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Stress-Adaptation and Stress-Induced Changes in Campylobacter Jejuni

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STRESS-ADAPTATION AND STRESS-INDUCED CHANGES IN *CAMPYLOBACTER JEJUNI*

STRESS-ADAPTATION AND STRESS-INDUCED CHANGES IN *CAMPYLOBACTER JEJUNI*

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Poultry Science

By

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ABSTRACT

The foodborne pathogen *Campylobacter jejuni* is one of the leading causes of human gastroenteritis. This bacterium is not a robust organism compared with many other foodborne pathogens and requires special conditions in the laboratory for its growth. In nature, however, this organism is able to survive in very diverse and hostile environments and produce disease in humans. The different mechanisms by which *C. jejuni* survives stressful conditions in the environment remain unclear. Adhesion and invasion are thought to be important factors for the colonization of *C. jejuni* in the intestinal tracts of hosts. Previous research in our laboratory showed that *C. jejuni* has the ability to induce an adaptive tolerance response to stresses like acid and aerobic conditions. The objectives of this research were to determine the influence of acid-adaptation on the virulence and antibiotic profiles of *Campylobacter jejuni*. In experiment 1, the effect of acid-adaptation on virulence was studied by determining the adhesion and invasion of human intestinal cells by different strains of *Campylobacter jejuni*. Different human and poultry isolates of *C. jejuni* were exposed to an acid stress (pH 5.5) and further subjected to different secondary stresses such as an acid pH of 4.5, starvation, and salt (3%). After exposure to the secondary stress, the adhesion and invasion abilities of the isolates were evaluated in vitro using an INT 407 cell line. Acid-adapted *C. jejuni* showed an increase in adhesion and invasion when exposed to the secondary stresses of acid, starvation and salt. The degree of adhesion and invasion varied with strains of *C. jejuni*, the time of adaptation to acid, as well as exposure time to the second stress. These data indicate that some stresses may increase the virulence of *C. jejuni*. In experiment 2, the effect of acid adaptation on the antibiotic sensitivity of different strains of *C. jejuni* were studied. The antibiotic

profiles of *C. jejuni* after stress-adaptation were compared with antibiotic profiles of non-stressed *C. jejuni* using the Kirby Bauer agar disc diffusion assay. The antibiotic profiles of *C. jejuni* were found to change when the acid-adapted bacteria were subjected to further stresses such as a lethal acidic pH of 4.5, aerobic atmosphere and starvation. In the majority of the cases antibiotic-resistant *C. jejuni* strains were found to become sensitive to antibiotics after stress-adaptation, but in a few cases *C. jejuni* showed increased resistance. These results indicate that increasing various stresses in *C. jejuni* may, in some cases, reduce antibiotic resistance.

This dissertation is approved for recommendation
to the Graduate Council.

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DEDICATION

I dedicate this dissertation

to

my

Late Father K. R. Sanal Kumar

and

Mother Radha Sanal Kumar

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Chapter 1

INTRODUCTION

Campylobacter is one among the leading causes of human bacterial gastroenteritis throughout the world. In the United States alone, campylobacteriosis is estimated to affect approximately 850,000 persons annually as per the reports of Centers for Disease Control. Although the *Campylobacters* do not produce a high mortality rate, it is estimated that they can cause around 8000 hospitalizations and 76 deaths per year (Scallan *et al.,* 2011). The word *kampylos* is derived from Greek meaning curved. Even though the genus *Campylobacter* comprises of many species only 2 of these species, namely *C. jejuni* and *C. coli* are found to be associated with gastroenteritis in humans (Vandamme *et al.,* 2008). *Campylobacters* are spirally curved rods, gram-negative and slender. They have a single polar flagellum on one or both ends that gives the organism a characteristic darting or cork-screw like motility. The main reservoirs for these bacteria are chicken, ruminants, pigs, and wild birds (Gras *et al.,* 2012). Poultry are found to harbor these bacteria in large numbers in their intestines. This might be due to the higher body temperature of poultry which is a suitable growth temperature for *Campylobacter* (Skirrow, 1977). Even though there are several sources of *Campylobacter*, the disease campylobacteriosis is mainly believed to be caused by the consumption of undercooked meat and/or handling of poultry meat (Wingstrand *et al.,* 2006; Humphrey *et al.,* 2007).

Microbial contamination of foods with spoilage and pathogenic bacteria has always been the greatest concern to the food industry (Ray & Bhunia, 2008). With the goal of pathogen reduction and safer food, various methods are employed in the food industry which includes the use of heat, pressure, osmotic shock, acids, salts, antimicrobials, radiation and biological control methods. Recent findings indicate that foodborne

pathogens are capable of surviving these stresses by the mechanism of adaptive tolerance response which is commonly referred to as ATR. Exposure of bacteria to sub-lethal stresses induces an adaptive tolerance response in many bacteria which provides protection towards subsequent exposure to more severe stresses that they might encounter in the environment (Yousef and Courtney, 2003). Many studies have been carried out regarding ATR in *Salmonella, Listeria, Escherichia coli* and *Vibrio cholerae* (Bacon *et al.,* 2003; Koutsoumanis *et al.*, 2003; Tosun and Gonul, 2005). The survival and existence of pathogens in the food production chain is largely dependent on their ability to induce such an ATR or stress response. Stress adaptation has been observed when foodborne bacteria are exposed to the same (homologous) stresses or different (heterologous) stresses. Specifically, the acid tolerance response has been recognized as an important strategy employed by foodborne pathogens to counteract acid stress. *Salmonella, Listeria*, and *E. coli*, all have enhanced survival abilities during exposure to a lethal acid challenge, when exposed to prior sublethal acid conditions (Koutsoumanis and Sofos, 2004; Zhao and Houry, 2010).

Even though *C. jejuni* is a fragile organism that requires fastidious growth conditions in the laboratory, it is able to persist and survive in the environment and continue to be a serious public threat. After emerging from its natural niche in the cecum and crop of poultry, these organisms are exposed to a series of stresses and unfavorable conditions before colonizing and causing disease in humans (Humphrey *et al.,* 2007). These organisms are susceptible to many of the environmental conditions and are less tolerant to environmental stresses when compared to other foodborne pathogens (Park,

2002). *Campylobacter* lacks the usual stress response factors which are normally present in most of the gram negative bacteria necessary for survival in adverse environmental conditions. But still these bacteria are capable of persisting and surviving in the environment with the mechanisms of survival remaining an enigma. The physiology of this organism, including stress response is not well studied. *C. jejuni* has been found to survive different stresses in the environment by methods such as conversion to a viable but non-culturable (VBNC) state, transition from a rod to a coccoid shape, high degree of genetic heterogeneity, formation of biofilms and an adaptive tolerance response (Murphy *et al.,* 2006).

Acid is a key stress encountered while passing through the human gastrointestinal tract. *C. jejuni* encounters both organic and inorganic acids not only in the gastrointestinal tracts of the hosts but also in the food and poultry processing environments. *Campylobacter*s are found to survive the different stresses used in poultry processing and persist on poultry and poultry products (Keener *et al.,* 2004). Given the prevalence, yet the fastidious nature of *C. jejuni,* the question of how this organism is surviving these harsh conditions is unknown to the industry and scientists. It may be assumed that when exposed to a stress such as heat or acids, the organism acquires some kind of adaptive tolerance response which helps it to survive the rest of the stresses. *C. jejuni* must survive the acid pH conditions of the stomach to be able to cause disease in humans. The normal pH of empty stomach is around 2.0 and this can increase up to about 7.0 after the consumption of a full meal (Dressman *et al.,* 1990). So an acid adapted strain of *C. jejuni* has an increased possibility of survival at these pH conditions. Also the low infective dose

of *C. jejuni* might be an indication that the organism can adapt to these low pH conditions. *C. jejuni* has been shown to induce such an adaptive tolerance when exposed to acid and/or aerobic conditions (Murphy *et al.,* 2003). Other studies regarding ATR in *C. jejuni* proves that this bacteria is capable of resisting oxidative stress when exposed to a low temperature (Garénaux *et al.,* 2008) and has thermal resistance when starved (Klančnik *et al.,* 2006). Low temperature was found to give cross-protection to *C. jejuni* when exposed to oxidative stress, while the optimal growth temperature of 42^{o}C did not (Garénaux *et al.,* 2008). But the exact mechanisms behind such a tolerance are still not clear.

At the molecular level the stress responses in bacteria may include many changes such as up-regulation of genes and subsequent transcription of proteins required for their survival. Understanding the stress survival related gene response and survival mechanisms will provide valuable information into controlling this pathogen, reducing contamination, and treating campylobacteriosis. It has been shown that the virulence of foodborne pathogenic bacteria depends on their adaptation to and survival in the stressful conditions encountered within the host (Gahan and Hill, 2003). In fact, a stress response by the bacteria may not only enable survival under more extreme conditions or resistance to subsequent stressful conditions, but also enhance virulence. Multiple stress response genes have been found to be involved in adaptation and regulation of virulence factors (Reid *et al.*, 2008). Virulence genes are any genes expressed by the bacteria in an effort to enhance growth and survival during interactions with the host and environment. The virulence genes and mechanisms of pathogenesis of *C. jejuni* are still ambiguous. There have been four factors shown to be involved in causing symptoms of campylobacteriosis including

motility, adhesion, invasion, and toxin production (Zheng *et al.,* 2006). Motility and chemotaxis are important for seeking out the mucus layer of the intestines, and critical in moving away from a stressful environment. Adhesion and invasion are also thought to be important in colonization and could possibly be cued by stress. Toxin production is important for host tissue destruction. The destruction of host cells releases valuable nutrients for the bacteria to employ in an otherwise stress starved environment. A better understanding of any correlation between stress and the virulence factors of *C. jejuni* may give information that could facilitate the treatment of campylobacteriosis, reduce the prevalence of foodborne illness and make poultry products safer for consumers. Study of the microbial physiology after exposure to various stresses used in the food industry such as acid, salt and low temperature can lead to a more genuine evaluation of food safety concerns and thus increase food safety. This will lead to the development of better strategies which can increase the sensitivity of *C. jejuni* to the stresses used in the food processing industry.

JUSTIFICATION:

Stress adaptation results in complex changes in cell composition and regulation, which help the microorganisms to maintain the physiology of the cell resulting in survival and growth after exposure to various stresses. This adaptation and survival of the microorganisms are due to various molecular mechanisms such as gene amplification and production of stress proteins. Understanding the mechanisms which help *C. jejuni* in stress adaptation will help in the control of this pathogen in the farm, processing plant and food industry environments.

At present the food industry and processing plants employ different types of hurdle technologies to reduce the incidence of *C. jejuni* in food and poultry. Knowledge about the mechanisms helping this organism in producing an adaptive response mechanism can help in assessing and strengthening the intervention methods and thus reduce/ eliminate the organism in food. Stress-adaptation especially acid adaptation was found to increase the expression of virulence genes in many bacterial species. Thus, understanding the mechanisms involved in stress-adaptation of *C. jejuni* will also help in the study of the pathogenesis of the bacteria and help in the development of new treatment strategies to control the disease in humans.

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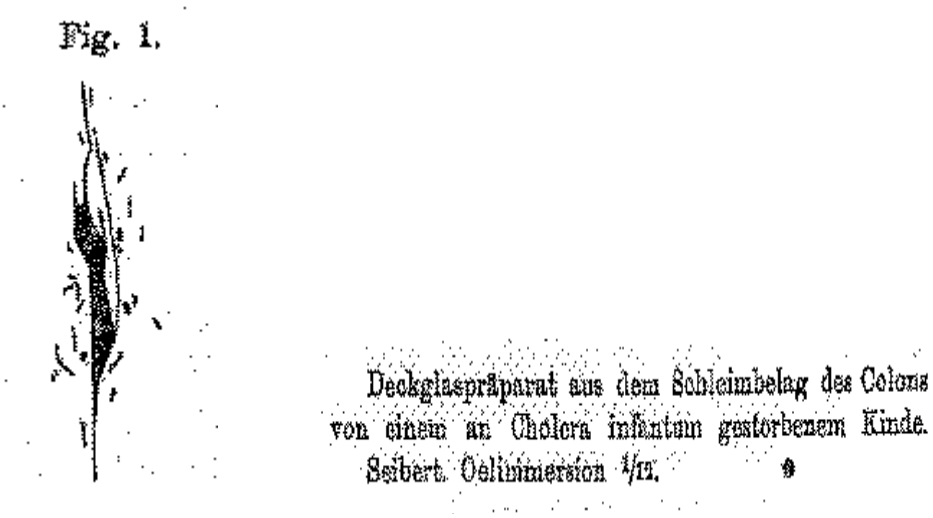
Chapter 2

LITERATURE REVIEW

2.1 *Campylobacter*

The name *Campylobacter* is derived from the Greek word "Kampylos" meaning curved. The first report of *Campylobacter*s dates back to 1886, when the scientist Escherich observed spiral organisms microscopically in the stool samples of children with diarrhea and colons of children who had died from "cholera infantum" (fig.2.1). Even though he tried to grow these organisms on solid medium his attempts were unsuccessful and so he did not consider them to be of any clinical importance (Escherich, 1886; Kist, 1986; Siegel *et al.*, 1990; Butzler, 2004). The first isolation of the bacteria was in 1906 when McFadyean and Stockman were able to isolate a vibrio-like bacterium from aborted sheep fetuses (fig. 2.2) and these bacteria were assigned as *Vibrio fetus* in 1919 by Smith and Taylor (McFadyean & Stockman, 1913; Smith, 1919; Smith & Taylor, 1919; Skirrow, 2006; Zilbauer *et al.*, 2008). The first documented outbreak of *Campylobacter* infection in humans occurred in the year 1938 in Illinois due to consumption of milk contaminated by the organism (Levy, 1946). Later, in 1947, Vinzent and co-workers reported the isolation of *Vibrio fetus* from the blood of three pregnant women having fever. But it was not until 1963 that these bacteria were given the name "*Campylobacter*" by Sebald and Veron. They found that these bacteria had a low G + C content and their growth requirements were also different from the various species of bacteria in the *Vibrio* genus (Sebald & Veron, 1963). Even though *Campylobacter* was first isolated and cultured from human feces in 1968 by Dekeseyer and Butzler in Brussels (Butzler *et al.*, 1973), it was not until the mid-80's that *Campylobacter jejuni* began to be recognized as the most common cause of bacterial gastroenteritis in humans (Alter and Scherer, 2006).



(Oil immersion slide preparation from the mucous lining of the colon of a child who had died of cholera infantum)

Fig 2.1: Theodore Escherich's drawing of *Vibrio*-like bacteria. Escherich observed these bacteria in the stool samples of children with diarrhea and colons of children who had died of *cholera infantum*. Even though he could observe these bacteria, he was not able to culture them and so he did not consider these bacteria to be of any clinical importance [Adapted from Escherich, 1886].

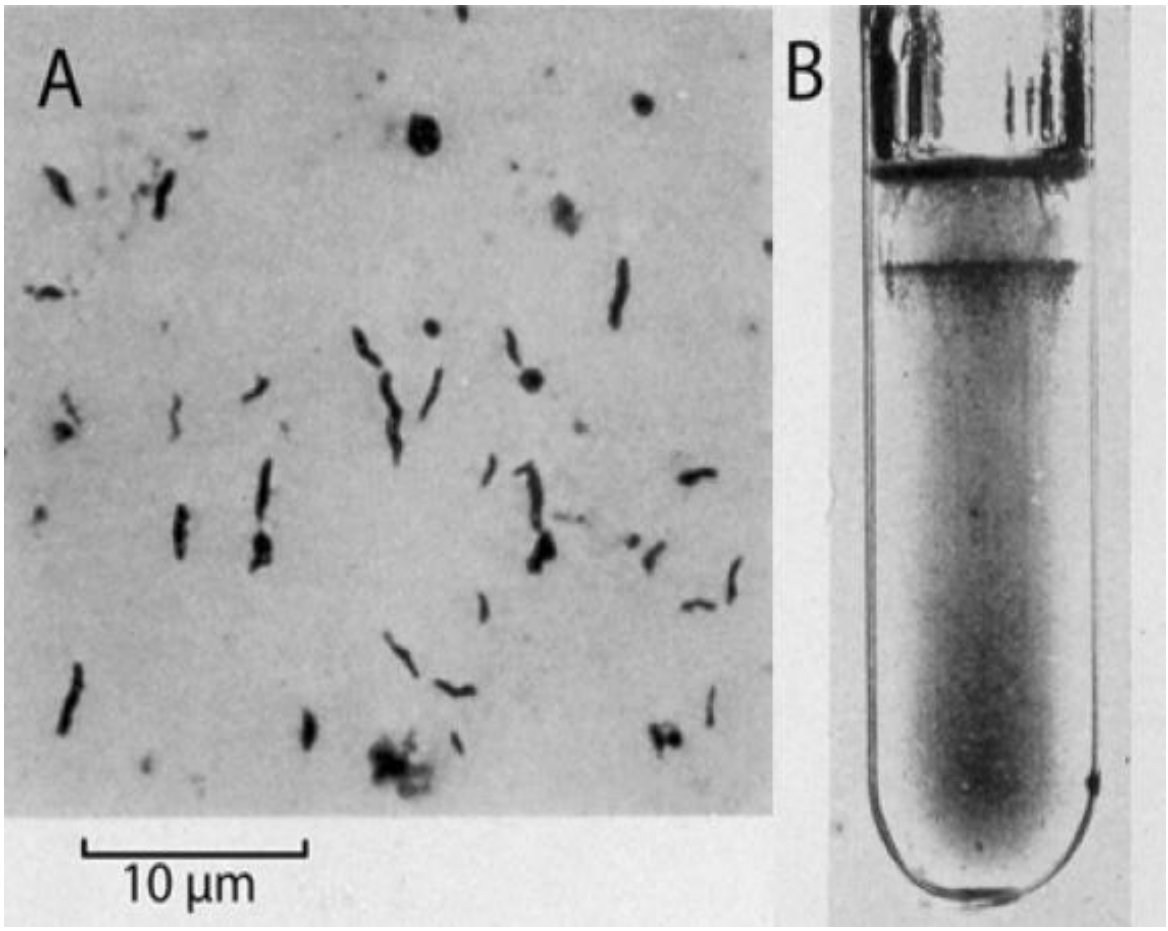


Fig 2.2: John McFadyean and Stewart Stockman's photographs of the *"Vibrio"* species in smears of uterine exudate from an infected ewe *(A)* and a culture of the *"Vibrio"* species in agar *(B),* which had been inoculated and mixed while liquid and then solidified [1]. The characteristic subsurface band of growth, where there is reduced oxygen tension, resembles that of *Brucella abortus.* [Adapted from Skirrow, 2006].

