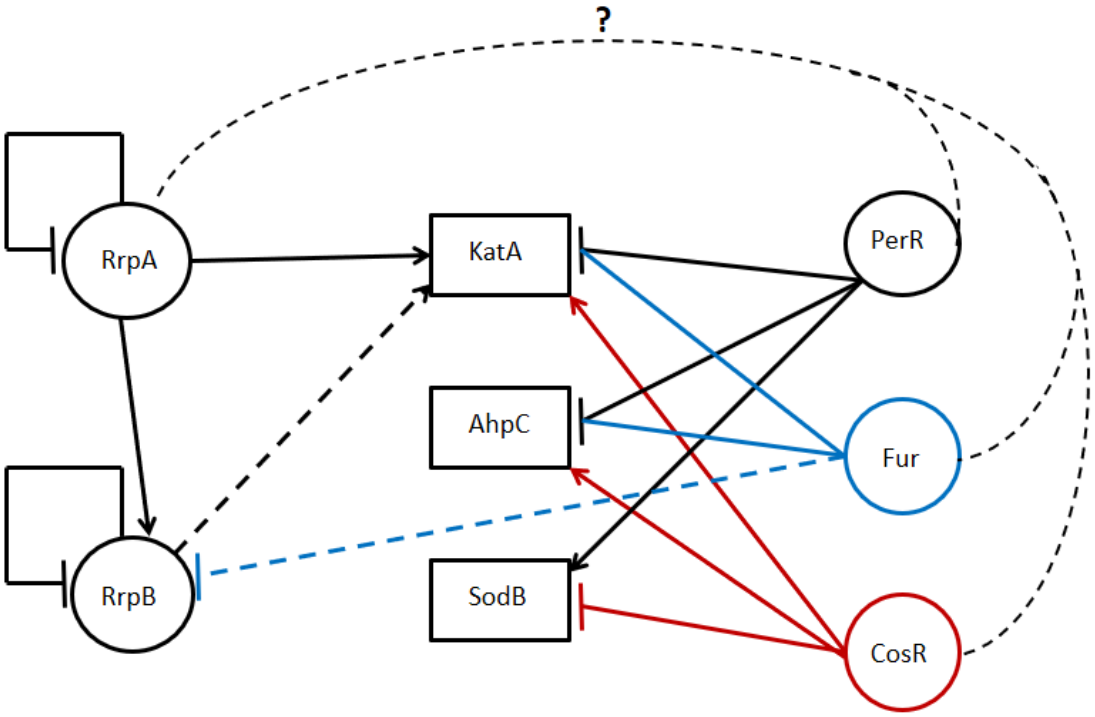

Figure 7.1 Schematic diagram of the roles of RrpA, RrpB and other oxidative stress regulators. Circles indicate transcriptional regulators, and squares indicate enzymes. Arrows indicate positive regulation. Lines with blunt ends indicate negative regulation. Dash lines indicate putative regulation.

The *rrpAB* double mutant demonstrated the opposite phenotype compared to the single mutants. The double mutant exhibited increased resistance to hydrogen peroxide, menadione and cumene hydroperoxide. However, the double mutant did not exhibit an increase in either KatA activity or SodB activity. Furthermore, mutation of both

rrpA and *rrpB* does not seem to affect the expression of *katA, sodB* or *ahpC.* The RNA seq analysis performed on the *rrpAB* double mutant also did not indicate any effect on expression of these three genes. However, the RNA isolation was performed after microaerobic growth under non-oxidative stress conditions. *rrpA* and *rrpB* expression are probably both induced in the presence of ROS and/or under aerobic conditions. Therefore, RNA-seq should also be performed on the wild-type strain and *rrpA, rrpB* and *rrpAB* mutants after exposure to oxidative stress and after growth under aerobic conditions in order to investigate further changes in gene expression in the different mutants and also whether *rrpA* and *rrpB* expression will be affected in the wild-type strain.

7.2 Variation in the presence of RrpB

Horizontal gene transfer is important for bacterial evolution and genetic diversity providing niche adaptation advantages (Wiedenbeck and Cohan, 2011). The evolution from non-pathogenic to pathogenic bacteria occurs through the acquisition of virulence genes via horizontal gene transfer (Maurelli, 2007). Gene transfer is highly related to sequence similarity and to bacteria belonging to the same environmental niche (Polz *et al.*, 2013). Horizontal gene transfer and gene loss are mechanisms that can lead to genome differences between two closely related bacterial species (Polz *et al.*, 2013). Closely related genomes can have genomic differences due to evolution based on the ecological niche adaptation of a local population (Polz *et al.*, 2013). Bacteria can become more pathogenic or better adapted to a specific environment through gain or loss of genes (Maurelli, 2007).