2.2 Family *Campylobacteraceae*

Members of this family occur primarily as commensals or parasites in humans, domestic animals and birds. This family includes three genera – *Campylobcater*, *Arcobacter* and *Sulfurospirillum* with the type genus as *Campylobacter*. This family also includes the generally misclassified species *Bacteroides ureolyticus*. These bacteria are also gram-negative bacteria with microaerobic requirements and low G + C content. The two genera, *Helicobacter* and *Wolinella* were removed from this family and accommodated in the newly created family *Helicobacteraceae* (Vandamme *et al.,* 2008).

2.3 Genus *Campylobacter*

Campylobacter cells are slender and are seen mostly as curved or S-shaped or as spiral rods. For some species the cells are seen as straight rods. But as the cultures grow older these cells acquire a spherical or coccoid shape. These bacterial cells generally are 0.5 – 5 um in length and have a width of 0.2 – 0.8 um. They are gram-negative, non-spore forming and have a single, polar and unsheathed flagellum at one or both ends. The presence of this flagellum gives these bacteria a darting type of motility called as the cork-screw motility which is characteristic of campylobacters. Campylobacters are not able to ferment or oxidize carbohydrates and they get their energy from either amino acids or tricarboxylic acid cycle intermediates. These bacteria prefer a growth temperature ranging from 30^{o}C to 37^{o}C with the thermophilic campylobacters like *C. jejuni* and *C. coli* preferring a growth temperature of 42^{o}C (Vandamme *et al.,* 2008). The genus at present comprises of 18 species of which only 12 species are found associated with diseases in

humans with *C.jejuni* and *C.coli* accounting for more than 95% of those infections (Vandamme *et al.,* 2008; Humphrey *et al.,* 2007).

2.4 *Campylobacter jejuni*

Campylobacter jejuni are naturally found as commensals in the intestinal tract of warm blooded birds and animals such as poultry, wild birds, cattle, sheep, pigs, dogs and cats (Altekruse *et al.,* 1994; Stern 1992). The natural and most common habitat for these organisms is the intestine of poultry, which might be due to the higher body temperature (Skirrow, 1977). *Campylobacter* rapidly colonizes in chicks usually by the age of 3 weeks and high levels of the organism are found in the ceca of these birds (Corry and Atabay, 2001). Even though the primary site of colonization is the intestinal crypts in the cecum, these bacteria are also found to colonize the large intestine and cloaca and microbial biosynthesis occurs at all these locations as they are rich in amino acids (Beery *et al.,* 1988). The organism has been found to colonize the ceca of poultry at very high concentrations ranging from 10^5 to $>10^9$ CFU/g (Jacobs-Reitsma, 2000). *C. jejuni* has also been isolated from pet animals like cats, dogs, monkeys, turtles and hamsters (Fox *et al.,* 1983; Harvey and Greenwood 1985; Fang *et al.,* 1991; Tresierra-Ayala and Fernandez 1997).

2.5 Campylobacteriosis:

2.5.1 Incidence:

Campylobacter jejuni is one of the most important foodborne bacteria and is considered as the leading cause of human bacterial gastroenteritis throughout the world.

An estimated 850,000 cases of human campylobacteriosis and over 8000 hospitalizations occur each year in the United States (Scallan *et al.,* 2011). The Foodborne Diseases Active Surveillance Network estimates approximately 13 cases of campylobacteriosis per 100,000 people even with many of the cases being undiagnosed or unreported (Centers for Disease Control, 2010). Although the campylobacters do not produce a high mortality rate, it is estimated that they can cause approximately 76 deaths per year. The high frequency of *C. jejuni* infections has led to significant economic losses with the annual cost associated with this disease estimated at 1.5 billion dollars in the United States **(**Scharff, 2012).

2.5.2 Disease:

Foodborne illness caused by *C. jejuni* is mostly associated with the consumption of food animals, especially poultry. Even low numbers of 500 organisms or fewer can cause gastrointestinal disease (Black *et al.,* 1988). These low infective doses are more commonly seen in milkborne and waterborne campylobacteriosis which might be due to buffering action or rapid passage through the stomach (Skirrow and Blaser, 2000). The incubation period of foodborne campylobacteriosis is usually 4-5 days but can range from 1 – 10 days (Humphrey *et al.,* 2007). The symptoms associated with this disease include fever, diarrhea, headache, abdominal pain, myalgia, vomiting and blood in feces (Poly *et al.*, 2005). In majority of *C. jejuni* illnesses, the use of antimicrobials is not necessary as the infections are usually self-limiting within 5 – 8 days (Black *et al.,* 1988). In some cases, however, the infection does not subside within 3 – 4 days and antibiotic therapy may be indicated. Other possible consequences of *C. jejuni* infections include Guillain Barré Syndrome (GBS), reactive arthritis, and irritable bowel syndrome (Blaser and Engberg,

2008). It has been estimated that 1 in 1000 *C. jejuni* infections can lead to GBS (Nachamkin, 2002). GBS is a form of ascending paralysis affecting peripheral and cranial nerves especially the facial nerve (Zilbauer *et al.,* 2008). Studies showed the presence of cross-reactive determinants in glycoproteins from human peripheral nerves and a frequently isolated serotype of *C. jejuni* (serotype O: 19), which suggests the existence of a molecular mimicry between the two types of glycoproteins resulting in the development of GBS in patients with history of previous *C. jejuni* infection (Brezovska *et al.,* 2011).

2.5.3 Sources of Infection:

A majority of human cases of foodborne illness due to *Campylobacter spp* is attributed to the consumption of poultry and poultry products as these are major sources of the organism (Nachamkin, 2001; Tauxe, 1992; Adak *et al.,* 1995). Studies have shown that the colonization of these bacteria in poultry is difficult to prevent even with proper preventive measures in place. During processing the poultry carcasses may become contaminated with these bacteria due to rupture of intestinal tract and they get attached to the poultry skin (Davis and Conner, 2007). Even though the various decontamination techniques used during the slaughtering of birds has been found to reduce the *Campylobacter* levels, fresh poultry has been found to harbor *C. jejuni* at levels ranging from 10^2 to 10^5 per carcass (Jacobs-Reitsma, 2000). Campylobacteriosis is mainly seen as sporadic cases with few isolated outbreaks. Large outbreaks of this disease have been usually reported with consumption of water or milk (Gubbels *et al.,* 2012; Biswas *et al.,* 2007; CDC, 2002). Contamination of water supply can occur by excretion of animals and birds. *C. jejuni* has also been found to associate with protozoans such as ciliate protozoa

like *Tetrahymena* and freshwater amoebae (Snelling et al., 2005). Surveys of retail vegetables and mushrooms have also reported the presence of *C. jejuni* on these products (Doyle and Schoeni, 1986; Federighi *et al.*, 1999; Chai *et al.*, 2007).

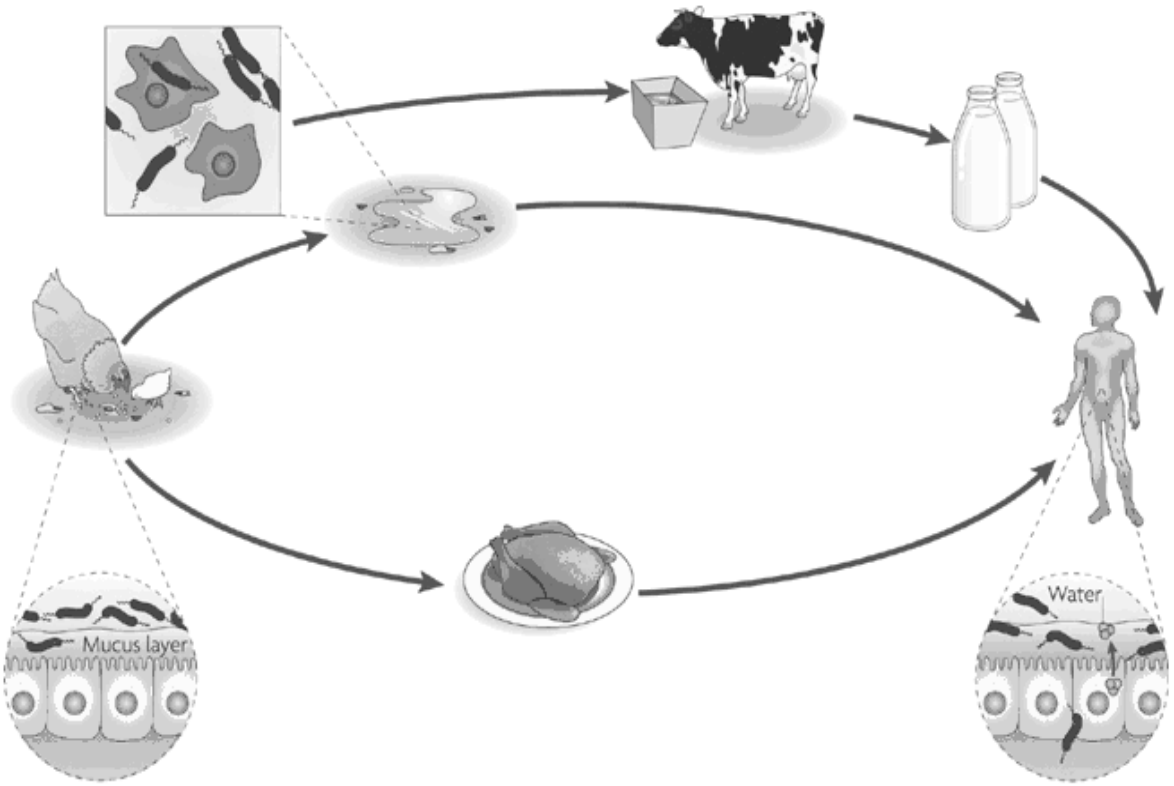


Figure 2.3: The sources and outcomes of *Campylobacter jejuni* infection (Adapted from Young *et al.*, 2007).

2.6 Existing Approaches in Eliminating *Campylobacter jejuni* from Meat Products

2.6.1 Scalding:

This process helps in breaking down the proteins that hold feathers in place and opens the feather follicles which results in easy removal of feathers. Chicken skin harbors *C. jejuni* in large numbers and has been found to support the survival of the organism (Lee *et al.,* 1998). Studies have shown that the *Campylobacter* numbers decrease significantly in the carcass rinses and skin samples after scalding, but are not eliminated completely (Keener *et al.,* 2004). Scalding serves as an intervention method only when the pH in the scald water is maintained towards an acidic pH (3-4) which helps in lowering microorganisms. *Campylobacter* has been shown to be resistant toward heat at a neutral pH of 7.0 (Humphrey & Lanning, 1987). Studies by Berrang *et al.*, 2011 shows that to reduce the numbers and prevalence of Campylobacter on broiler carcasses scalding treatment with a high pH of 9.89 is more efficient than the control treatment of scalding with a pH of 6.88. Very high temperature in the scalding tank causes the carcasses to become oily resulting in easier attachment of bacterial pathogens.

2.6.2 Carcass washers:

The National Chicken Council (NCC) recommends whole carcass rinses with water containing 20 ppm available chlorine. Carcass rinses help in removing any loose material sticking to the surface of carcasses after evisceration and also helps in decreasing the microbial load on the surface (Waldroup *et al.,* 1992). Another study has shown that the currently used inside – outside washers in poultry processing have limited effectiveness in reducing the *Campylobacter* counts on carcasses as the study found only 0.5 log reductions

in *C. jejuni* counts using the carcass washers without any processing aids (Bashor *et al.*, 2004).

2.6.3 Chilling:

Poultry carcasses need to be rapidly cooled to prevent bacterial growth. Two types of chillers are commonly used in poultry processing – immersion chilling and air chilling. Water chillers are most commonly used in many of the processing plants. Studies show water chillers as a source of potential cross-contamination for *Campylobacter* (Keener *et al.*, 2004). Chlorination of chiller water with 20-50 ppm chlorine and an optimum pH of 6.0 are two important factors that help in the control of bacterial pathogens in the water chillers. Freezing has been found to reduce *C. jejuni* counts on poultry, but this reduction was found to depend on other factors such as temperature, storage time and freezing conditions (Zhao *et al.*, 2003).

2.6.4 Processing aids:

2.6.4.1 Water:

Washing carcasses with hot water helps in reducing bacterial load on the surface. A hot water spray at 60^{o}C was found to reduce *Campylobacter* levels as compared to 20^{o}C water sprays (Li *et al.*, 2002). Another study with water spray at 70^{o}C for 40 s was found to cause a 1.6 log 10 reductions in *Campylobacter* without any harmful effects on the chicken skin (Purnell *et al.*, 2004). Currently many poultry processing plants use warm water rinses to wash the carcasses and it is hypothesized that the water rinses reduce the

surface tension of water which helps in increased removal of bacteria and fecal matter (Keener *et al.,* 2004).

2.6.4.2 Organic acids:

Many organic acids such as lactic, acetic, citric, malic and succinic have been shown to be effective as antimicrobials in poultry processing (Keener *et al.,* 2004). Lactic acid is present naturally in meat at low concentrations which is partly responsible for the flavor of meat and also increases shelf life by its antimicrobial effects. It has been also observed that the use of lactic acid at 1 – 2% concentrations did not affect the color and flavor of meat and helped to reduce the bacterial load on poultry carcasses immediately after slaughter and during storage (Bolder, 1997). Treatment of breast skin from chilled broiler carcasses with organic acids such as acetic, citric, lactic, malic, mandelic and tartaric acid was found to cause yellow discoloration of the skin (Bilgili *et al.,* 1998). Studies report varying results as to the use of organic acids as antimicrobial agents. Treatment of chicken wings with acetic acid (0.5, 1.0, 1.5, and 2.0%) was found to reduce *C. jejuni* counts significantly in comparison to a 1% lactic acid treatment (Zhao and Doyle, 2006). Lactic acid (2.5%) did not produce a significant decrease in *C. jejuni* on chicken skin and meat samples as compared to treatment with water alone (Riedel *et al.,* 2009). Applying a 5 % lactic acid solution on to chicken skin surface for 1 minute did not produce any significant reduction in *C. jejuni* counts, but researchers found that the treatment prevented an increase in counts even after storage for 7 days (Lecompte *et al.,* 2009). A recent study showed that dipping of leg and breast meat samples in lactic acid (3%) and

acetic acid (2%) produced a significant decrease in *C. jejuni* counts by 0.36 -1.98 log cfu/cm^2 as compared to tap water dipped controls (Coşansu & Ayhan, 2010).

2.6.4.3 Chlorine and chlorine compounds:

Poultry processing plants use chlorine to reduce spoilage bacteria as well as foodborne bacterial pathogens and to prevent accumulation of microorganisms on working surfaces and equipment (Bailey *et al.,* 1986). Chlorine is added to the processing water either in the form of chlorine gas directly or in the form of sodium hypochlorite, which forms hypochlorous acid in water (Keener *et al.,* 2004). The USDA, Food Safety and Inspection Service (FSIS) permits the use of chlorine in poultry processing at levels up to 50 ppm for carcass washing and for use in chiller water (Russell, 2007). The addition of 25 ppm chlorine to water in the final carcass washing equipment was found to reduce *C. jejuni* counts significantly (Whyte *et al.,* 2001). But chlorination of chilled water was not found to effectively reduce *C. jejuni* attached on to chicken skins (Yang *et al.,* 2001). They hypothesized that chlorine was unable to reduce *C. jejuni* as the oil on the skin surface prevented chlorine from coming in contact with the surface. Spray washing of poultry carcasses with chlorinated water (50 ppm) at different temperatures (21.1^{o}C, 43.3^{o}C, 54.4^{o}C) was found to produce no effect on the numbers of *Campylobacter* recovered from the whole carcass rinses after treatment (Northcutt *et al.,* 2005).

2.6.4.4 Trisodium phosphate:

Use of trisodium phosphate (TSP) in decontamination of poultry carcasses started in the year of 1992 with the approval of the U. S. Department of Agriculture. TSP is

mainly used as a dip after water chilling or before air chilling. The bactericidal property of TSP is due to its high pH which causes lysis of bacterial cells (Dincer and Baysal, 2004). Dipping of poultry carcasses in a 10% TSP solution at 50^{o}C and consequent storage for a period of 1-6 days before rinsing was found to significantly decrease *Campylobacter* counts (Slavik *et al.*, 1994). Increasing the treatment times with TSP to 5 min and 15 min was found to cause additional decrease in *C. jejuni* counts by approximately 1 log as compared to treatments for 1 min or 15 sec (Riedel *et al.*, 2009).

2.6.4.5 Acidified sodium chlorite (ASC):

High pressure spraying of poultry carcasses with ASC at concentrations of 100 ppm for a period of 15 minutes during pre-chill and post-chill operations was found to decrease *C. jejuni* counts by 1 log compared to controls (Chantarapanont *et al.*, 2004). Another study of post-chill application of ASC (600 – 800 ppm at pH 2.5 – 2.7) for only a period of 15 sec was found to significantly reduce *Campylobacter spp* in commercial broiler operations (Oyarzabal *et al.*, 2004). Addition of ASC in drinking water at a level of 600 ppm prior to slaughter of broilers for 24 h or 5 days was found to reduce *Salmonella* in the crop. However this treatment did not have any effects in reducing *C. jejuni* (Mohyla *et al.*, 2007). Dipping of poultry carcasses in a solution of ASC for 20 s immediately after chilling was found to reduce the prevalence of pathogens such as *Escherichia coli*, *Salmonella* spp and *Campylobacter* spp (Sexton *et al.*, 2007).

2.6.4.7 Cetylpyridinium chloride (CPC)

Cetylpyridinium chloride (1-hexa-decyl pyridinium chloride) is a quaternary ammonium compound which has antimicrobial properties. This compound is stable, non-volatile and soluble in water with a neutral pH and is widely used in mouthwashes due to its bactericidal properties (Breen *et al.,* 1995). Quaternary ammonium compounds are microbial agents acting on the bacterial cell membrane by lowering surface tension of the cells and thus resulting in a loss of selective permeability of the bacterial cell membrane (McDonnell and Russell, 1999). CPC has been approved by the U. S. Food and Drug Administration to treat surface of poultry carcasses or giblets before or after chilling (USDA, FSIS, 2012). CPC is commonly used as a spray or rinse or a solution for dipping. CPC has been found to be effective against pathogens such as *Escherichia coli* O157:H7, different species of *Salmonella* and *Listeria* monocytogenes in a wide variety of foods such as poultry (Breen *et al.,* 1995; Kim *et al.,* 1996; Breen *et al.,* 1997; Wang *et al.,* 1997, Xiong *et al.,* 1998), beef (Cutter *et al.,* 2000; Singh *et al.,* 2005) and fresh-cut vegetables (Wang *et al.,* 2001). Studies by Arritt *et al.,* 2002 found that 0.5% CPC is an effective antimicrobial agent for inactivating, reversing the attachment as well as inhibiting attachment of *Campylobacter jejuni* to chicken skin. A one minute dip in 0.5 % CPC was found to cause more than 4 logs reduction of *C. jejuni* populations on chicken skin and meat samples (Riedel *et al.,* 2009). However CPC was found to be less effective when the treated samples were stored at 5^{o}C for 24 h. In-line spraying of poultry carcasses with a 0.5 -0.7 % CPC was found to reduce the incidence of *Salmonella* and *Campylobacter* on broiler carcasses (Zhang *et al.,* 2011). Addition of CPC (5.5%) in brines was found to produce below detection level reductions in *E.coli* at 4^{o}C and 15^{o}C (Adler *et al.,* 2011).

2.6.4.6 Irradiation:

Different types of radiation such as gamma, X-rays and UV rays are being used in food processing to reduce/eliminate foodborne bacterial pathogens. *Campylobacter*s are very sensitive to gamma irradiation having D-values between 0.12 – 0.25 kilograys (Humphrey *et al.*, 2007). UV radiations at a wavelength of 254 nm and dose range of 9.4 – 32.9 mV/sec/cm^2 were found to produce little significant reductions (0.4 – 0.7 log) in *C. jejuni* counts on boiler carcasses (Isohanni and Lyhs, 2009). They hypothesized that UV irradiation alone or in combination with activated oxygen cannot be used as the sole method for decontaminating poultry carcasses and that for the effective removal of *C. jejuni* from carcasses; this method has to be combined with other decontamination techniques.

2.7 Survival Mechanisms of *C. jejuni* to Environmental Stresses

C. jejuni are extremely fragile foodborne bacteria requiring fastidious growth conditions such as a microerophilic atmosphere, a neutral pH and a temperature of 37˚C to 42˚C for growth in the laboratory. During its transmission from the natural hosts (poultry) to humans, during poultry processing, and during storage at retail markets, *C. jejuni* have to survive a wide range of environmental stresses such as nutrient limitation, high or low temperatures, low pH and exposure to atmospheric oxygen. These bacteria are very sensitive to stresses used in food and poultry processing such as a low pH below 5, temperatures below 30^{o}C, a salt level of more than 2.5%, high oxygen levels, high temperature and drying (Ray and Bhunia, 2008; Solomon and Hoover, 1999). However, studies show that at its optimum growth temperature, *C. jejuni* is able to grow at pH

ranging from 5.5 to 8.0 (Kelana and Griffiths, 2003). Acid is a key stress encountered while passing through the human gastrointestinal tract. *C. jejuni* encounters both organic and inorganic acids not only in the human gastrointestinal tract, but also in the chicken gut as well as in the processing plant, from foods and sanitizers. *Campylobacters* are found to survive the high temperature in the scalding tank as well as the use of common decontaminants (such as chlorine, acids, trisodium phosphate and acidified sodium chlorite) and still persist on the skin of poultry when it reaches the consumers (Keener *et al* ., 2004).

Campylobacter jejuni are generally slow to grow and do not grow well in many foods (Ray and Bhunia, 2008). They also do not compete well when growing with other bacteria. These bacteria are also susceptible to many of the environmental conditions and are less tolerant to environmental stresses as compared to other foodborne pathogens (Park, 2002). *Campylobacter* lacks the usual stress response factors necessary for survival in adverse environmental conditions. These bacteria do not possess the global stationary phase response factor (RpoS), the oxidative stress response factors (SoxRS and OxyR), the cold shock proteins (CspA) and the global heat shock response regulator (RpoH) which are normally present in other gram negative bacteria. They are found to possess only three sigma factors which are RpoD, FliA and RpoN (Parkhill *et al.*, 2000). How they still are capable of persisting and surviving in the environment remains an enigma. Many methods have been proposed as helping *C. jejuni* to survive the environmental stresses such as a viable but non-culturable state (VBNC), conversion from a rod to a coccoid shape, a high

degree of genetic heterogeneity, formation of biofilms and an adaptive tolerance response (ATR).

2.7.1 Viable but non-culturable state:

It has been shown that *C. jejuni* has the ability to convert into a viable non-culturable state (VBNC) when exposed to adverse environmental stress conditions, which also helps in the survival of the organism (Rollins and Colwell 1986; Korhonen and Martikainen 1991). The VBNC state is defined as a state of dormancy during which the bacteria will remain viable with reduced metabolic activity, but will not grow on any growth media normally used to grow the bacteria (Barer and Harwood, 1999; Moore, 2001; Oliver, 2005). Viability assays which measure metabolic activity or cellular integrity are used to differentiate whether an organism is viable or dead (Oliver, 2005). These assays determine the capacity of the viable bacterial cell to reduce tetrazolium salts to formazans. The viable count is then measured as a fraction of the total bacterial count which can be determined by staining with DAPI (4',6-diamidino-2-phenylindole) or acridine orange (Kogure *et al.,* 1979; Boucher *et al.,* 1994). A recent study reports the detection of VBNC bacteria on chicken carcasses using a combination of real-time quantitative PCR (Q-PCR) and propidium monoazide (PMA) treatment of samples. This method was able to detect the VBNC bacteria with an intact membrane within a short time of less than 3h and did not detect any DNA from dead bacteria (Josefsen *et al.,* 2010).

Many studies have examined the ability of the VBNC state to return to a normal metabolically active state. Upon entry into the VBNC state *C. jejuni* has been found to

lose motility as well as assume a coccoid shape from the regular rod shape (Boucher *et al.,* 1994; Medema *et al.,* 1992). The ability of the VBNC state of *C. jejuni* to colonize is controversial. Studies by Pearson *et al.,* in 1993 claimed that VBNC state was able to colonize chickens while studies by Ziprin and Harvey in 2004 and Ziprin *et al.,* in 2003 showed that VBNC forms were unable to colonize in chickens. Even though the role of VBNC state in production of the disease is controversial, studies report that the bacteria retain its virulence in this state (Skovgaard, 2007). Studies by Baffone *et al.,* 2006 shows that the VBNC cells of *C. jejuni* were able to regain their culturability after an in vivo passage through mice and that it was dependent on the number of respiring bacteria in the VBNC state. A recent study shows that *C. jejuni* was able to enter a VBNC state upon prolonged incubation at 4^{o}C and even though the transcription level of virulence genes was lowered in this state, the bacteria was able to retain its ability to invade Caco-2 human intestinal epithelial cells (Chaisowwong *et al.,* 2012).

2.7.2 Transition from a rod to a coccoid shape:

Transition of the organism from a rod to coccoid shape also has been reported in helping the organism survive hostile environments (Moran and Upton, 1987). A decrease in culturability of the bacterial cells has been reported along with this transition to the coccoid shape (Ng *et al.,* 1985; Chou *et al.,* 1983). It has also been reported that the transition from rod to a coccoid shape does not involve gene transcription and protein synthesis and so has been described as a passive process (Boucher *et al.,* 1994; Hazeleger *et al.,* 1995; Hudock *et al.,* 2005; Thomas *et al.,* 1999). Studies also report that the coccoid

form of *C. jejuni* is less virulent than the spiral form based on cell-culture assays (Nannapaneni *et al.,* 2001).

2.7.3 Genetic heterogeneity:

Genotypic plasticity in *C. jejuni* has also been suggested as another mechanism helping the organism in surviving adverse conditions (Murphy *et al.,* 2006). The molecular mechanisms controlling this genomic diversity in *C. jejuni* have not been fully understood. According to Arber, 2000 genetic diversity is caused by any of the following 3 mechanisms: (1) local sequence change in DNA brought about by substitutions, insertions or deletions of nucleotides (2) rearrangement of DNA sequences by recombination of DNA segments and (3) horizontal gene transfer by means of transformation, conjugation or transduction. Studies report genomic rearrangements as well as intra and interspecies exchange of genetic material occur very frequently in *C. jejuni* (Dingle *et al.,* 2001; de Boer *et al.,* 2002; Ridley *et al.,* 2008; Wilson *et al.,* 2009; Biggs *et al.,* 2011). Parkhill *et al.,* in 2000 identified several hyper-variable regions in *C. jejuni* genome which are also thought to be a reason for genetic diversity. These hyper-variable regions contain short runs of the same nucleotide (homo-polymeric tracts). This kind of repetition leads to slip stranded mutations within the homo-polymeric tracts resulting in changing the expression of many genes. Majority of these genes are primarily involved in the expression of surface structures such as flagella, capsule and lipo-oligosaccharide (Hendrixson, 2006; Karlyshev *et al.,* 2002; Park *et al.,* 2000; Parkhill *et al.,* 2000).

2.7.4 Formation of Biofilms:

Biofilms have been proposed as helping *C. jejuni* to survive stresses in the environment (Joshua *et al.,* 2006, Gunther IV and Chen, 2009). Pre-established biofilms also have been shown to help *C. jejuni* in attaching to surfaces (Hanning *et al.,* 2008). *Enterococcus faecalis* and *Staphylococcus simulans* are commonly found in the poultry processing environment. These two organisms were found to enhance biofilm formation with *C. jejuni* under the microaerobic conditions though *C. jejuni* is not a strong biofilm maker. This ability of *C. jejuni* to form biofilms with *E. faecalis* and *S. simulans* may be due to quorum sensing or antimicrobial activities or interspecies competition within the biofilm community. However, *Pseudomonas aeruginosa*, which is usually a strong biofilm maker, was not recovered from this mixed microbial population under the conditions used in the study (Teh *et al.,* 2010). Recently, it has been shown that *C. jejuni* is able to exist as metabolic commensals along with *Pseudomonas* species and this commensalism helps *C. jejuni* to survive in conditions with high oxygen concentrations (Hilbert *et al* ., 2010). Aerobic conditions have shown to increase the biofilm formation in *C. jejuni* helping the bacteria to survive in stress conditions and such biofilms have been shown to function as a reservoir of viable planktonic cells (Reuter *et al.,* 2010). Recent studies also have shown that *C. jejuni* can form microcolonies and biofilms on human intestinal tissue which may be important in the pathogenesis of the disease (Haddock *et al.,* 2010).

2.7.5 Adaptive tolerance response (ATR):

Exposure to sublethal stresses has been found to induce an adaptive tolerance response (ATR) in many bacterial species and this kind of a response provides protection to the bacteria when they are exposed to subsequent homologous or heterologous stresses (Yousef and Courtney, 2003). An adaptive tolerance response has been shown to be induced in *C. jejuni* CI 120, a chicken isolate, when acid and/or aerobic-adapted cells were exposed to further acid stress conditions (Murphy *et al.,* 2003a). *C. jejuni* was found to secrete a heat stable extracellular protein during growth which was found to provide protection against acid and heat stresses (Murphy *et al.,* 2003b). Studies by Ma *et al.,* in 2009 show that exposure to stresses such as acid, aerobic atmosphere and starvation produced an adaptive tolerance response in *C. jejuni* isolates when they were further exposed to secondary acid or starvation stresses. Even though *C. jejuni* is a micro-aerobic bacteria sensitive to exposure to high levels of oxygen, it was found that this organism was more resistant to oxidative stress at 4^{o}C than at its normal growth temperature of 42^{o}C. This might be due to a cross-protection offered by the stress proteins produced by the organism in response to exposure to cold environment (Garénaux *et al.,* 2008). Increased expression of proteins involved in stress responses, especially the oxidative proteins, was observed when the organism was recovered after exposure to high pressure treatment (Bieche *et al.,* 2010). Thus, adaptive tolerance response has been shown as one of the survival mechanisms of *C. jejuni* to survive environmental stresses.

2.8 Stress Responses of *C. jejuni* to Environmental Stresses

2.8.1 Cold stress:

C. jejuni have been found to survive well under refrigeration and freezing temperatures (Chan *et al.,* 2001; Lazaro *et al.,* 1999). Even though thermophilic *Campylobacter*s are not able to multiply at temperatures of 30^{o}C and below, metabolic activities such as oxygen consumption, ATP generation and protein synthesis were found to function at temperatures as low as 4^{o}C (Park, 2002). The inability of these organisms to multiply below 30^{o}C may partly be explained by the fact that they do not produce cold shock proteins (Parkhill *et al.,* 2000). It was also found that the cells had high catalase activity at 4^{o}C which is able to catalyze hydrogen peroxide formed as a result of respiration to water and oxygen. Accumulation of hydrogen peroxide is toxic to the cells as it oxidizes the SH groups (Hazeleger *et al.,* 1998). Studies have shown that freezing helps in destroying only a part of the *C. jejuni* population present on chicken meat and the rest of the population usually survives with sub-lethal injuries or without injuries (Sampers *et al.,* 2010). *C. jejuni* in chicken meat preparations was found to survive refrigeration temperature (4^{o}C), freezing temperature (22^{o}C) and a salt level of 1.5 % (Sampers *et al.,* 2010). Exposure of *C. jejuni* to cold shock results in the overproduction of the stress-related proteins such as SodB (Stinzi and Whitworth, 2003). The *sodB* gene was also found to provide protection when the organism undergoes freeze thaw treatments (Garénaux *et al.,* 2009). A recent study shows the role of polynucleotide phosphorylase (PNPase) in helping the low temperature survival of *C. jejuni*, as researchers found that a mutation in the *pnp* gene lowered the survival of the organism by 2 to 4 logs (Haddad *et al.,* 2009). Another study reveals that *luxS* gene expression increases in *C. jejuni* when the

organism survives in chicken meat juice at low temperatures (5^{o}C). Quorum sensing controlled *luxS* gene up-regulates genes involved in O-linked flagellin glycosylation and down regulates the expression of haemin uptake and peroxide stress response genes. Thus, *C. jejuni* is able to show a limited transcriptional response in chicken meat juice that helps its prolonged survival at low temperatures (Ligowska *et al.,* 2010).

2.8.2 Heat stress:

At a high temperature of 55^{o}C, *C. jejuni* were found to survive for only about 1 minute because these organisms lack the heat shock response factors such as $\boldsymbol{\sigma^{32}}$ or $\boldsymbol{\sigma^{B}}$ present in other gram negative bacteria (Park, 2002). Other alternative mechanisms proposed for the heat shock response in *C. jejuni* are the RacRS regulon, a two-component regulatory system, responsive to temperature and colonization, and other orthologues of HrcA and HspR (Park, 2002). Recent studies show that racR and racS mutants had defective growth, were unable to survive at 42^{o}C, had reduced motility and were not able to colonize in chicks. These findings reveal the importance of the RacRS regulatory system in surviving stresses associated with heat shock response (Apel, 2012). Exposure to sublethal levels of TSP was found to increase the heat resistance in *C. jejuni* (Riedel *et al.*, 2012). When *C. jejuni* cells were exposed to a temperature of 48^{o}C, the viability of cells pretreated with TSP was found to decrease suddenly compared to the unstressed cells. They also found that the cation / proton antiporters (NhaA1/NhaA2) as well as the multidrug efflux pumps of the RND (resistance-nodulation-cell division) superfamily help in inducing tolerance to sublethal levels of TSP. Also, *C. jejuni* was shown to have only a

limited transcriptional response that mainly is associated with transport processes on exposure to sublethal TSP levels.

2.8.3 Oxidative stress:

The oxidative stress regulators such as SoxRS, OxyR and SlyA which are present in *Salmonella* and *E.coli* are not found in *C. jejuni*. The enzyme alkyl hydroperoxide-reductase (AhpC) has been found to confer aerotolerance to *C. jejuni* and, thus, helps the organism survive in oxygen rich environments (Baillon *et al.*, 1999). Recently, two other enzymes, the thiol peroxidase (Tpx) and the bacterioferrin comigratory protein (Bcp) have been found to provide protection to *C. jejuni* against oxidative stress caused by excess oxygen and exogenous peroxides. The enzymes Tpx and Bcp are found to be localized in the cytoplasm and are found to provide protection to *C. jejuni* especially against molecular oxygen during the exponential growth phase (Atack *et al.*, 2008). The global post-transcriptional regulator, *csrA* was found to be playing a vital role in the regulation of genes required for the survival of *C. jejuni* when exposed to high oxygen atmospheres. A *csrA* mutant strain was found to be very sensitive when exposed to atmospheric oxygen with more than 99 % loss in viability when compared to the controls grown under microaerobic conditions. The mutant strain was also found to be a poor biofilm former and had reduced motility and adhesion to intestinal epithelial cells *in vitro*. This study thus shows that the *csrA* gene may be having a regulatory role in the pathogenesis of *C. jejuni* (Fields and Thompson, 2008). A putative transcriptional regulator of *C. jejuni* known as Cj1556 was also found to be involved in oxidative and aerobic stress responses.

2.8.4 Acid stress:

C. jejuni requires an optimum pH of 6.5 – 7.5 for its growth in the laboratory and are very sensitive to a pH below 4.9 (Solomon and Hoover, 1999). But these bacteria have been shown to grow well within the pH range of 5.5 – 8.0 (Kelana and Griffiths, 2003). These bacteria often have to encounter acid stress in the food and poultry processing environments as well as in the gastrointestinal tracts of hosts. It was shown that *C. jejuni* had the ability to induce an adaptive tolerance response upon exposure to acid and/or aerobic conditions when they were initially adapted to a mild stress such as an acid pH of 5.5 (Murphy *et al.,* 2003a). A heat stable component suggested as a protein produced by *C. jejuni* during the growth phase was found to play a key role in the induction of ATR (Murphy *et al.*, 2003b). Research shows that *C. jejuni* has the capacity to sense and respond to acid stress very well. Studies by Le and coworkers (2012) showed that exposure to *C. jejuni* to an acid pH of 5.0 was found to increase its invasion of mouse intestinal crypt cells. They found that on exposure to acid, *C. jejuni* was down-regulating genes involved in cell division and replication and up-regulating flagellar and stress response genes.

2.9 Virulence Factors of *C. jejuni*

Many virulence factors have been proposed in the survival of *C. jejuni* and establishment of the disease in humans. The organism may be causing the disease by any of the three following methods: 1) production of toxins by the ingested and colonized bacteria; 2) invasion of the intestinal epithelial cells and subsequent damage to the cells, or 3) migration to extraintestinal sites through the lymphatic vessels (Zheng *et al.*, 2006).

Depending on the status of the host and the strain involved in infection, one or more of the above described methods may be used. Even though the organism has many virulence factors, the four factors that have been shown to be involved in causing symptoms of campylobacteriosis are motility, adhesion, invasion, and toxin production.