Speciation is the irreversible process where a bacterial lineage splits, which can occur through the acquisition or loss of genes that will differentiate a bacterial ecological niche (Wiedenbeck and Cohan, 2011). This may be a subtle difference in ecological requirements and bacterial capabilities (Wiedenbeck and Cohan, 2011). However, it can lead to independent evolutionary trajectories of a population (Polz *et al.*, 2013). The genomic changes occur due to mutation of single nucleotides or amino acids, or genomic insertions and deletions (Maurelli, 2007). *C. jejuni* and *C. coli* descended from the same ancestor. *C. jejuni* and/or *C. coli* may have acquired or lost genes that then separated them into two different species. *C. jejuni* and *C. coli* possess core genes

and dispensable genes (Lefebure *et al.*, 2010). Bacterial species are differentiated based on the core genes (Lefebure *et al.*, 2010). *C. jejuni* and *C. coli* have developed a small number of unique core genes (Lefebure *et al.*, 2010). The recombination of core genes between the two species is not a common feature (Lefebure *et al.*, 2010).

Variation in the distribution of *rrpB* was observed amongst *C. jejuni* strains with *rrpB* found to be present in roughly 50% of the strains. The vast majority of *C. jejuni* strains contain *rrpA,* which is located in a conserved region of the genome. Only very few *C. coli* strains contain *rrpA.* Therefore, we can speculate that *C. jejuni* acquired *rrpA* after the species split or that *C. coli* may have lost *rrpA* in the evolutionary process. Gene loss is a type of genetic change which has great potential for bacterial diversity and adaptation (Albalat and Canestro, 2016). Loss of a gene or reduced expression sometimes confer beneficial effect to the bacteria (Koskiniemi *et al.*, 2012). *C. jejuni* and *C. coli* genomic differences lead to differences in prevalence in animal host species and environmental sources (Lefebure *et al.*, 2010). This was observed in the MLST analysis which demonstrated specific clonal complexes associated to each species.

Normally, new genes are acquired through horizontal gene transfer from highly related organisms (Nasvall *et al.*, 2012). The *rrpB* gene was identified within a genetic plasticity region containing an R-M system. R-M systems are acquired through horizontal gene transfer of mobile elements (Furuta *et al.*, 2010). R-M systems can move independently from the rest of the genome. It is suggested that R-M systems generate bacterial diversity by promoting homologous recombination and genomic rearrangements, such as deletions and insertions (Kobayashi, 2001, Furuta *et al.*, 2010). *rrpB* may have been acquired together with the R-M system genes through horizontal gene transfer or through recombination. Mobile genetic elements are important for adaptation to a specific niche, which is also a model for bacteria speciation (Wiedenbeck and Cohan, 2011). Genetic variable regions can be acquired through homologous recombination, when this variable region is located between two conserved regions (Koskiniemi *et al.*, 2012). This is in agreement with the variable region observed containing R-M system and *rrpB,* which was located in between two conserved regions. This contributes to the presence of variable islands within core regions in the bacterial genome (Koskiniemi *et al.*, 2012). However, new genes can also arise from copies of duplicated genes (Nasvall *et al.*, 2012). The extra copy is

then able to acquire one or more mutations providing a new beneficial function (Nasvall *et al.*, 2012). Another possibility is that *rrpB* is a duplication of *rrpA*, and that accumulated mutations differentiated *rrpB* from *rrpA.* RrpA has 43.6% identity and 58.4% similarity to RrpB. If two sequences share more than 40% identity, it is very likely that they have similar function (Pearson, 2013). However, change of a few residues can cause dramatic differences in enzyme activity (Pearson, 2013). Depending on the environmental pressure, the duplication can be maintained or lost (Nasvall *et al.*, 2012). Normally, redundant gene copies tend to undergo deleterious mutations more often than beneficial ones, leading to inactivation of the duplicated gene (Nasvall *et al.*, 2012). Many *C. jejuni* strains lacking *rrpB* did not seem to have any disadvantage compared to strains containing *rrpB.* Two *C. jejuni* reference strains containing both *rrpA* and *rrpB* were shown to be naturally more sensitive to H_2O_2 stress compared to reference strains containing only *rrpA.* Analysis of a greater number of *C. jejuni* wild-type strains also demonstrated that strains containing only *rrpA* have a tendency to be more resistant to H_2O_2 (Gundogdu *et al.*, unpublished data). Therefore, strains lacking *rrpB* have a tendency to be more resistant to peroxide stress. However, RrpB may have had a more essential role under different environmental conditions due to selective pressure. As it was observed, selective pressure was probably the reason why strains containing *rrpB* were conserved to just a few specific MLST clonal complexes. Duplicated genes may be dispensable if they are involved in redundant metabolic processes (Nasvall *et al.*, 2012). RrpB may have a redundant or complementary function to RrpA and is not essential for *C. jejuni* strains. Loss of *rrpB* may give an advantage to the *C. jejuni* strains to survive oxidative stress or the presence of RrpB may confer an advantage in colonising specific niches.

7.3 Analysis of Brazilian *C. jejuni* strains

The analysis of the Brazilian *C. jejuni* strains highlighted both phenotypic and genomic differences compared to other *C. jejuni* strains in this study. Brazilian *C. jejuni* strains may have adapted differently due to distinct environmental pressures and niche adaptations. It was observed that the variation in the presence of *rrpB* in the Brazilian strains did not appear to influence the strains resistance to H_2O_2 stress. The Brazilian isolates exhibited variable resistance to H_2O_2 stress, which did not correlate

to the presence or absence of *rrpB.* The opposite was observed for other *C. jejuni* strains, where the presence of *rrpB* influenced resistance to H_2O_2 stress.

The genomic structure of the Brazilian strains containing *rrpB* was very similar to NCTC 11168. However, strains lacking *rrpB* were shown to have very different genetic structure compared to other *C. jejuni* strains distributed worldwide. The number of Brazilian isolates analysed in this study was not large, further investigation with a greater number of Brazilian strains will be necessary to confirm whether the resistance to H_2O_2 stress is related to the presence or absence of *rrpB.* Strains from different parts of Brazil should also be analysed to observe if this feature is restricted to a region of Brazil or if that is a trend across the whole country.

Genomic typing techniques, such as MLST, are important tools to better understand *C. jejuni* epidemiology. *C. jejuni* epidemiology is still not very clear, and there is evidence that this bacterium behaves differently in developed and developing countries. There are not many studies using MLST in Brazil. Therefore, more investment for genome typing and epidemiology studies are necessary. The use of tools such as MLST is crucial for an understanding of *C. jejuni* epidemiology and evolution (Maiden, 2006).

MLST can achieve high levels of discrimination (Perez-Losada *et al.*, 2013). It also gives precise locality of data related to strain distribution (Perez-Losada *et al.*, 2013). MLST is a widespread technique that can be easily reproduced worldwide. The information obtained by the MSLT can be uploaded and shared throughout the world via the Internet. Thus, the data generated can be readily compared among laboratories on publicly available databases (Maiden, 2006, Perez-Losada *et al.*, 2013).

This study discovered new STs based of MLST technique. The new Brazilian STs are going to be uploaded to the online database where they will be assigned a unique ST number based of each allele fragment sequenced (Maiden, 2006). This information will be available for any researcher worldwide. This information can be useful as researchers can compare their own profiles to the profiles presented in this study. MLST can also detect evolutionary changes in the genome of populational studies. With this technique, ST profiles can be tracked globally for similar genotypes. MLST can identify lineages most linked with livestock, humans or both sources, and also identify which strains are more virulent (Maiden, 2006).

It is important to integrate all the information acquired from the isolates analysed by MLST. If researchers combine genomic and epidemiological data with the geographic information, they will have a clear picture of the pathogens analysed and resources to develop methods to prevent the spread of infectious pathogens (Perez-Losada *et al.*, 2013).