2.9.1 Motility and chemotaxis:

Motility and chemotaxis are important for the organism in the initial colonization of intestinal cells (Bhavsar and Kapadnis 2007). *C. jejuni* exhibit the typical cork-screw type motility with the help of a single polar unsheathed flagellum at one or both ends. The flagellar regulatory system in *C. jejuni* co-ordinates the production of colonization and virulence determinants, which together with the flagella are required for the bacteria's interactions with diverse hosts. Two structural genes namely *flaA* and *flaB* are involved in the expression of the flagellum. The expression of flagellar genes in *C. jejuni* is controlled by the two alternative sigma factors which in turn are controlled by the flagellar type III secretion system. σ^{28} is required for the expression of the major flagellar gene *flaA* and filament genes whereas σ^{54} is required for the expression of flagellar rod and hook genes (Lertsethtakarn *et al.,* 2011). Studies show one of the invasion genes CiaI and four other genes called as **feds** (flagellar coexpressed determinants) to be involved in colonization. These genes were found to be dependent upon the sigma factor σ^{28} and were seen co-expressed with flagella even though they are not required for motility (Barrero-Tobon and Hendrixson, 2012). The flagellum aids *C. jejuni* in penetrating the intestinal mucus layer and binding to the epithelial cells (Szymanski *et al.,* 1995). A mutation in *flaA* gene was shown to inhibit colonization in chicks and invasion into human epithelial cells (Fields and

Swederlow 1999). *C. jejuni* show a strong positive chemotaxis toward mucins (Hugdahl *et al.*, 1988).

2.9.2 Adhesion and invasion:

Interaction of *Campylobacter jejuni* and the host cells is a complex process involving cell surface structures of the bacteria and the host cell receptors (Rubinchik *et al.*, 2012). Adhesion and invasion are thought to be two important factors involved in the process of disease production by *C. jejuni*. Once the organism is ingested orally through any of the common food vehicles, the organism attaches or adheres to the intestinal epithelial cells, which is considered as the first step in the pathogenesis of the organism. *C. jejuni* requires adhesins such as CadF (*Campylobacter* adhesion to fibronectin) and PEB1 (major cell binding factor of *C.jejuni*) for adhering to the intestinal epithelial cells. CadF is a 37 kDa outer-membrane protein of *C. jejuni* which binds with the host fibronectin, a component of the extracellular matrix, which results in adhesion of the organism to the cells (Monteville *et al.*, 2003). Once the organism is able to attach to the intestinal epithelial cells, next step is the invasion of these cells. Adhesion to cells by means of binding with fibronectin initiates a cascade of host cell signaling events and synthesis of many new proteins by the organism which leads to internalization of the organism (Nachamkin 2001). During the process of invasion *C. jejuni* synthesizes and secretes a set of proteins called as the *Campylobacter* invasion antigens (Cia) that are required for the maximal inavasion of host cells (Malik-Kale *et al.*, 2008). It has been found that *C. jejuni* is capable of secreting these invasion antigens only when they have a fully functional flagellum (Konkel *et al.*, 2004). Scientists have been able to identify only

one of these invasion antigens named CiaB. The bile acid deoxycholate has been found to increase the pathogenicity of *C. jejuni* by stimulating the synthesis of Cia proteins (Malik-Kale *et al.,* 2008). Another study reveals that a response regulator CbrR modulates the resistance of *C. jejuni* towards sodium deoxycholate present in bile and that this response regulator contributes to colonization of chickens (Raphael *et al.,* 2005). Studies by Novik *et al.,* 2010 have identified three genes *aspA, aspB* and *sodB* by transposon mutagenesis screening to be involved in *C. jejuni* entry, invasion and survival within the host cells. Similarly another gene cj1136 which encodes for a galactosyltransferase involved in the synthesis of LOS (lipooligosaccharide) in *C. jejuni* has been shown to be important in the invasion of human intestinal cells in vitro and for in vivo colonization of chicken gut (Javed *et al.,* 2012).

Upon infection to human intestinal epithelial cells certain virulence genes of *C. jejuni* are up-regulated. One among those genes is the *spoT* gene which was isolated for the first time by Gaynor *et al.,* in 2005. This was shown to produce a stringent response towards specific stresses (low CO_2, high O_2 concentrations), transmission and antibiotic resistance (rifampicin). Studies by Gilbert and Slavik, 2005 showed that there were differences in adhesion and invasion ability for the different human and poultry isolates *in vitro* using the intestinal epithelial cells (INT 407). *In vitro* invasion of *C. jejuni* in various intestinal cell lines has been found to be very low compared to the starting level of inoculum, which does not correlate with the level of *C. jejuni* invasion found in clinical cases in humans (Friis *et al.,* 2005). This may be due to the presence of high levels of atmospheric oxygen in comparison to the low-oxygen environment in the intestinal lumen.

Research by Mills *et al.,* 2012 found that the use of a vertical diffusion chamber (VDC) increased the invasion of *C. jejuni* by 80 % as the VDC was able to provide a microaerobic atmosphere.

2.9.3 Toxins:

Six different cytotoxins have been proposed to be produced by *Campylobacter*s, of which the cytolethal distending toxin (CDT) is the best characterized toxin of *C. jejuni*. The production of CDT by *C. jejuni* was first reported by Johnson and Lior in 1988. They observed that a toxin present in the cell culture supernatants was capable of distending HeLa cells, Chinese hamster ovary cells, HEp-2 cells and Vero cells, ultimately resulting in death. The CDT causes arrest of eukaryotic cells in the G_2 phase of the cell cycle by preventing the activation of CDC2/ cyclin B1 (Whitehouse *et al.,* 1998). CDC2/cyclin B1 has to be dephosphorylated at the specific tyrosine residue, Tyr 15 for the cells to enter mitosis. The structural genes encoding this toxin, *cdtA*, *cdtB* and *cdtC* were isolated and characterized by Pickett *et al.,* in 1996. These three genes are highly conserved and any mutations in these genes results in CDT-negative strains, which were also found to cause the disease (AbuOun *et al.,* 2005). The A and C subunits are required for binding to the cells while the B subunit has the toxic activity (Lara-Tejero and Galan 2001). Even though the cdt genes are present in majority of the *C. jejuni* strains, the role of CDT in the pathogenesis of the organism is not clear (Eyigor *et al.,* 1999).

2.10 Stress-induced Virulence Gene Expression

It has been shown that the virulence of pathogenic bacteria depends on their adaptation to and survival in the stressful conditions encountered within the host (Abee and Wouters 1999). Stress responses by the bacteria may not only enable survival under more extreme conditions or resistance to subsequent stressful conditions, but also enhance their virulence. Virulence genes are any genes expressed by the bacteria in an effort to enhance growth and survival during interactions with the host and environment. Starvation stress in *C. jejuni* was found to decrease the transcription levels of the cytolethal distending toxin B (*cdtB*) and the *Campylobacter* invasion antigen B (*ciaB*) genes while increasing the level of *cadF* (Ma *et al.,* 2009). A recent study reports that lipooligosaccharide (LOS) genes such as *waaF* and *lgtF* are responsible for pathogenesis and stress survival of *C. jejuni* as these are the core-specific moieties that contribute to host colonization (Naito *et al.,* 2010). Multiple stress response genes were found to be involved in adaptation and regulation of virulence factors of *C. jejuni*. Recent studies show that the transcription of virulence and stress response genes were induced by the temperature shifts associated with food storage and was found to vary with the strains (Poli *et al.,* 2012).

2.11 Antimicrobial Susceptibility Testing:

Different methods are employed to study the antimicrobial susceptibility patterns in bacteria such as agar dilution, broth macrodilution, broth microdilution, disc diffusion and epsilometer (E) tests. The agar dilution considered as golden standard is performed by mixing two-fold concentrations of the antibiotics with the agar growth medium which are dispensed into individual petri plates and bacterial culture added on to them to see the

lowest concentration of the antibiotic which will prevent visible growth of bacteria. The broth macrodilution also works the same way as agar dilution except that in place of agar the liquid growth medium is used. Broth microdilution also uses the same procedures as broth macrodilution but growth medium is in smaller quantities (100 µl) and the test is performed in 96 well plates. The E-test makes use of the principle of an antimicrobial gradient in an agar medium by way of placing a strip with antibiotic concentration gradient and the intersection of the strip and the inhibition zone gives the MIC. Disc diffusion test is one of the simplest but most reliable methods. This test is performed by inoculating a standard inoculum onto the Mueller Hinton (MH) agar plate and placing the antibiotic discs on the inoculated surface of the agar. The Clinical Laboratory Standards Institute (CLSI, formerly known as NCCLS) has approved the agar dilution, disk diffusion and broth microdilution methods for the antimicrobial susceptibility testing of *Campylobacter spp* (Fitzgerald *et al.*, 2008).

2.11.1 Mechanisms Involved in Antibiotic Resistance in *C. jejuni*

Increasing antibiotic resistance in *Campylobacter jejuni* worldwide is raising serious concerns over the treatment of *Campylobacter* infections in humans. Antibiotic resistance develops in bacterial populations over the course of time through mutations that are chromosomally mediated and thus can be passed on to the next generation (Cirz *et al.*, 2005). *C. jejuni* has developed resistance to numerous antibiotics such as fluoroquinolones, macrolides, aminoglycoides, B-lactams, tetracycline and ampicillin have been reported (Zhang and Plummer, 2008). The general resistance mechanisms of *C. jejuni* to important antibiotics are shown in figure 2.4 (Jeon *et al.*, 2010). Fluoroquinolone

resistance in *Campylobacter jejuni* was first reported in the late 1980s (Allos 2001). The resistance to this class of antibiotics was observed at the same time that these antibiotics were introduced in animal production for prophylactic and therapeutic purposes (Aarestrup and Engberg, 2001). Resistance to fluoroquinolones is mainly caused by spontaneous point mutations occurring in the quinolone resistance-determining region (QRDR) of the gyrA gene in *C. jejuni* which encodes for the A subunit of the enzyme DNA gyrase (Engberg *et al.,* 2001; Fabrega *et al.,* 2008). These mutations thus results in reduced affinity of fluoroquinolones to DNA gyrase which in turn produces resistance in *C. jejuni* towards fluoroquinolones. The high mutation frequency in *C. jejuni* for fluoroquinolones is modulated by Mfd, a transcription-repair coupling factor involved in DNA repair (Han *et al.,* 2008). Mutations in the 23S rRNA subunit of *C. jejuni* is found to cause resistance to macrolide antibiotics (Gibreel *et al.,* 2005), but the development of this type of resistance requires long-term exposure to antibiotics (Lin *et al.,* 2007).

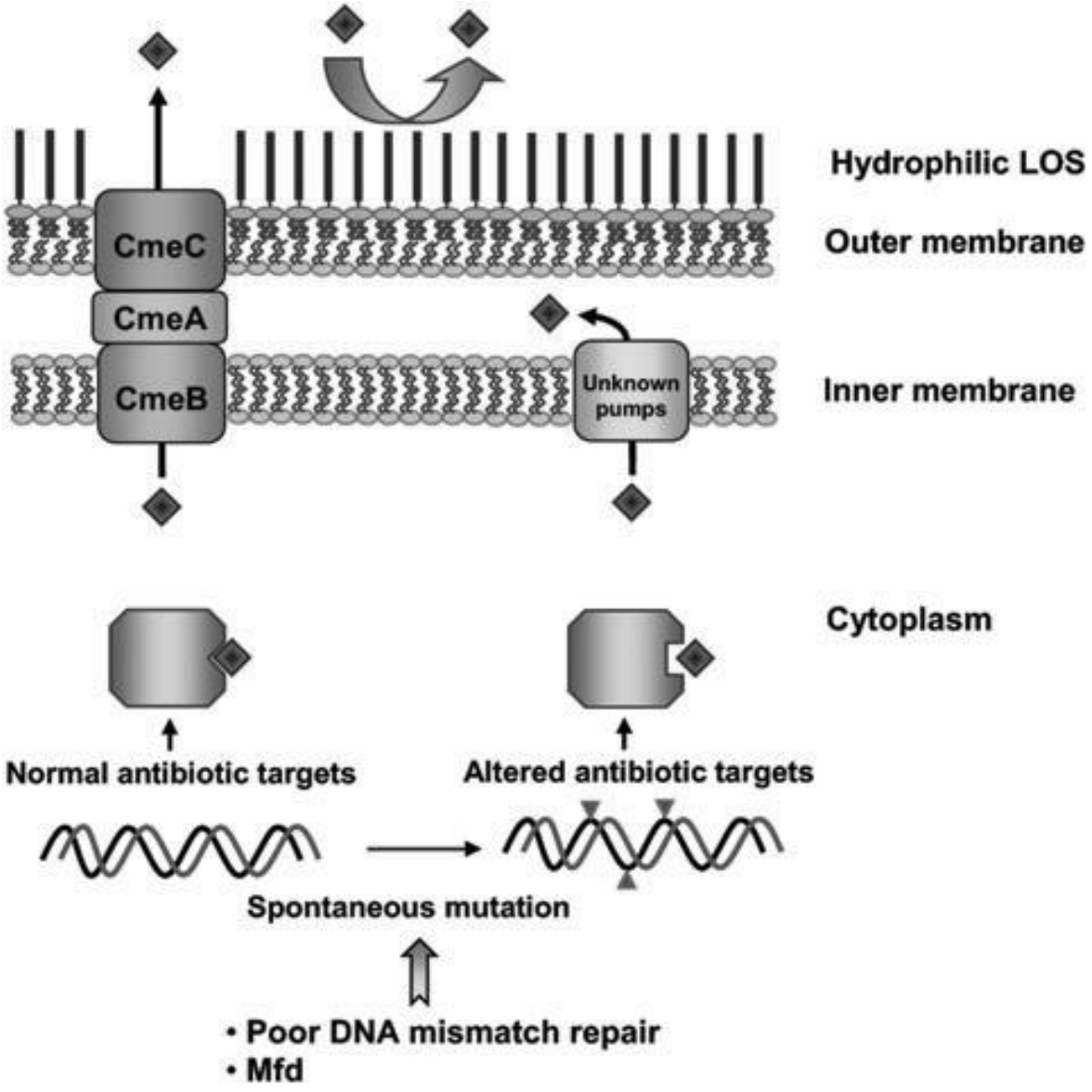


Fig. 2.4: Mechanisms associated with *Campylobacter* resistance to macrolide and fluoroquinolone antibiotics. LOS reduces the uptake of hydrophobic antibiotics (e.g. macrolide); efflux pumps (such as CmeABC and other uncharacterized efflux transporters) decrease the intracellular concentration of antibiotics; and chromosomal mutations reduce the affinity of antibiotics to their targets. Mfd and the lack of an intact mismatch repair system enhance the spontaneous mutation rate in *Campylobacter* (Adapted from Jeon *et al.*, 2010).

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CHAPTER 3

INFLUENCE OF ACID-ADAPTATION OF *CAMPYLOBACTER JEJUNI* ON ITS ADHESION AND INVASION OF INT 407 CELLS

ABSTRACT

The mechanisms of survival of *Campylobacter jejuni* inside and outside the host are not fully understood. *C. jejuni* has to survive the acid conditions in food and poultry processing as well as in the human stomach and colonize in the intestine to cause disease in humans. The aim of this study was to determine the influence of acid-adaptation on the survival as well as adhesion and invasion of human intestinal cells by nine *Campylobacter jejuni* strains after exposure to different stress conditions. Acid-adapted and non-adapted *C. jejuni* were exposed to different secondary stress conditions such as acid (pH 4.5), starvation (phosphate buffered saline, pH 7.2), or salt (3% w/v NaCl). After exposure to the secondary stress, the adhesion and invasion abilities of the isolates were evaluated *in vitro* in tissue culture using INT 407 cell line. The survival rates of acid-adapted cells of some strains of *C. jejuni* exposed to different secondary stresses were found to be significantly higher than the non-acid-adapted cells. Similarly, some strains also showed an increase in adhesion and invasion ($P < 0.05$) when acid-adapted *C. jejuni* were exposed to stresses such as acid, starvation, or salt as compared to non-acid-adapted *C. jejuni*. However, the survival rates as well as the degree of adhesion and invasion were found to vary with the strain of *C. jejuni*, the time of adaptation to acid, the type of the secondary stress and exposure time to the secondary stress. Thus, in our research we found that adaptation to acid stress can enhance the survival of *C. jejuni* when exposed to secondary stresses and thereby increase adhesion and invasion of human intestinal cells *in vitro*. These results show that adaptation to stresses could influence virulence of *C. jejuni* and that acid adaptation of *C. jejuni* could cause an increase in its virulence.

INTRODUCTION

Campylobacter jejuni is a leading cause of human bacterial gastroenteritis throughout the world. An estimated 850,000 cases of human campylobacteriosis occur each year in the United States (Scallan *et al.,* 2011). Foodborne illness caused by *C. jejuni* is associated with the consumption of food animals, especially undercooked poultry. Even numbers as low as 500 *C. jejuni* or fewer can cause gastrointestinal disease (Black *et al.*, 1988). If *C. jejuni* on contaminated food is ingested and reaches the intestinal tract, adherence to the intestinal epithelial cells can occur. Adhesion and invasion of the intestinal cells are two major steps in the pathogenesis of this disease. The symptoms associated with this disease include fever, diarrhea, headache, abdominal pain, myalgia, vomiting and blood in feces (Poly *et al*. 2005). The disease is usually self-limiting, but occasionally the symptoms may persist for 2 weeks or more, necessitating treatment with antibiotics. In some cases, *C. jejuni* may also produce complications such as Guillain–Barré syndrome (GBS), reactive arthritis, and hemolytic anemia. It has been estimated that 1 in 1000 infections can lead to GBS (Nachamkin, 2002), an autoimmune disease involving the nervous system, which can develop because certain structures in the lipo-oligosaccharides (LOS) of *C. jejuni* mimick the gangliosides in human nerve tissue leading to production of cross-reactive antibodies that cause GBS (Godschalk *et al.,* 2004).

C. jejuni are considered to be relatively fragile organisms requiring fastidious growth conditions in the laboratory. These organisms also lack the usual stress response factors necessary for survival in adverse environmental conditions such as the global stationary phase stress response factor (RpoS), oxidative stress response factor (SoxRS),

major cold shock proteins and heat shock response regulators (Park, 2002). But despite these characteristics, *C. jejuni* can survive harsh conditions in the environment and persist on poultry and poultry products. Foodborne bacterial pathogens may encounter a variety of sub-lethal stresses when outside a host in foods and the environment. Exposure of foodborne bacteria to sub-lethal stresses has been found to induce a tolerance response helping them to become more tolerant toward severe stresses in the environment. This kind of a tolerance response has been termed as the adaptive tolerance response or ATR. Acid is a key stress encountered while passing through the human and avian gastrointestinal tract as well as in food processing environments. Hence, the acid adaptive tolerance response has been specifically recognized as an important strategy employed by foodborne pathogens to counteract acid stress. *Salmonella, Listeria,* and *Escherichia coli,* all have enhanced survival abilities during exposure to a lethal acid challenge, after exposure to prior sub-lethal acid conditions (Bacon *et al.*, 2003; Koutsoumanis *et al*, 2003; Tosun and Gonul, 2005). *Campylobacter* also has been shown to induce such an adaptive tolerance when exposed to acid and/or aerobic conditions, cold conditions and high pressure treatment (Murphy *et al.,* 2003; Shaheen *et al.*, 2007; Garénaux *et al.*, 2008; Ma *et al.,* 2009; Bieche *et al.,* 2010).