Brazil is the second major chicken meat producer and the major exporter in the world (Clements, 2016, Workman, 2016). However, Brazil does not have legislation to control or to monitor *Campylobacter* in poultry products. Currently, biosecurity measures are used by farmers to control *Campylobacter* in flocks. However, these measures are not enough to avoid flock contamination. Slaughter houses also apply strict measures of disinfection and decontamination of all their equipments to avoid cross contamination. However, these measures are also not enough to control *Campylobacter* in the industry. Therefore, multiple measures should be taken to reduce the level of *Campylobacter* contamination in poultry before slaughter to try to reduce the levels of contamination in the industry, and thus, reduce the risk of human infection. The Brazilian government needs to invest more in *Campylobacter* research and to create surveillance programs to clarify the dimension of Campylobacteriosis in Brazil.

It is essential to study *C. jejuni* physiology and the mechanisms of survival in hostile environments to fully comprehend the epidemiology of this human pathogen for the development of effective intervention strategies.

RNA seq is an excellent technique to investigate changes in gene expression under optimal conditions and under extreme conditions. The next analysis should use RNA seq to investigate changes in gene expression under different types of oxidative stress. Investigating these changes in gene expression under different conditions will give a clearer picture of how oxidative stess regulators change the gene expression to survive under hostile conditions. These changes in gene expression should be compared to gene expression under standard growth conditions in microaerobic conditions to understand which genes are essential for survival in hostile environments. RNA seq together with population genetic analysis will, hopefully, provide valuable information that can be paramount to develop essential control measures for *C. jejuni*, and efficiently reduce the number of human infections.

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Appendix 1

Strains used in this study

***C. jejuni* strain**	**Description**	**Reference**
11168H	A hypermotile derivative of the original sequence strain NCTC 11168 that shows higher levels of ceacal colonisation in a chick colonization model	Karlyshev et al., 2002 Jones et al., 2004
11168H *rrpA* mutant	Kanamycin cassette inserted into the *rrpA* gene	Gundogdu et al., 2001
11168H *rrpB* mutant	Kanamycin cassette inserted into the *rrpB* gene	Gundogdu et al., 2001
11168H *rrpAB* double mutant	Kanamycin cassette inserted into the *rrpA* gene and Chloramphenicol cassette inserted into the *rrpB* gene	Gundogdu et al., 2001
11168H *katA* mutant	Kanamycin cassette inserted into the *katA* gene	LSHTM *Campylobacter* Resource Facility
11168H *perR* mutant	Kanamycin cassette inserted into the *perR* gene	LSHTM *Campylobacter* Resource Facility
11168H *ahpC* mutant	Kanamycin cassette inserted into the *ahpC* gene	LSHTM *Campylobacter* Resource Facility
11168H *rrpB perR* double mutant	Kanamycin cassette inserted into the *perR* gene and Chloramphenicol cassette inserted into the *rrpB* gene	This study

81116	Genetically stable strain which remains infective in avian models	Wassenaar et al., 1991
81116 *rrpA* mutant	Kanamycin cassette inserted into the *rrpA* gene	LSHTM *Campylobacter* Resource Facility
81-176	Highly virulent and widely studied laboratory strain, isolated from milkborne outbrake in USA	Korlath et al., 1985
81-176 *rrpA* mutant	Kanamycin cassette inserted into the *rrpA* gene	LSHTM *Campylobacter* Resource Facility
81-176 *rrpB* mutant	Kanamycin cassette inserted into the *rrpB* gene	LSHTM *Campylobacter* Resource Facility
M1	Isolated from human with diarrhea	Champion et al., 2005
M1 *rrpA* mutant	Kanamycin cassette inserted into the *rrpA* gene	LSHTM *Campylobacter* Resource Facility
11919	Isolated from chicken	Champion et al., 2005
11973	Isolated from chicken	Champion et al., 2005
12450	Isolated from chicken	Champion et al., 2005
12487	Isolated from chicken	Champion et al., 2005
12912	Isolated from bovine	Champion et al., 2005
13040	Isolated from chicken	Champion et al., 2005
13249	Isolated from chicken	Champion et al., 2005

13713	Isolated from bovine	Champion et al., 2005
30280	Isolated from human with diarrhea	Champion et al., 2005
47886	Isolated from human with septicemia	Champion et al., 2005
12241	Isolated from ovine	Champion et al., 2005
31481	Isolated from human asymptomatic	Champion et al., 2005
33106	Isolated from human asymptomatic	Champion et al., 2005
34007	Isolated from human with septicaemia	Champion et al., 2005
40917	Isolated from human with bloody diarrhea	Champion et al., 2005
44119	Isolated from human with septicaemia	Champion et al., 2005
47693	Isolated from chicken	Champion et al., 2005
62914	Isolated from human vomiting	Champion et al., 2005
64555	Isolated from human with bloody diarrhea	Champion et al., 2005
CCAMP 487	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)