The exact mechanisms involved in the pathogenesis of *C. jejuni* infections in humans are not fully understood. Many virulence factors have been proposed in the survival of *C. jejuni* and establishment of the disease in humans. *C. jejuni* may cause disease either by invasion of the intestinal epithelial cells and subsequent changes to host cells, production of toxins by the ingested and colonized *C. jejuni*, or damage to extra-

intestinal sites by *C. jejuni* passing through the lymphatic vessels (Zheng *et al.*, 2006). Depending on the status of the host and the strain involved in infection, one or more of the above described methods may be involved. Invasion of intestinal epithelial cells was found to be reduced when *C. jejuni* was exposed to stresses such as low temperature, heat shock and starvation. Exposure to atmospheric oxygen, however, increased the invasion ability (Mihaljevic *et al.*, 2007). Exposure to oxygen was also found to increase the invasion of murine macrophages by *C. jejuni* as well as increase their intracellular survival when compared to other stresses such as starvation and heat shock (Pogacar *et al.*, 2009). To the best of our knowledge, the effect of stress-adaptation on the adherence and invasion of *C. jejuni* exposed to further secondary stresses has not been studied. In our research, the effects of acid-adaptation on *C. jejuni* adherence and invasion using a human intestinal epithelial cell model were studied.

The objectives of this study were as follows: (1) to determine whether acid-adaptation in *Campylobacter jejuni* increased the survivability when the bacteria are further exposed to secondary stresses such as acid (pH 4.5), starvation (phosphate buffered saline, pH 7.2) or salt (3% w/v NaCl); and (2) to compare any changes in adhesion and invasion of INT 407 cells by *C. jejuni*, with and without acid-adaptation, after exposure to secondary stresses.

MATERIALS AND METHODS

Bacterial strains:

A total of nine *C. jejuni* strains, isolated from human and poultry, were used for this study. The six human isolates included 81-176, the strain known to produce disease in human volunteers, NCTC 11168, and four isolates 70 H, 71 H, 87 H and 1023 H obtained from humans exhibiting campylobacteriosis. The three poultry isolates used were obtained from different stages of processing including pre-chilled chicken carcass isolate (PRCC 3), post-chilled chicken carcass isolate (POCC 13), and retail chicken carcass isolate (RECC 3). The four *C. jejuni* human isolates were kindly donated by Dr. Donald Cave, University of Arkansas for Medical Sciences, Little Rock, AR; strain 81-176 was donated by Dr. Michael Johnson, University of Arkansas, Fayetteville, AR; NCTC 11168 was obtained from Qijing Zhang, Iowa State University, Ames, IA. The isolates were stored at -80^{o}C in *Campylobacter* enrichment broth, Bolton's (Acumedia®) supplemented with glycerol and sub-cultured prior to the stress experiments. Frozen stock cultures were passed twice on *Campylobacter* blood agar plates and then inoculated into *Campylobacter* enrichment broth, Bolton's (CEB) and incubated in a micro-aerobic atmosphere consisting of 5% oxygen, 10% carbon dioxide and 85% nitrogen at 42^{o}C for 18 h to obtain the early stationary phase cultures for the experiments.

Objective 1: Survivability of acid-adapted *C. jejuni* exposed to different secondary stresses:

Early stationary phase (18 h) cultures in *Campylobacter* enrichment broth, Bolton's (CEB) were divided into two portions and centrifuged at 8000 x g for 5 min and

subsequently resuspended in either acid broth (pH 5.5) to obtain acid-adapted cells or in CEB broth to obtain cells not acid stressed. Acid broth was prepared by adding hydrochloric acid (HCl) directly to the CEB broth. After an adaptation time of 2 h both acid-adapted cells and non-acid-adapted cells were exposed to the following stresses for a period of 2 h: 1. acid stress (4.5) by resuspending both the treatment groups in CEB broth with a pH of 4.5; 2. starvation stress by resuspending acid-adapted cells and non-acid-adapted cells in phosphate buffered saline (PBS) with a pH of 7.2; 3. salt stress by resuspending cells from both treatment groups in 3% NaCl solution (w/v). At 2 h post-exposure to the secondary stresses surviving culturable *C. jejuni* was evaluated by standard plating methods. The survival percentages were calculated by dividing the cfu / mL after acid adaptation and exposure to secondary stresses by the cfu / mL at time zero and multiplying by 100. All the experiments were repeated three times. We also examined the effects of a 3 h acid-adaptation on the survivability *C. jejuni* when further exposed to acid stress (pH 4.5). These experiments were done with four isolates which included one human isolate (81-176) and 3 poultry isolates (PRCC 3, POCC 13 and RECC 3).

Acid-adaptation studies using lactic acid were also done by the addition of lactic acid to CEB broth and exposure of the acid-adapted strains to the secondary stress of acid (pH4.5). These experiments were done with four isolates which included one human isolate (81-176) and 3 poultry isolates (PRCC 3, POCC 13 and RECC 3). To determine the effect of time of exposure to the secondary stress, acid-adapted strains were subjected to a secondary stress of acid (pH 4.5) and samples were taken for adhesion and invasion assays at 0, 2, 4, 6, 12, 24 and 48 h post exposure to the secondary acid stress. These

experiments were done using two *C. jejuni* strains 81-176, the human strain and PRCC, the poultry strain from pre-processed chilled chicken carcass. Stress-adaptation protocol is described in the form of a flow chart in Appendix A1.

Objective 2: Adhesion and invasion assays:

The INT 407 cell line (human embryonic intestinal cells) used for this study was kindly donated by Dr. Jody Lingbeck, University of Arkansas Fayetteville, AR. The tissue culture cells were grown in Basal Medium Eagle (BME) (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Sigma) and 2mM L-glutamine (Sigma) in 75 cm^2 tissue culture flasks at 37˚C in a 5% CO_2 atmosphere until cell layers were confluent. The cells were seeded into 24-well tissue culture plates at a concentration of 10^5 cells per well and plates were incubated in a 5% CO2 atmosphere at 37˚C for 24 h to allow cell attachment before adhesion and invasion assays.

Adhesion and invasion assays were performed as previously described using INT 407 cells (Gilbert & Slavik, 2005). The protocol is described a step-wise manner in appendix A2. The INT 407 cells were grown in 75 cm^2 tissue culture flask using Basal Medium Eagle's (Sigma-Aldrich, St. Louis, USA) as the growth media. When the cells reached 75-80% confluency, the media was poured off and the cells were collected using trypsin solution (0.25%; Sigma). The cell concentration was determined using a hemocytometer and cells were re-suspended in fresh media to a final concentration of 10^5 cells / ml and subsequently seeded on to 24-well tissue culture plates at the rate of 1 ml per well. The plates were incubated for 24 h in a 5% CO_2 atmosphere at 37˚C and washed 3

times with sterile PBS before they were used for the adhesion and invasion assays. Acid-adapted and non-acid-adapted *C. jejuni* cells were exposed to further stress as described in objective 1 and subsequently washed twice with PBS before challenging the intestinal cells on the tissue culture plates. The multiplicity of infection was assessed by determining the cfu / ml of the cultures used for infecting the intestinal cells. Plates then were incubated for 2 h to allow adherence and invasion of tissue culture cells. After 2 h, all plates were washed three times with PBS to remove any non-adhering bacteria before the addition of fresh medium containing 100μg ml-1 gentamicin (to measure invasion alone) or, in the duplicate plates, PBS with 0.01% Triton X-100 (to measure adherence and invasion). Plates with media plus gentamicin were incubated in a 5% CO_2 at 37˚C for an additional 2 h, while plates with PBS plus 0.01% Triton X-100 (Mallinckrodt-Baker, Phillipsburg, NJ) were gently swirled for 30 minutes at room temperature to lyse the intestinal *cells* to release internalized bacteria. After the 2 h incubation, tissue culture plates with media plus gentamicin, plates were washed three times with PBS prior to the addition of PBS with 0.01% Triton X-100. Cells were lysed using the same method as described above.

To determine the colony forming units (cfu) of bacteria adhering and invading the tissue culture models, ten-fold dilutions were made of the lysed cells in sterile PBS and plated onto CE agar. The cfu of adhering bacteria was determined by using the formula: cfu of non-gentamicin treated culture (adherent and invasive) – cfu of gentamicin treated culture (invasive) = cfu of adherent bacteria.

Statistical analysis:

The experiments were conducted in three independent replicates and were analyzed by a one way ANOVA and Student's t-test using the JMP statistical software package 9.0.2. The results were considered statistically significant with p-values reported at $P<0.05$.

RESULTS

Survivability of acid-adapted *C. jejuni* exposed to different secondary stresses:

Survivability of *C. jejuni* strains were assessed in this study by determining the survival percentages of viable and culturable *C. jejuni* cells after acid-adaptation and exposure to the secondary stresses of lethal acid (pH 4.5), starvation (phosphate buffered saline, pH 7.2) or salt (3 % w/v NaCl). Non-acid-adapted cells exposed to the secondary stresses served as the control group. Acid-adapted cells exposed to the secondary stress of lethal acid for 2 h were found to exhibit a significantly higher survival percentage ($P>0.05$) than their respective controls for the human isolates 81-176, NCTC 11168, and 87 H and the poultry isolates PRCC 3 and RECC 3 (Fig 3.1). The remaining three human isolates 70 H, 71 H and 1023 H showed no significant differences in survival percentages between acid-adapted and non-acid-adapted cells when exposed to the secondary stress of acid. Increasing the acid adaptation time to 3 h and exposing to the secondary stress of acid was found to increase the survival percentages of acid-adapted cells for only two of the three poultry isolates, POCC 13 and RECC 3. The human isolate 81-176 and the poultry isolate PRCC 3 did not show any significant differences between the treatment groups (Fig 3.4).

Similarly, after acid adaptation when *C. jejuni* cells were exposed to a secondary stress of starvation for 2 h, five out of the nine isolates showed higher survival percentages for the acid-adapted cells than their respective controls. These isolates were 81-176, NCTC 11168, 70 H, PRCC 3 and POCC 13 (Fig 3.7). A secondary stress of salt was found to increase the survival percentages of acid-adapted cells for only two of the poultry isolates POCC 13 and RECC 3 (Fig 3.10). These results show that an acid-adaptation helps the early stationary phase cells of *C. jejuni* to enhance their survivability after exposure to secondary stresses which varied among the isolates used in our study.

Effects of acid adaptation of *C. jejuni* on adhesion and invasion of INT 407 cells:

A plate count assay was used to determine the adhesion to and invasion of INT 407 cells by *C. jejuni* after acid-adaptation and exposure to secondary stresses including acid (pH 4.5), starvation (phosphate buffered saline, pH 7.2) or salt (3 % w/v NaCl). Non-acid-adapted cells exposed to further stresses served as the control group. Some acid-adapted *C. jejuni* isolates were found to have increased adhesion and invasiveness, but this varied with the strains, the time of adaptation of acid and the time of exposure to the secondary stress. With a 2 h acid-adaptation, the isolate from the pre-chilled chicken carcass (PRCC 3) showed increased adhesion as compared to its control with no acid-adaptation when exposed to the secondary stress of acid. Similar results were also obtained for two of the human isolates NCTC 11168 and 87 H (Fig 3.2). When the acid-adaptation time was increased to 3 h, two poultry isolates (POCC 13 and RECC 3) were found to show significant increases in adhesion compared to the control group after exposure to further

acid stress (Fig 3.5). A corresponding increase was seen in the invasion of all the isolates with increased adhesion (Fig 3.3 and 3.6).

Acid-adapted cells of *C. jejuni* after exposure to a secondary stress of starvation also showed an increase in adhesion for the poultry isolates PRCC 3 and POCC 13 as well as for the human isolates 81176 and NCTC 11168, whereas the non-acid-adapted cells of the human isolate 87 H were found to have significantly higher adhesion and invasion (Fig 3.8). However, this increased adhesion could be seen only 2 h post-starvation in our study. Similarly, when acid-adapted *C. jejuni* were exposed to a secondary stress of salt for a period of 2 h, only two of the poultry isolates POCC 13 and RECC 3 were found to have significantly higher rates of adhesion than the controls. On the contrary, however, the human isolate 81-176 and the poultry isolate PRCC 3 were found to exhibit higher adhesion rates for the control group (Fig 3.11). The rest of the human isolates did not show any difference between the two treatment groups. We found similar observations in the invasion rates of the strains after exposure to the secondary stresses of starvation and salt (Fig 3.9 and 3.12). Invasiveness of the strains was dependent on their adhesion, with higher invasion percentages seen for those strains with higher adhesion rates.

The percentage of adhesion and invasion was found to vary with the time of exposure to the secondary stress of acid (pH 4.5). Acid-adapted *C. jejuni* strains, 81-176 and PRCC were found to have increased adhesion rates at 2 h, 4 h, 12 h and 24 h post exposure to the secondary acid stress of pH 4.5 (Figure 3.13). Similar increases were also observed in the invasion rates of both the isolates at these time points. However, no

significant differences were observed in the percentage of adhesion and invasion between the acid-adapted and non-acid-adapted treatment groups when lactic acid-adapted *C. jejuni* strains were further exposed to acid pH of 4.5.

DISCUSSION

Adaptation to stress is among the survival methods for pathogenic organisms including foodborne bacteria (Ray and Bhunia, 2008). Recent studies have shown the effects of various stress conditions on the survival of *C. jejuni in vitro* in various tissue culture cell lines (Pogacar *et al.*, 2009 and 2010). To our knowledge, there is no previous record on the adhesion and invasion abilities of *C. jejuni* after acid-adaptation. The results of our study indicate that *C. jejuni* is capable of inducing an adaptive tolerance response (ATR) on exposure to an acidic pH of 5.5 which might be helping in increasing the survival rates when exposed to secondary stresses including acid, starvation or salt. It was also found that the adhesion / invasion of *C. jejuni* to human intestinal cells (INT 407) were dependent on the survival percentages. However, we found variations among the strains used in our study, indicating that only some strains have the ability to induce an ATR under the conditions used in our study. The induction of an ATR, as well as adhesion and invasion abilities, were found to vary not only with the strain of *C. jejuni* but also with the time of exposure to a sub-lethal pH for adaptation, time of exposure to the secondary stress as well as the type of secondary stress. *C. jejuni* isolates used in the present study were shown to have difference in adhesion and invasion rates under normal conditions without exposure to any stress in earlier studies in our laboratory (Gilbert and Slavik, 2005). Other studies have also observed that different isolates of *C. jejuni* show variation

in their ability to adhere to and invade tissue culture cells under normal conditions of growth (Zheng *et al.,* 2006; Coote *et al.,* 2007). This difference in adhesion and invasion among different *C. jejuni* strains may be one of the reasons for the different adhesion and invasion rates observed in our study. The adhesion and invasion rates also might be dependent on the survival percentages of *C. jejuni* surviving further secondary stresses. Studies have shown variations among the strains of *C. jejuni* in the survival ability to survive further stresses after adaptation to a sub-lethal stress (Murphy *et al.,* 2003; Shaheen *et al.,* 2007). Thus, the variations observed in our results might be due to the difference in the isolates, adaptation time to the mild acid stress and the different secondary stresses to which the acid-adapted cells were exposed.

In our research, we found that an acid-adaptation helps *C. jejuni* to increase survivability when exposed to secondary stresses and also to increase their virulence by increasing their adhesion and invasion of intestinal epithelial cells. Our results are consistent with the results of previous research conducted with other foodborne bacterial pathogens. *Salmonella* strains were shown to induce an ATR following acid-adaptation at pH 4.3 followed by subsequent exposure to a pH of 3.0 (Bacon *et al.,* 2003). The virulence as determined by invasion of acid-adapted *Salmonella typhimurium* in INT-407 cells was found to be increased on further exposure to an acidic pH of 3.8 (Wilmes-Riesenberg *et al.,* 1996). An adaptation to acid or salt and subsequent exposure to acid or salt stress was also found to increase the virulence potential of *Escherichia coli* strains as measured by adhesion to various tissue culture cells (House *et al.,* 2009; Olesen and Jespersen, 2010).

The induction of an ATR could possibly help *C. jejuni* to survive stresses in the food and poultry processing environment. *C. jejuni* also has to survive the acidic conditions in human stomach to cause the disease. It has been shown that the pH in the human stomach can vary approximately from 2 to 7 depending on gastric secretions and contents in stomach (Dressman *et al.,* 1990). A very low infectious dose for *C. jejuni* (Black *et al.,* 1988) suggests that this organism is capable of survival in acidic environment.

The virulence of pathogenic bacteria depends on their adaptation to and survival in the stressful conditions encountered within the host (House *et al.,* 2009) and *C. jejuni* has also been shown to have evolved specific adaptations to survive in the host cells. One such adaptation is the survival of *C. jejuni* in intestinal epithelial cells wherein vacuoles containing *C. jejuni* were found to deviate from the endocytic pathway and thus avoid delivery into lysosomes (Watson and Galan, 2008). Pathogenesis of the gastrointestinal disease produced by *C. jejuni* is not only dependent on the ability to survive stresses in the human stomach, but also on the interactions of the bacteria with the intestinal cells. Four factors were shown to be involved in causing symptoms of campylobacteriosis including motility, adhesion, invasion, and toxin production (Young et al., 2007). Motility and chemotaxis are important for seeking out the mucus layer of the intestines, and critical in moving away from a stressful environment. Adhesion and invasion are also thought to be important in colonization and could possibly be influenced by stress. It has been shown that a brief exposure to aerobic stress (atmospheric oxygen) for 5 h can increase the survival of *C. jejuni* in murine macrophages and pig intestinal epithelial cells (Pogacar *et*

al., 2009 and 2010). Stress response genes in *C. jejuni* were found to be up-regulated in response to *in vitro* as well as *in vivo* acid shock for a period of 20 min (Reid et al., 2008). Adaptation to acid and sodium chloride stress and further exposure to stresses was found to increase the invasion ability of *Listeria* in Caco-2 cells (Olesen *et al.,* 2009). The results from this study also indicate that adaptation to sub-lethal stresses may increase the virulence of *C. jejuni* by increasing the adhesion and invasion abilities.

CONCLUSIONS

The isolates used in the study varied in their production of an ATR, as well as in their adhesion and invasion abilities. The fact that acid-adapted cells of *C. jejuni* were able to have increased survival rates and increased adhesion and invasion abilities compared to non-acid-adapted cells is significant because as few as 500 *C. jejuni* cells can cause disease in humans. A stress response by the bacteria may not only enable survival under more extreme conditions, but also increases its resistance to subsequent stressful conditions which could enhance the virulence. The results of our study show the effect on the survival and virulence of stress-adapted *C. jejuni* after exposure to secondary stresses. Other factors, such as production of stress proteins, as well as up-regulation of virulence genes might also be involved in the pathogenesis of *C. jejuni*. Because studies have demonstrated a variation among *C. jejuni* isolates in response to stress, caution should be taken when applying the results of this study to other *C. jejuni* strains.