CCAMP 488	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 489	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 491	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 492	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 499	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 500	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 505	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 507	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 511	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 593	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)

CCAMP 600	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 607	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 609	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 611	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 677	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 679	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 680	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1479	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1490	Isolated from human faeces	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 463	Isolated from poultry drinking water	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)

CCAMP 830	Isolated from creek stream water from sewage treatment station	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 831	Isolated from creek stream water from sewage treatment station	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1013	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1014	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1015	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1016	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1018	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1019	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1020	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1021	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)

CCAMP 1022	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1023	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1024	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1032	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1050	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1051	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1052	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1053	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1054	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1055	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)

CCAMP 1056	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)
CCAMP 1057	Isolated from poultry meat	Oswaldo Cruz Foundation (FIOCRUZ – Brazil)

Appendix 2

Amino Acids Alignments using ClustalW2

RrpA - *Cj1546*

```
CG8421_          -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
1336             -MTKENSQCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
LMG9872          -MTKENSQCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
140-16_          -MTKENSQCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
HB93-13          -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
1213             -MTKENSQCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
81116_           -MTKENSQCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
23264            -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
S3_              -MTKENSQCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
RM1221           -MTKENSQCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
260.94           -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
2008-988         -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIMRYNELKRFLSSISFKTLTN 59
81-176           -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
11168            -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
11168-BN148      -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
ICDCCJ07001      -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
PT14             -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
60004            -MTKENSPCNFEECGFNYTLALINGKYKMSILYCLFRYEIVRYNELKRFLSSISFKTLTN 59
414              MKNTKNSTCNYQEYGFNYTLALISGKYKMSVLYCLYKDKIVRYNELKRILNPISFKTLTN 60
                   ..:** **::* *********.******:****:: :*:********:*..********

CG8421_          TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMCDWEEENKKLQGK--- 113
1336             TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMCDWEEENKKLQGK--- 113
LMG9872          TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMCKWGEKDKKGKNA--- 113
140-16_          TLRELEYDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMCKWGEKDKKGKNA--- 113
HB93-13_         TLRELENDGLIIRKEYAQIPPKVEYNLSKRGQSLIPILQAMCKWGEKDKKEKNA--- 113
1213             TLRELENDGLIIRKEYAQIPPKVEYNLSKRGQSLIPILQAMCKWGEKDKKEKNA--- 113
81116            TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMCKWGEKDKKEKNA--- 113
23264            TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMCKWGEKDKKGKNA--- 113
S3_              TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN- 115
RM1221           TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN- 115
260.94_          TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN- 115
2008-988         TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN- 115
81-176           TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN- 115
11168            TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN- 115
11168-BN148      TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN- 115
ICDCCJ07001      TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN- 115
PT14             TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKKCLN- 115
60004            TLRELENDGLIIRKEYAQIPPKVEYSLSKRGQSLIPILQAMSKWGKKDKKGKNA--- 113
414              VLRELENAGLIIRKEYPQIPPKVEYSLSKKGQSFIPILEAMCDWGRRKQKINILKIY 117
                 .*****  ********.********.***:***:****:**..* ...:* :
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Primers

Forward: CGFNYT - TGY GGN TTY AAY TAC ACN

Reverse: PPKVEY - CCN CCN AAR GTN GAR TAC (Original)

GGN GGN TTY CAN CTY ATG (Complementary)

GTA YTC NAC YTT NGG NGG (Reverse)

RrpB - *Cj1556*

```
LMG23357          MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISSTKNQNISQNVLTQNL 60
LMG9872           MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISSTKNQNISQNVLTQNL 60
81-176            MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
87330             MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
129-258           MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
305               MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
IA3902            MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
CF93-6            MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
84-25             MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
LMG9879           MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
11168             MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
11168-BN148       MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
LMG23269          MKKYHSLCPIETTLNLIGNKWKILIIRDLLQGTKRFGELRKSISFTKNQNISQNVLTQNL 60
                  ************************************************ ***************