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FIGURE LEGEND

Fig 3.1. Survival % vs. different strains of *C. jejuni*. Survival of acid-adapted and non-stressed *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of acid pH of 4.5 for 2 h. The survival percentages of acid-adapted *C. jejuni* were significantly higher for the strains 87 H, NCTC 11168, 81-176, PRCC and RECC ($p<0.05$).

Fig 3.2. Adhesion % vs. different strains of *C. jejuni*. Adhesion of acid-adapted and non-stressed *C. jejuni* strains with a 2 h adaptation time when exposed to secondary stress of acid pH of 4.5 for a period of 2 h.

Fig 3.3. Invasion % vs. different strains of *C. jejuni*. Invasion of acid-adapted and non-stressed *C. jejuni* strains with a 2 h adaptation time when exposed to secondary stress of acid pH of 4.5 for a period of 2 h.

Fig 3.4. Survival % vs. different strains of *C. jejuni*. Survival of acid-adapted and non-stressed *C. jejuni* with an adaptation time of 3 h and subsequent exposure to secondary stress of acid pH of 4.5 for 2 h. The survival percentages of acid-adapted *C. jejuni* were significantly higher for only two strains POCC and RECC ($p<0.05$).

Fig 3.5. Adhesion % vs. different strains of *C. jejuni*. Adhesion of acid-adapted and non-stressed *C. jejuni* strains with a 3 h adaptation time when exposed to secondary stress of acid pH of 4.5 for a period of 2 h.

Fig 3.6. Invasion % vs. different strains of *C. jejuni*. Invasion of acid-adapted and non-stressed *C. jejuni* strains with a 3 h adaptation time when exposed to secondary stress of acid pH of 4.5 for a period of 2 h.

Fig 3.7. Survival % vs. different strains of *C. jejuni*. Survival of acid-adapted and non-stressed *C. jejuni* strains with a 2 h adaptation time when exposed to secondary stress of starvation for a period of 2 h. The survival percentages of acid-adapted *C. jejuni* were significantly higher for the strains 70 H, NCTC 11168, 81-176, PRCC and POCC ($p<0.05$).

Fig 3.8. Adhesion % vs. different strains of *C. jejuni*. Adhesion of acid-adapted and non-stressed *C. jejuni* strains with a 2 h adaptation time when exposed to secondary stress of starvation for a period of 2 h.

Fig 3.9. Invasion % vs. different strains of *C. jejuni*. Invasion of acid-adapted and non-stressed *C. jejuni* strains with a 2 h adaptation time when exposed to secondary stress of starvation for a period of 2 h.

Fig 3.10. Survival % vs. different strains of *C. jejuni*. Survival of acid-adapted and non-stressed *C. jejuni* strains with an adaptation time of 2 h when exposed to a secondary stress of 3 % NaCl for a period of 2 h. The survival percentages of acid-adapted *C. jejuni* were significantly higher for only two strains POCC and RECC ($p<0.05$).

Fig 3.11. Adhesion % vs. different strains of *C. jejuni*. Adhesion of acid-adapted and non-stressed *C. jejuni* strains with an adaptation time of 2 h when exposed to a secondary stress of 3 % NaCl for a period of 2 h.

Fig 3.12. Invasion % vs. different strains of *C. jejuni*. Invasion of acid-adapted and non-stressed *C. jejuni* strains with an adaptation time of 2 h when exposed to a secondary stress of 3 % NaCl for a period of 2 h.

Fig 3.13. Adhesion of acid-adapted and non-acid-adapted *C. jejuni* human strain 81-176 (panel A) and *C. jejuni* poultry strain PRCC (panel B) with an adaptation time of 2 h and subsequent exposure to secondary stress of acid pH of 4.5 for different periods of time ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of acid (pH 4.5) for 2h

% Survival vs. Strain of *C. jejuni*

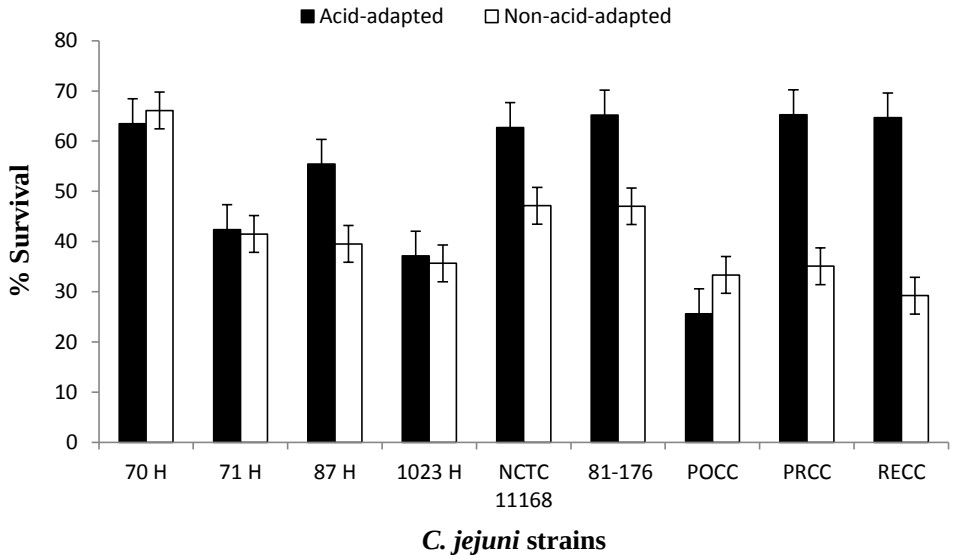

Fig 3.1: Survival of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of acid pH of 4.5 for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of acid (pH 4.5) for 2h

% Adhesion vs. Strain of *C. jejuni*

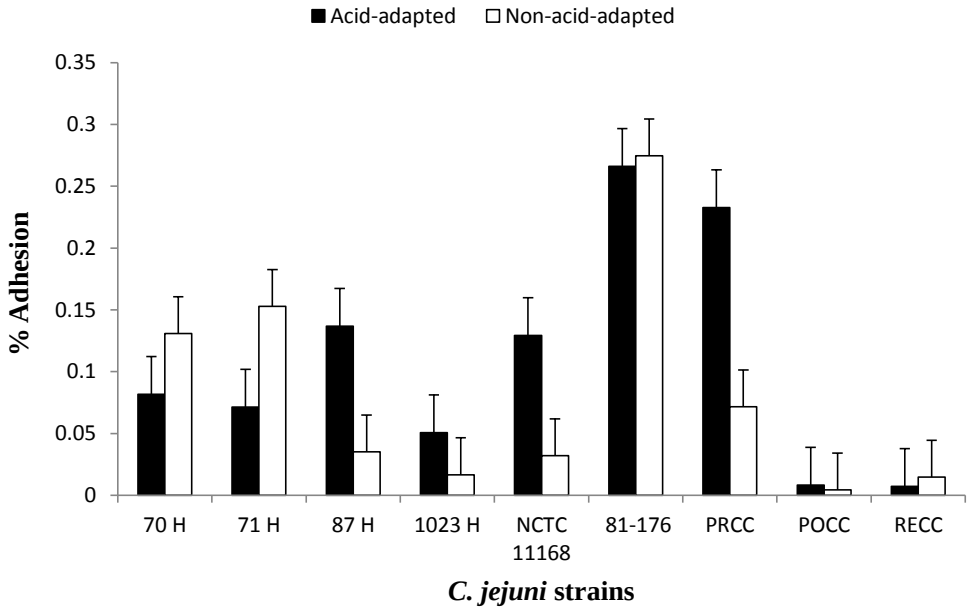

Fig 3.2: Adhesion of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of acid pH of 4.5 for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of acid (pH 4.5) for 2h

% Invasion vs. Strain of *C. jejuni*

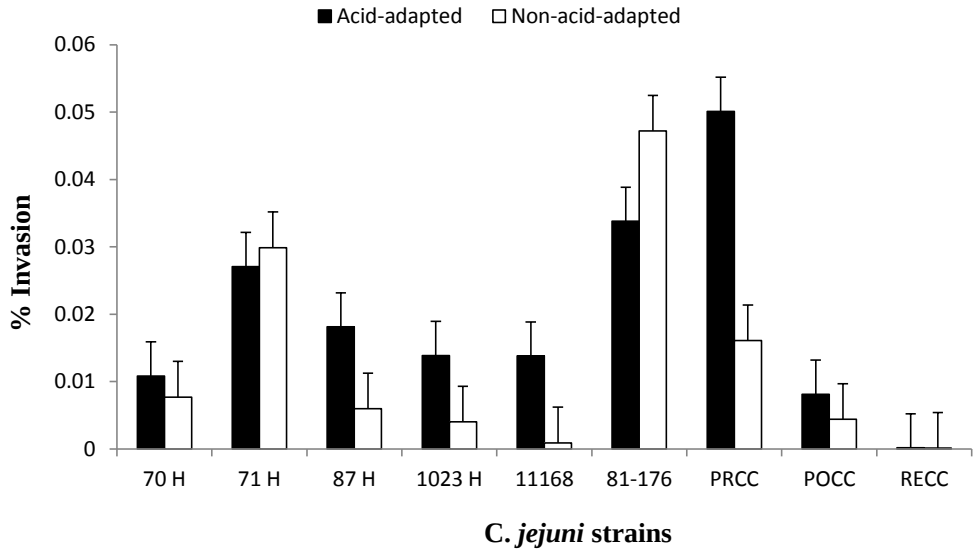

Fig 3.3: Invasion of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of acid pH of 4.5 for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 3 h and exposure to a secondary stress of acid (pH 4.5) for 2 h

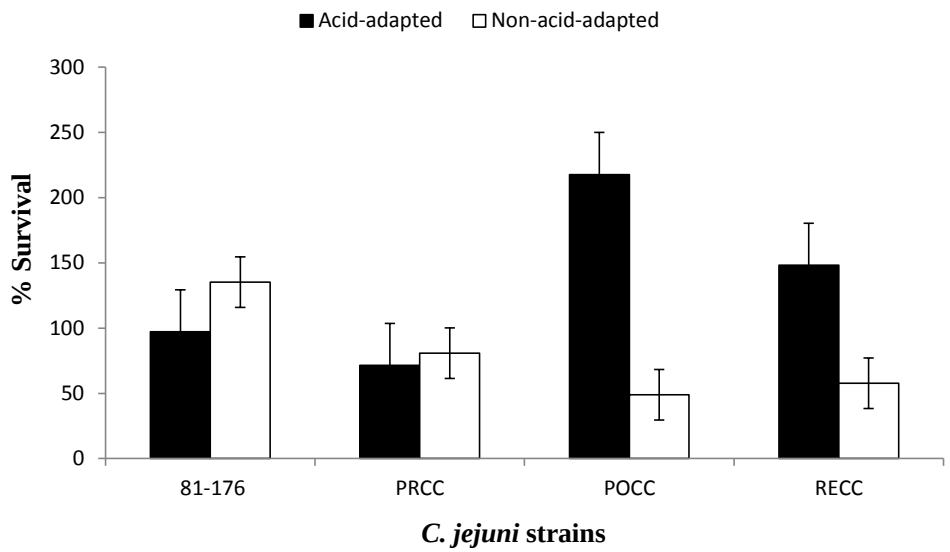

Fig 3.4: Survival of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 3 h and subsequent exposure to secondary stress of acid pH of 4.5 for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 3 h and exposure to a secondary stress of acid (pH 4.5) for 2 h

% Adhesion vs. Strain of *C. jejuni*

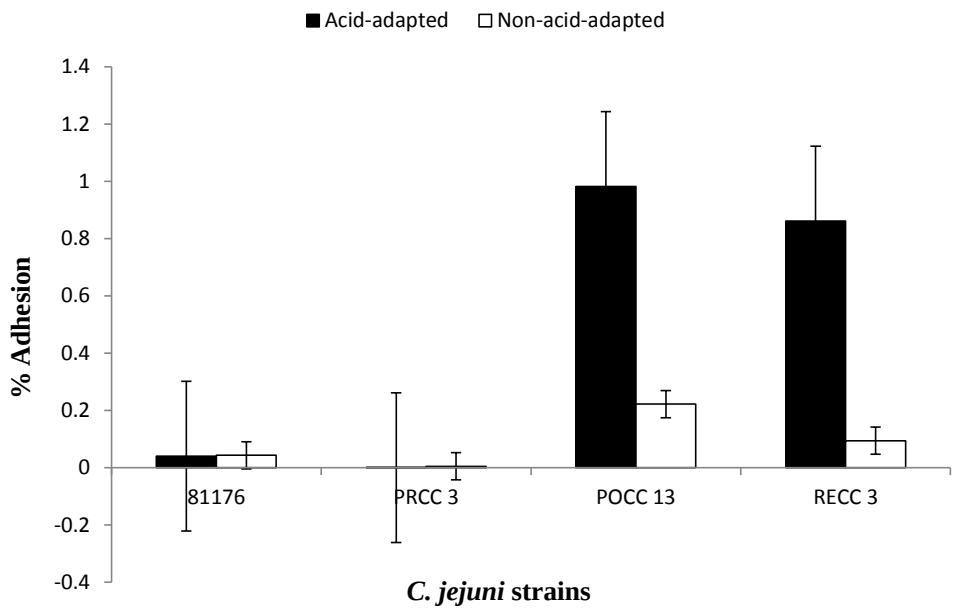

Fig 3.5: Adhesion of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 3 h and subsequent exposure to secondary stress of acid pH of 4.5 for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 3 h and exposure to a secondary stress of acid (pH 4.5) for 2 h

% Invasion vs. Strain of *C. jejuni*

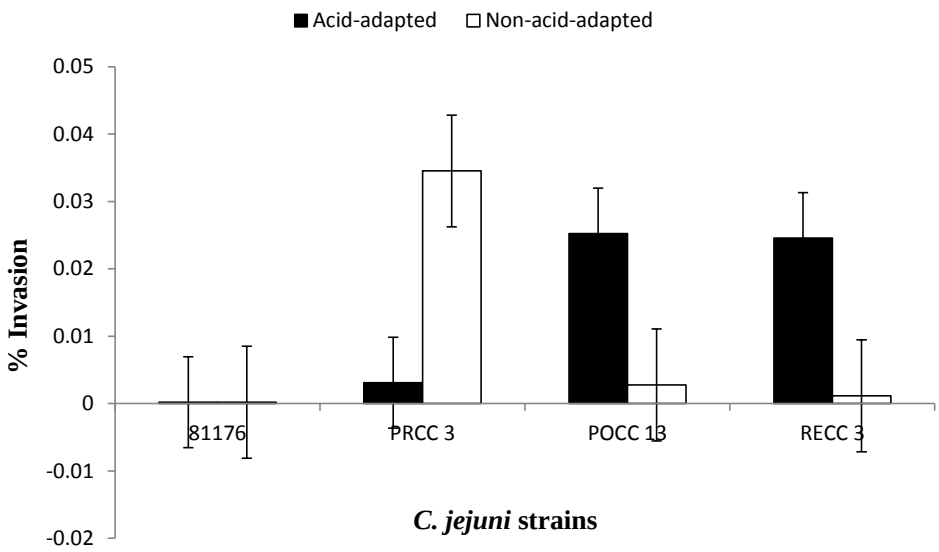

Fig 3.6: Invasion of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 3 h and subsequent exposure to secondary stress of acid pH of 4.5 for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of starvation for 2 h

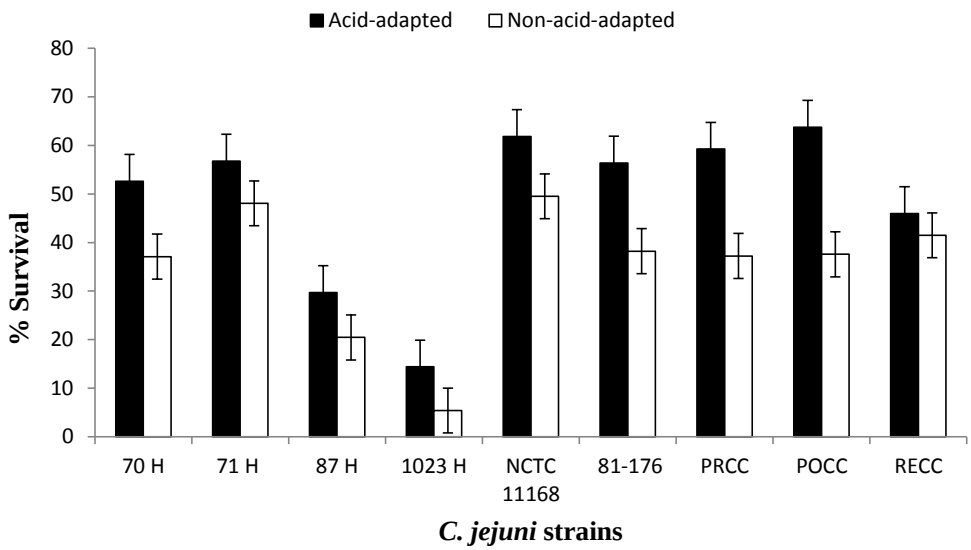

Fig 3.7: Survival of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of starvation for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of starvation for 2 h

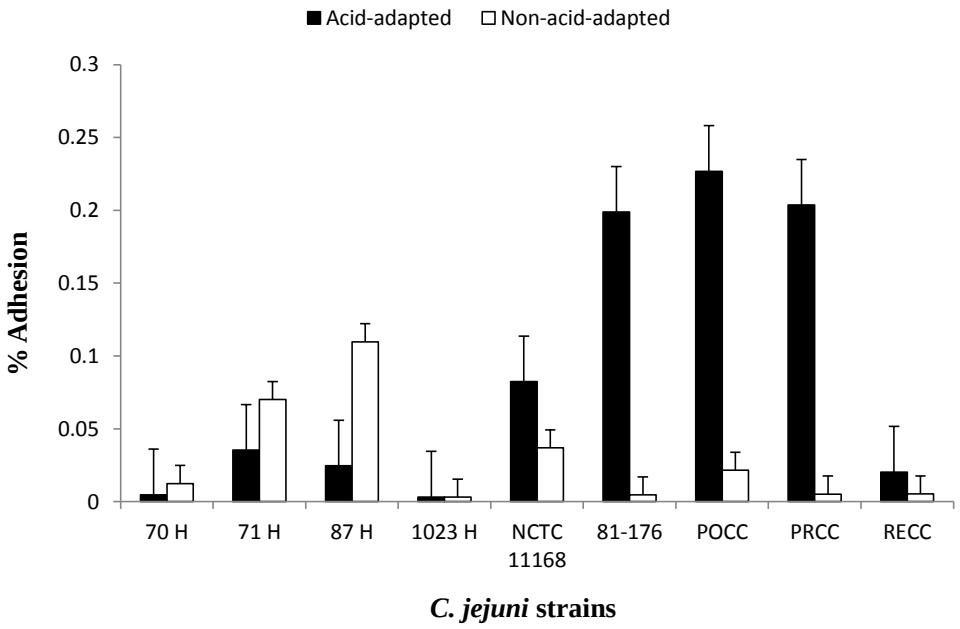

Fig 3.8: Adhesion of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of starvation for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of starvation for 2 h

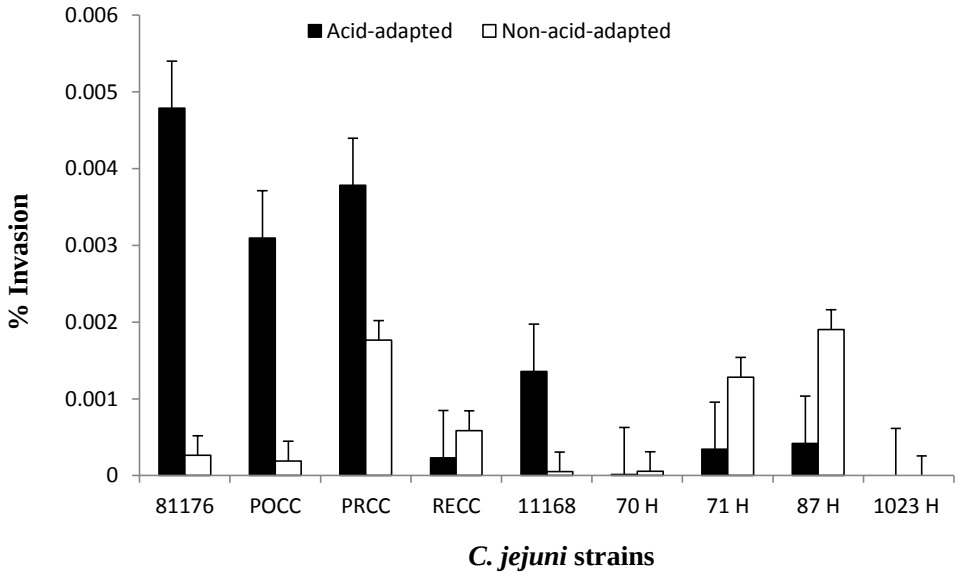

Fig 3.9: Invasion of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of starvation for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of 3% salt for 2 h

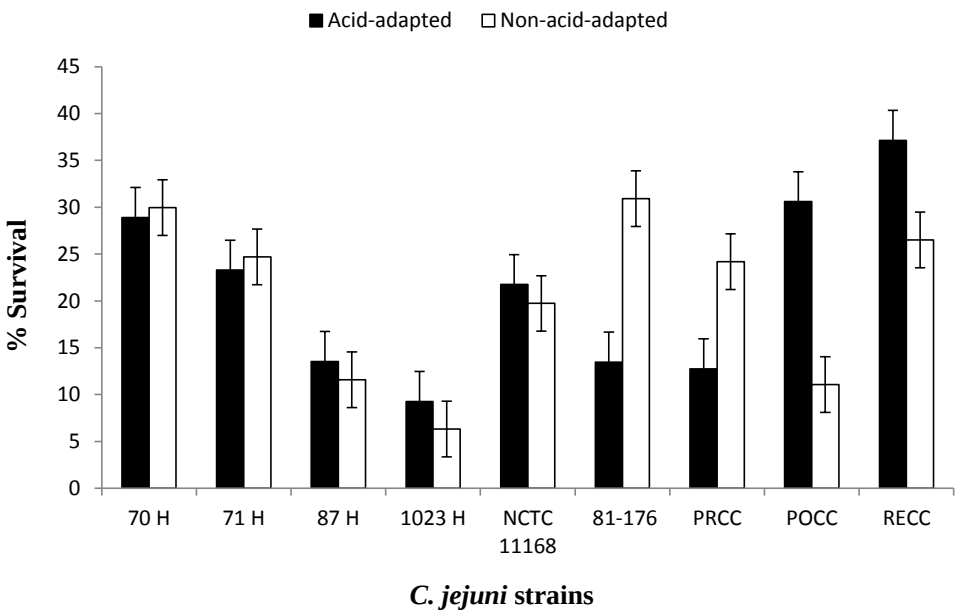

Fig 3.10: Survival of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of 3 % salt for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of 3% salt for 2 h

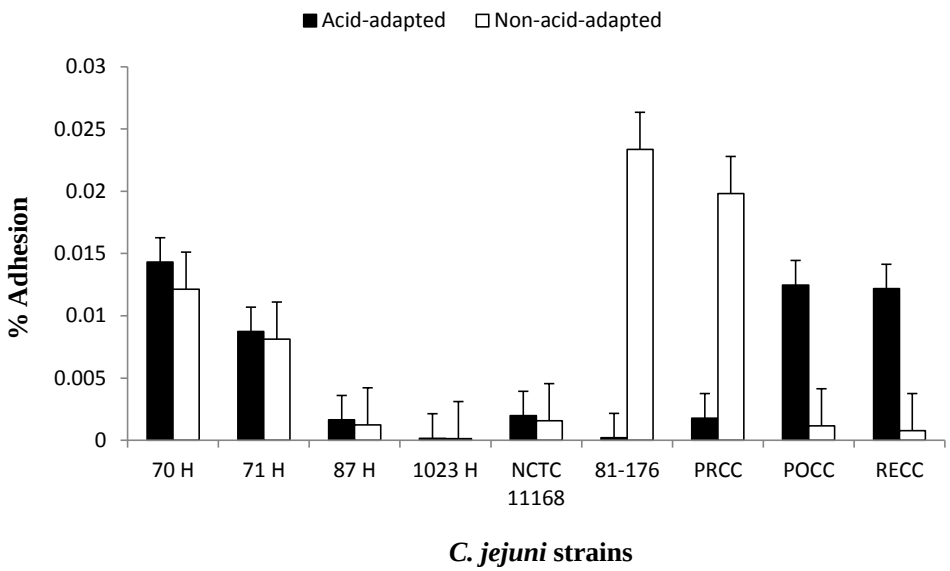

Fig 3.11: Adhesion of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of 3 % salt for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of 3% salt for 2 h

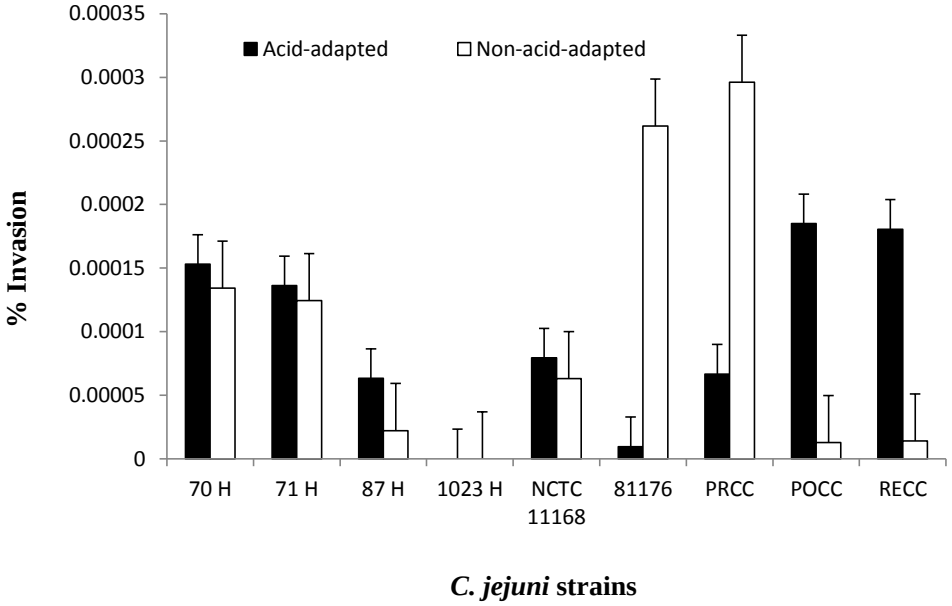

Fig 3.12: Invasion of acid-adapted and non-stressed strains of *C. jejuni* with an adaptation time of 2 h and subsequent exposure to secondary stress of 3 % salt for 2 h ($p<0.05$).

Adaptation to primary stress of acid (pH 5.5) for 2 h and exposure to a secondary stress of acid (pH 4.5) for 2h

% Adhesion vs. time of exposure to secondary acid stress

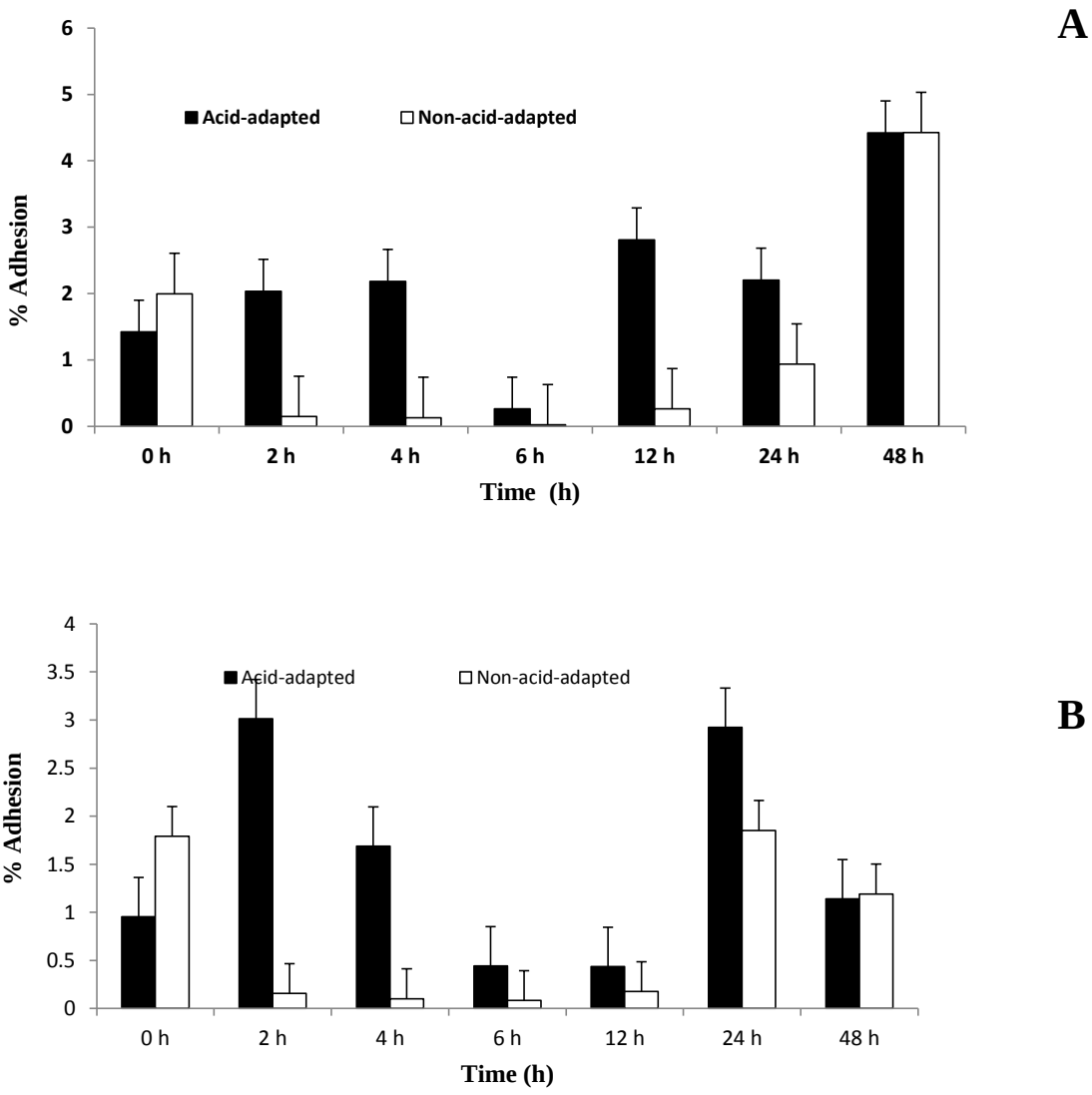

Fig 3.13: Adhesion of acid-adapted and non-acid-adapted *C. jejuni* human strain 81-176 (panel A) and *C. jejuni* poultry strain PRCC (panel B) with an adaptation time of 2 h and subsequent exposure to secondary stress of acid pH of 4.5 for different periods of time ($p<0.05$).

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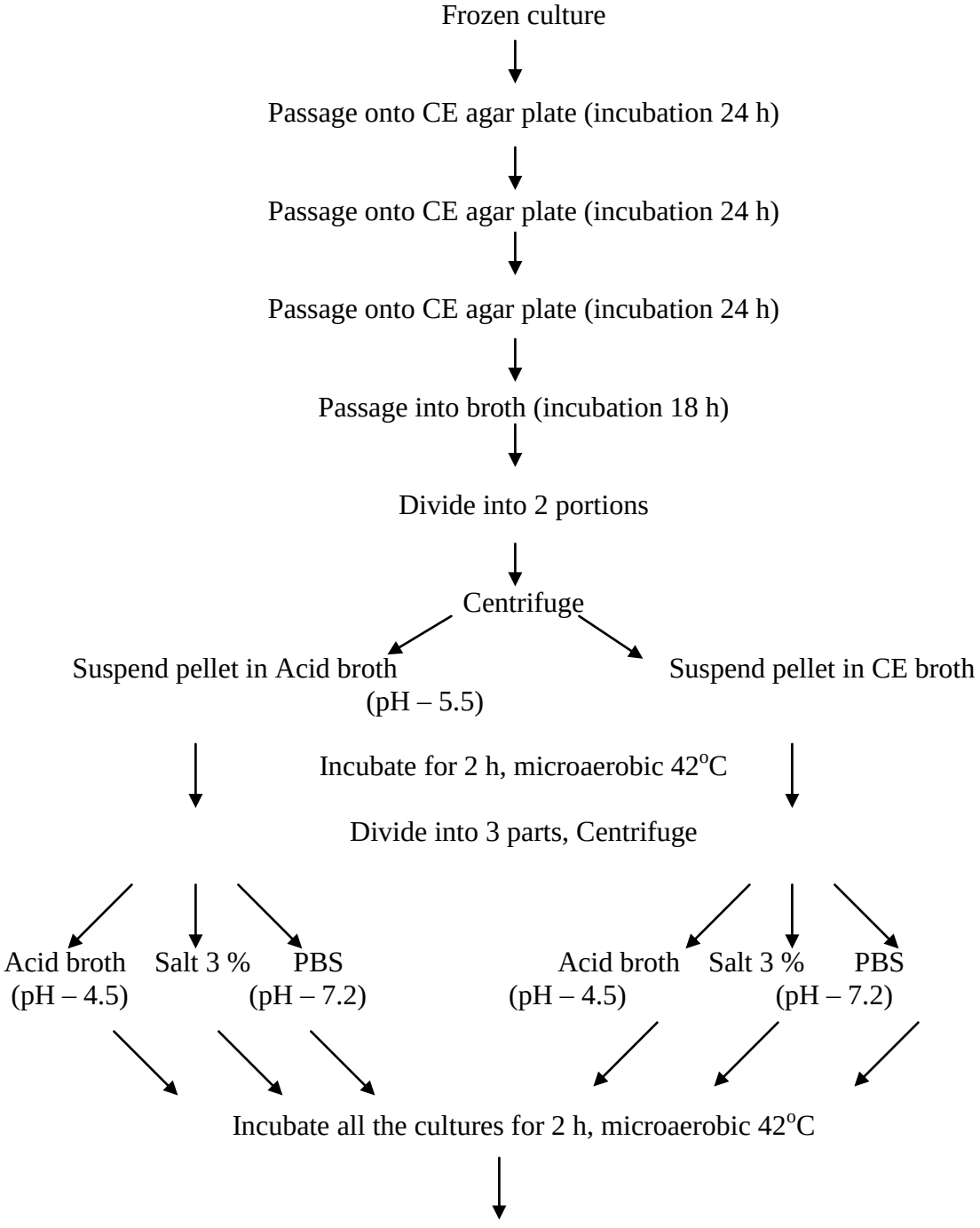
APPENDIX A1

Flow chart of acid-adaptation and exposure of *C. jejuni* cultures to different secondary stresses.



After 2 h all cultures were centrifuged and re-suspended in Basal Medium Eagle (BME) at 1: 10 dilution and these were used for adhesion and invasion assays

APPENDIX A2

Protocol for Adhesion and Invasion of *C. jejuni*

Adhesion:

1. Seeding of 24 well TC plates with 10^5 cells/ well
2. Incubation for 18- 24 h
3. Washing of plates (2 X) with PBS to remove medium
4. Infection with different strains of *C. jejuni* (acid-adapted and non-acid-adapted *C. jejuni* after exposure to different secondary stresses).
5. Incubation of infected INT 407 cells
   (2 h, 37^{o}C in CO_2 incubator)
6. Washing of plates (4 X) with PBS
7. Remove last wash and add 1ml of 0.01% Triton X-100 per well
   (30 min, room temp. with swirling)
8. Mix thoroughly with pipette
9. Determine bacterial count by Standard plating methods

Invasion:

Steps 1 to 6 are the same.

1. Remove last wash and add per well 1 ml of warm fresh BME with 10 % serum and gentamicin (100µg / ml)
2. Incubate plates (2 h, 37^{o}C in CO_2 incubator)
3. Wash twice with PBS
4. Remove last wash and add 1ml of 0.01% Triton X-100 per well
   (30 min, room temp. with swirling)
5. Mix thoroughly with pipette
6. Determine bacterial count by Standard plating methods

CHAPTER 4

EFFECT OF STRESS-ADAPTATION ON ANTIBIOTIC SENSITIVITY PROFILES OF *CAMPYLOBACTER JEJUNI*

ABSTRACT

Campylobacter jejuni is one of the leading causes of human gastroenteritis. *Campylobacter jejuni* requires special conditions and media in the laboratory for its growth. In nature, however, this organism is able to survive in very diverse and hostile environments and produce disease in humans and animals. The different mechanisms by which *C. jejuni* survives stressful conditions in the environment still remain unclear. Stress-adaptation may be one of the factors helping this organism to survive stresses. Some *C. jejuni* strains have been found to have increased antibiotic resistance in last several years. To determine the effect of acid adaptation on the antibiotic sensitivity of *C. jejuni*, 4 different strains of *C. jejuni* (a human isolate and 3 poultry isolates) were exposed to an acid pH of 5.5 and then re-challenged with different stresses. The antibiotic sensitivity profiles of *C. jejuni* after stress-adaptation were compared with antibiotic sensitivity profiles of non-stressed *C. jejuni* using the Kirby Bauer agar disc diffusion assay. The antibiotic sensitivity profiles of the *C. jejuni* isolates used in this study were found to change when the acid-adapted bacteria were subjected to further stresses such as an acidic pH of 4.5, aerobic atmosphere and starvation. In the majority of the cases, antibiotic-resistant *C. jejuni* strains were found to be more sensitive to antibiotics after stress-adaptation, but in a few cases *C. jejuni* showed increased resistance. These results indicate that increasing various stresses in a sequential pattern may, in some cases, reduce antibiotic resistance of *C. jejuni* strains.