LMG23357          RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
LMG9872           RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
81-176_           RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
87330             RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
129-258           RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
305               RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
IA3902            RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
CF93-6            RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
84-25             RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
LMG9879           RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
11168             RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
11168-BN148       RELEEAKLIKRKVYAEVPPKVEYSLTSLGNSLESILKSLENWGNSYKNIV 110
LMG23269          RELEEAKLIKRKVYAER----------LSTHLHL---------------- 84
                  ***************           *.. *.
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Primers

Forward: CPIETT – TGY CCN ATH GAR ACN ACN

Reverse: PPKVEY – CCN CCN AAR GTN GAR TAC (Original)

GGN GGN TTY CAN CTY ATG (Complementary)

GTA YTC NAC YTT NGG NGG (Reverse)

CosR – *Cj0355c*

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81116_          MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
11168-BN148     MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
81-176          MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
11168           MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
LMG23223        MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
414             MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGI 60
2008-872        MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLSDGDGA 60
RM1221          MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
IA3902          MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
S3              MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
PT14            MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
1997-10         MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
260.94_         MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
LMG23263        MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
doylei          MRILVIEDEISLNKTIIDNLNEFGYQTDSSENFKDGEYFIGIRHYDLVLANWTLPDGDGA 60
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81116_          ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVKKPLDFDILLARIEARLRLGG 120
11168-BN148     ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVKKPLDFDILLARIEARLRLGG 120
81-176          ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVKKPLDFDILLARIEARLRLGG 120
11168           ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVKKPLDFDILLARIEARLRLGG 120
LMG23223        ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKSGADDFVKKPLDFDILLARIEARLRLGG 120
414             ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVKKPLDFDILLARIEARLRLGG 120
2008-872        ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVKKPLDFDILLARIEARLRLGG 120
RM1221          ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVTKPLDFDILLARIEARLRLGG 120
IA3902          ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVTKPLDFDILLARIEARLRLGG 120
S3              ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVTKPLDFDILLARIEARLRLGG 120
PT14            ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVTKPLDFDILLARIEARLRLGG 120
1997-10         ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVTKPLDFDILLVRIEARLRLGG 120
260.94_         ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVAKPLDFDILLARIEARLRLGG 120
LMG23263        ELVNTIKHKSPRTSVMIMSSKTDKETEIKALKAGADDFVTKPLDFDILLARIEARLRLGG 120
doylei          ELVNTVKHKSPRTSVMIISSKTDKETEIKALKAGADDFVKKPLDFDILLARIEARLRLGG 120
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81116_          TNVIKIEDLVIDPDEEKITYKGQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
11168-BN148     TNVIKIEDLVIDPDEEKITYKGQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
81-176          TNVIKIEDLVIDPDEEKITYKGQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
11168           TNVIKIEDLVIDPDEEKITYKGQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
LMG23223        TNVIKIEDLVIDPDEEKITYKGQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
414             TNVIKIEDLVIDPDEEKITYKGQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
2008-872        TNVIKIEDLVIDPDEEKITYKGQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
RM1221          TNVIKIEDLVIDPDEEKITYKDQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
IA3902          TNVIKIEDLVIDPDEEKITYKDQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
S3              TNVIKIEDLVIDPDEEKITYKDQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
PT14            TNVIKIEDLVIDPDEEKITYKDQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
1997-10         TNVIKIEDLVIDPDEEKITYKDQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
260.94          TNVIKIEDLVIDPDEEKITYKDQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
LMG23263        TNVIKIEDLVIDPDEEKITYKGQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
doylei          TNVIKIENLVIDPDEEKITYKGQDIELKGKPFEVLTHLARHSDQIVSKEQLLDAIWEEPE 180
                *******:*************.************************************

81116_          LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
11168-BN148     LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
81-176          LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
11168           LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
LMG23223        LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
414             LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
2008-872        LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
RM1221          LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
IA3902          LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
S3              LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
PT14            LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
1997-10         LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
260.94          LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
LMG23263        LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
doylei          LVTPNVIEVAINQIRQKMDKPLNISTIETVRRRGYRFCFPKKS 223
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Primers

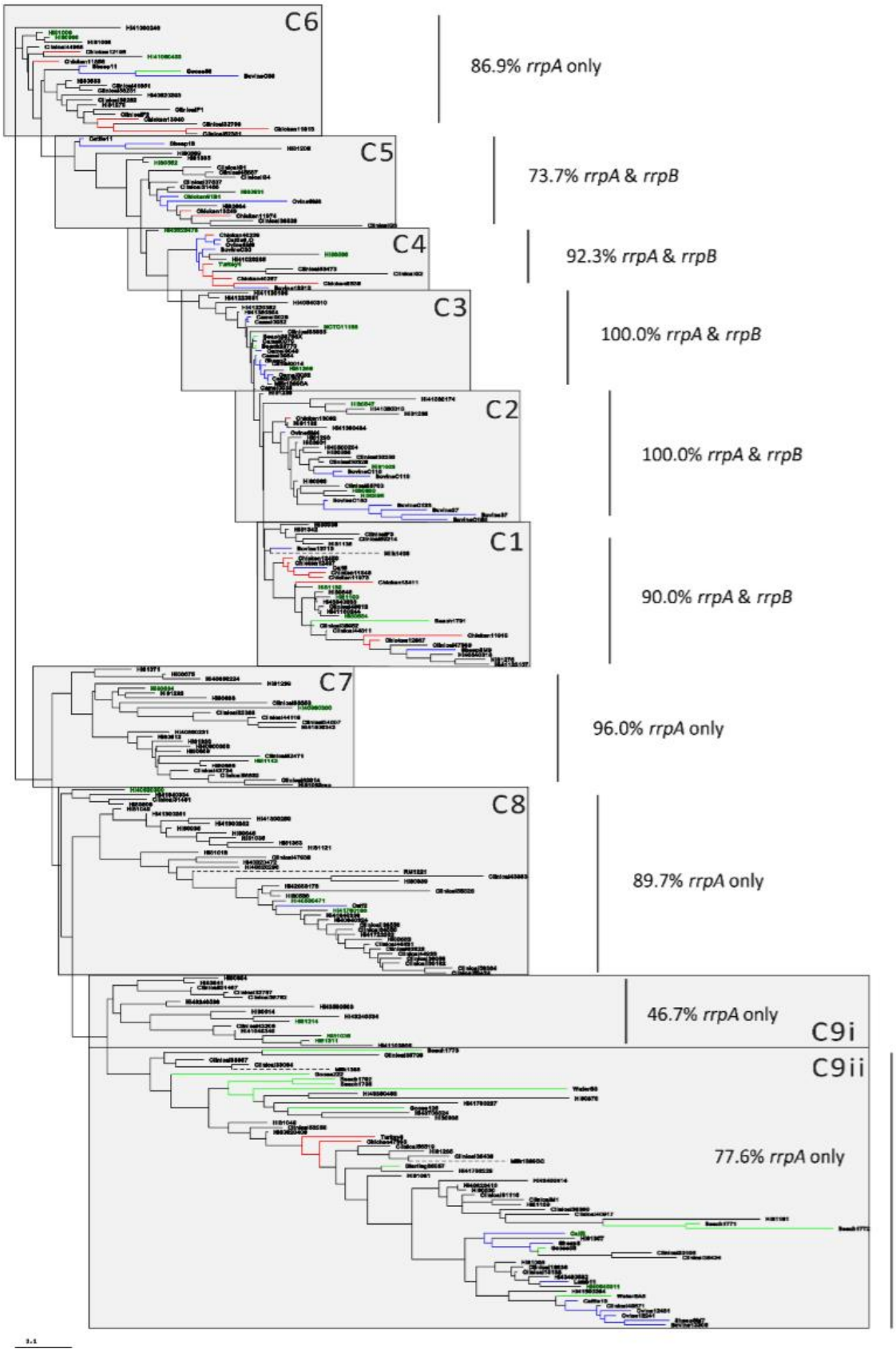
Forward: VIEDEI – GTN ATH GAR GAY GAR ATH

Reverse: FCFPKK – TTY TGY TTY CCN AAR AAR (Original)

AAR ACR AAR GGN TTY TTY (Complementary)

YTT YTT NGG RAA RCA RAA (Reverse)

Appendix 3. Prevalence of the *rrpA* and *rrpB* genes amongst 270 *C. jejuni* strains



MLST clonal complexes and prevalence of the *rrpA* and *rrpB* genes amongst 270 *C. jejuni* strains. Isolates are coloured according to clonal complex; ST-21 (red), ST-45 (green), ST-353 (blue), ST-257 (pink), ST-206 (orange), unassigned (black, bold) and untyped (black, unbold). Figure adapted from Stabler *et al.*, 2013.