KEYWORDS:

Campylobacter jejuni, stress, stress-adaptation, antibiotic resistance, antibiotics.

INTRODUCTION

Campylobacter jejuni is among the leading causes of foodborne bacterial diarrheal disease with approximately 850,000 cases per year in the United States with an associated economic loss estimated to be 1.5 billion dollars (Scharff, 2012). Other possible sequelae of *C. jejuni* infections include Guillain–Barré syndrome (GBS), reactive arthritis, and irritable bowel syndrome (Blaser and Engberg, 2008). Sources of infection for *C. jejuni* are primarily associated with poultry and poultry products since *C. jejuni* are often found as commensals in large numbers in the gastrointestinal tracts of birds (Jacobs-Reitsma, 2000; Corry and Atabay, 2001). *C. jejuni* is predominantly found to cause gastrointestinal enteritis in humans and even a very low dose, as few as 500 organisms, can cause infection (Black *et al.,* 1988). The incubation period of foodborne campylobacteriosis is usually 4 – 5 days but can range from 1 – 10 days (Humphrey *et al.,* 2007). The symptoms associated with this disease include fever, diarrhea, headache, abdominal pain, myalgia, vomiting and blood in feces (Poly *et al.,* 2005). In majority of the infections caused by *C. jejuni*, the use of antimicrobials is not necessary as infected persons usually recover within 5 – 8 days (Black *et al.,* 1988). But in some of the cases, where the infections are not found to subside within 3 – 4 days, antibiotic therapy may be indicated. Erythromycin is the drug of choice, but others such as ciprofloxacin, doxycycline and tetracycline are also used in the treatment of *C. jejuni* infections. Recently, increases in the antibiotic resistance patterns of *C. jejuni* against antibiotics such as ciprofloxacin, tetracycline and erythromycin have been found in *C. jejuni* isolates from food and water sources (Melero *et al.,* 2012; Garin *et al.,* 2012). Presence of such resistant strains in the food chain has raised concerns over the antibiotic treatment of *Campylobacter* infections.

Considering the highly fastidious growth requirements of *C. jejuni*, and the lack of genetic survival mechanisms, the ability of *C. jejuni* to survive outside the host and cause foodborne illness is perplexing. Research shows that some bacterial foodborne pathogens are capable of surviving many of the control measures employed in the food industry by a mechanism known as ***adaptive tolerance response***. Exposure of bacteria to stressful environment induces a response called as the adaptive tolerance response (ATR) which helps the bacteria to survive further homologous or heterologous stresses (Yousef and Courtney, 2003; Ray and Bhunia 2008). *C. jejuni* has been found to induce an adaptive tolerance response when exposed to acid or aerobic conditions (Murphy *et al.*, 2003; Ma *et al.*, 2009), but research involving effects of stress-adaptation on antibiotic sensitivity profiles after *C. jejuni* is exposed to secondary stresses is limited. Understanding stress response on a global level may give insight into how this fastidious organism survives outside the host. When bacteria are exposed to environmental stresses they may undergo phenotypic and genotypic changes to enhance their survival in the stressful environment (Storz and Hengge-Aronis; 2000). These changes may give rise to cross-protection when these bacteria are exposed to secondary stresses which may also subsequently change their antibiotic resistance profiles (Poole, 2012). Exposure of foodborne pathogens such as *E. coli*, *Salmonella Typhimurium* and *Staphylococcus* to sublethal food preservation stresses was found to change their antibiotic sensitivity profiles (McMahon *et al.*, 2007). By studying the effects of an adaptive tolerance response on the antibiotic sensitivity profiles of acid-adapted *C. jejuni* after exposure to various secondary stresses could provide information on the role of stress and stress-adaptation on antibiotic resistance. These results should help in developing better control strategies to reduce/eliminate *C. jejuni* in

processing environments. The aim of this research was to determine whether the ATR induced by different strains of *C. jejuni* on adaptation to a mild acid pH had any effects on the antibiotic sensitivity profiles after they were subjected to secondary stresses including acid, starvation and exposure to oxygen.

MATERIALS AND METHODS

Bacterial strains and growth conditions:

Four *C. jejuni* strains were selected for the present study including a human strain, 81-176, known to cause the disease in human volunteers and three poultry strains. The human strain 81-176 was donated by Dr. Michael Johnson, University of Arkansas, Fayetteville, AR. The poultry strains used were PRCC 3 (pre-chilled chicken carcass), POCC 13 (post-chilled chicken carcass) and RECC 3 (retail chicken carcass), which were obtained and isolated in our laboratory from different stages of poultry processing. Each isolate killed over 92% of HeLa cells when previously tested in an in vitro cytotoxicity assay (Gilbert and Slavik, 2004). The isolates were stored at -80^{o}C in *Campylobacter* enrichment broth, Bolton's (CEB) (Acumedia®) supplemented with glycerol and sub-cultured prior to the stress experiments. Frozen stock cultures were passed twice on *Campylobacter* blood agar plates and then inoculated into CEB broth and incubated in a micro-aerobic atmosphere consisting of 5% oxygen, 10% carbon dioxide and 85% nitrogen at 42^{o}C for 18 h to obtain the early stationary phase cultures for the experiments.

Acid-adaptation of C. jejuni and exposure to secondary stresses:

Early stationary phase (18 h) cultures in *Campylobacter* enrichment broth, Bolton's (CEB) were divided into two portions and centrifuged at 8000 x g for 5 min and subsequently re-suspended either in acid broth (pH 5.5) to obtain acid-adapted cells or in CEB broth to obtain non-stressed cells. Acid broth was prepared by adding hydrochloric acid (HCl) directly to the CEB broth. After an adaptation time of 2 h, the acid-adapted culture was divided into three portions, centrifuged and exposed to the following stresses for a period of 2 h: 1. acid stress (pH 4.5), by re-suspending in CEB broth with a pH of 4.5 and incubating in microaerobic atmosphere; 2. acid and aerobic stress, by re-suspending in CEB broth with a pH of 4.5 and incubating in aerobic atmosphere; 3. starvation stress by re-suspending cells in phosphate buffered saline (PBS) with a pH of 7.2. The non-stressed cultures of each isolate were centrifuged and re-suspended again in CEB to form the control group with no stress. The experimental design is given in the form of a flow chart in figure 4.1.

Determination of antibiotic sensitivity of C. jejuni using disc diffusion assay:

The acid-adapted cultures exposed to different secondary stresses and the non-stressed cultures of *C. jejuni* were used to determine antibiotic sensitivity by the Kirby Bauer agar disc diffusion assay as described by the Clinical and Laboratory Standards Institute (CLSI) using Mueller-Hinton (MH) agar (Difco®) plates. Briefly, the agar disc diffusion assay was performed as follows. Prior to performing the assay, all the cultures were centrifuged and re-suspended in normal saline and the concentration of the cultures were determined by serial dilution and plating on CE agar plates. For the assay, 100 ul of

cell suspension from each of the cultures was inoculated on to MH agar plates. Three to four antibiotic discs were evenly placed on the inoculated plates. Plates were incubated at 37^{o}C for 42-48 h under microaerobic conditions. *Escherichia coli* ATCC 25922, with known antimicrobial susceptibility, was used as the positive control for each of the antibiotics used. The antibiotic discs (BBL^{TM} Sensi-Disc^{TM}, Becton Dickinson) included in this study were ampicillin, 10 µg; chloramphenicol, 30 µg; ciprofloxacin, 5 µg; clindamycin, 2 µg; erythromycin, 15 µg; gentamicin, 10 µg; kanamycin, 30 µg; nalidixic acid, 30 µg; streptomycin 10 µg; tetracycline, 30 µg; and vancomycin, 30 µg. The antimicrobial inhibition zones were measured and interpreted as sensitive (S), intermediate (I) or resistant (R) according to CLSI standards and Huysmans and Turnidge, 1997. The measurement criteria used for measuring the antimicrobial inhibition zones for the different antibiotics used in this study are shown in Table 1.

Statistical analysis:

The experiments were conducted in three independent replicates and the mean antibiotic inhibition zones were calculated using the JMP statistical software package 9.0.2 and the means were compared using a Student's t-test. The results were considered statistically significant with p-values reported at $P<0.05$.

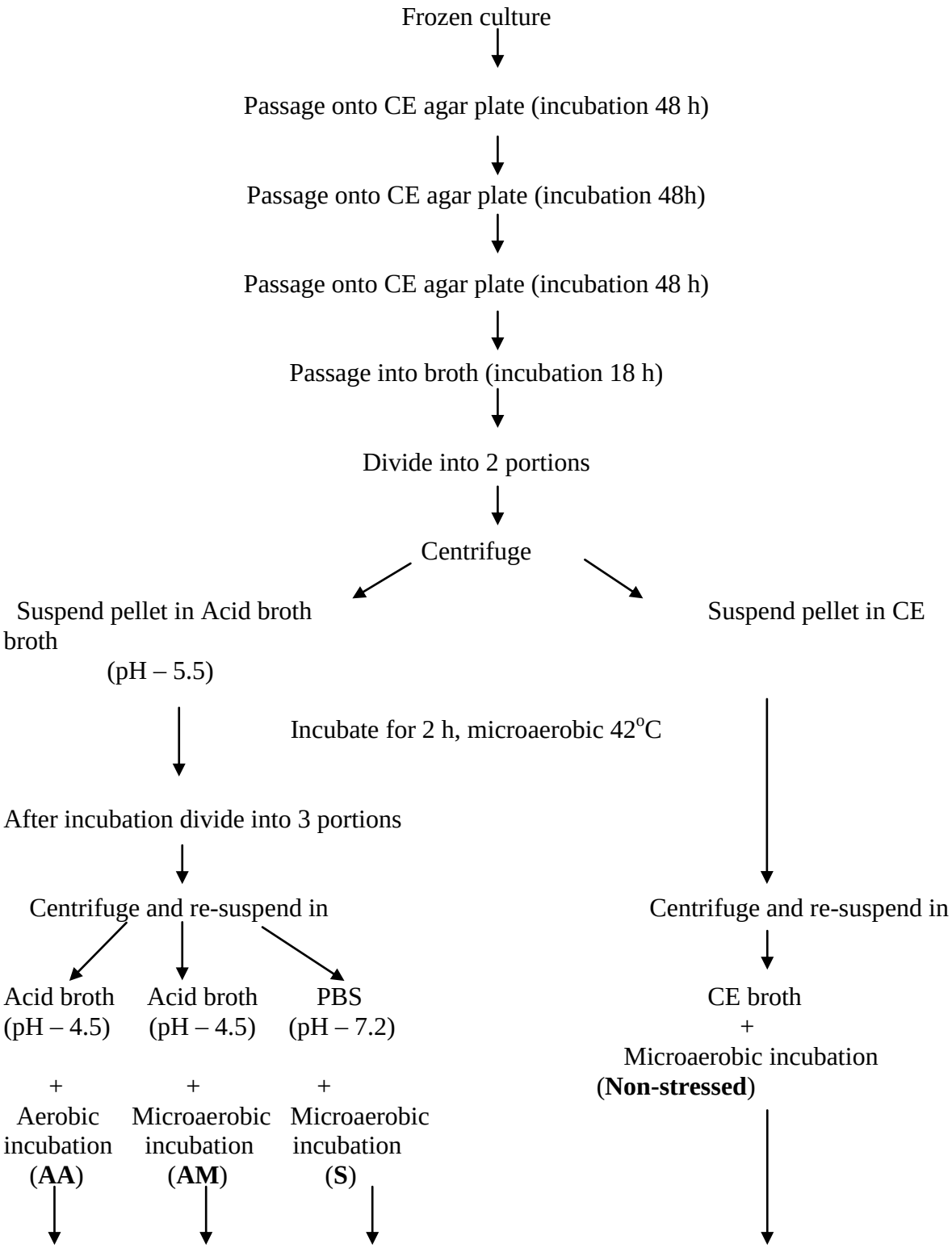


Fig. 4.1: Flow chart of acid-adaptation and exposure of *C. jejuni* cultures to different secondary stresses.

Antibiotics used	Zone diameter measured (mm)		
	Sensitive	**Intermediate**	**Resistant**
Ampicillin, 10 µg	≥ 10	N/A	≤ 9
Chloramphenicol, 30 µg	≥ 23	12-22	≤ 11
Clindamycin, 2 µg	≥18	16-17	≤ 15
Ciprofloxacin, 5 µg	≥ 24	19-23	≤ 18
Erythromycin, 15 µg	≥19	16-18	≤ 15
Gentamicin, 10 µg	≥ 23	N/A	≤ 22
Kanamycin, 30 µg	≥ 18	14-17	≤ 13
Nalidixic acid, 30 µg	≥ 15	N/A	≤ 14
Streptomycin, 10 µg	≥ 15	12-14	≤ 11
Tetracycline, 30 µg	≥ 33	16-32	≤ 15
Vancomycin, 30 µg			

Table 4.1: Disc diffusion criteria used in this study. The antimicrobial inhibition zones for the 11 antibioitcs used in this study were measured and recorded according to CLSI standards and Huysmans and Turnidge, 1997.

RESULTS

For each *C. jejuni* isolate antibiotic inhibition zones to the various antibiotics used were measured and recorded. Acid-adaptation and exposure to secondary stresses produced changes in the antibiotic sensitivity profiles of the human *C. jejuni* isolate, 81-176, for the antibiotics gentamicin, kanamycin, chloramphenicol and tetracycline compared to the control group with no stress (Table 2). All the stresses (starvation, acid and acid + aerobic exposure) changed the antibiotic sensitivity profiles of 81-176 for gentamicin and kanamycin from resistant and intermediate resistance to sensitive. However, for chloramphenicol and tetracycline the profiles were changed to sensitive from intermediate resistance and resistant in response to starvation and acid + aerobic exposure, respectively. Only with one antibiotic, nalidixic acid, did the sensitivity profile of 81-176 change to resistant from sensitive after starvation stress. The other two stresses did not produce any changes compared to the non-stressed 81176.

For the *C. jejuni* poultry isolate from pre-processed chicken carcass (PRCC), the antibiotic sensitivity profiles were changed with 5 of the 11 antibiotics used in the study compared to the control group after acid-adapted *C. jejuni* were given the secondary stresses of either acid or starvation or acid + aerobic exposure (Table 3). The antibiotic sensitivity profile of PRCC to ampicillin changed from sensitive to resistant with acid + aerobic exposure, whereas the acid stress produced resistance to gentamicin and intermediate resistance to kanamycin. Starvation stress was found to produce sensitive populations of PRCC to chloramphenicol, whereas both starvation and acid stresses changed the antibiotic sensitivity profile for nalidixic acid from resistant to sensitive.

Similarly, for the *C. jejuni* poultry isolate from post-processed chicken carcass (POCC), acid-adaptation and exposure to secondary stresses produced changes in the antibiotic sensitivity profiles for 5 of the 11 antibiotics used in the study compared to the control group. All stresses were found to change the resistant profile of non-stressed POCC to sensitive with the antibiotic kanamycin. For the antibiotic nalidixic acid the profile was changed from resistant to sensitive with acid stress, whereas both acid and starvation stresses changed the profile from intermediate resistance to sensitive for streptomycin. Acid + aerobic stress changed the sensitive profile of POCC towards gentamicin to resistant. The ciprofloxacin profile changed from sensitive to intermediate resistance with starvation and to resistant with acid + aerobic exposure.

The antibiotic sensitivity profiles for RECC, the *C. jejuni* poultry isolate from retail chicken carcass, changed with 7 of the 11 antibiotics used in this study compared to the control group after acid-adaptation and exposure to secondary stresses. Exposure to the secondary stress of starvation changed the antibiotic sensitivity profiles of non-stressed RECC from resistant to sensitive for ampicillin and nalidixic acid and to intermediate resistance with ciprofloxacin. For the antibiotics gentamicin and clindamycin, the secondary stresses of acid and acid + aerobic exposure changed the profile from sensitive to resistant, whereas for starvation stress the profile remained sensitive as with the non-stressed control. Acid-adapted RECC exposed to secondary stress changed the sensitive profile to resistant for erythromycin whereas both acid and acid + aeroic exposure changed the profile to intermediate with chloramphenicol.

Antibiotic disc diffusion assay results for *C. jejuni* human strain, 81-176

Antibiotics used	Concentration of antibiotics (in µg)	Non-stressed	Acid-adapted *C. jejuni* 81-176 exposed to different secondary stresses		
			Starvation + microaerobic incubation	Acid + microaerobic incubation	Acid + aerobic incubation
Ampicillin	10 µg	S	S	S	S
Chloramphenicol	30 µg	**I**	**S**	**I**	**I**
Clindamycin	2 µg	S	S	S	S
Ciprofloxacin	5 µg	S	S	S	S
Erythromycin	15 µg	S	S	S	S
Gentamicin	10 µg	**R**	**S**	**S**	**S**
Kanamycin	30 µg	**I**	**S**	**S**	**S**
Nalidixic acid	30 µg	**S**	**R**	**S**	**S**
Streptomycin	10 µg	S	S	S	S
Tetracycline	30 µg	**R**	**R**	**R**	**I**
Vancomycin	30 µg	R	R	R	R

Table 4.2: Antibiotic profiles of *C. jejuni* human isolate 81-176 (S-sensitive, I-intermediate or R- resistant) after acid-adaptation and exposure to secondary stresses. The profiles were compared with the control group with no stress exposure.

Antibiotic disc diffusion assay results for *C. jejuni* poultry strain, PRCC

Antibiotics used	Concentration of antibiotics (in µg)	Non-stressed	Acid-adapted *C. jejuni* PRCC exposed to different secondary stresses		
			Starvation + microaerobic incubation	Acid + microaerobic incubation	Acid + aerobic incubation
Ampicillin	10 µg	**S**	**S**	**S**	**R**
Chloramphenicol	30 µg	**I**	**S**	**I**	**I**
Clindamycin	2 µg	S	S	S	S
Ciprofloxacin	5 µg	S	S	S	S
Erythromycin	15 µg	S	S	S	S
Gentamicin	10 µg	**S**	**S**	**R**	**S**
Kanamycin	30 µg	**S**	**S**	**I**	**S**
Nalidixic acid	30 µg	**R**	**S**	**S**	**R**
Streptomycin	10 µg	S	S	S	S
Tetracycline	30 µg	R	R	R	R
Vancomycin	30 µg	R	R	R	R

Table 4.3: Antibiotic profiles of *C. jejuni* poultry isolate, PRCC (S-sensitive, I-intermediate or R- resistant) after acid-adaptation and exposure to secondary stresses. The profiles were compared with the control group with no stress exposure.

Antibiotic disc diffusion assay results for *C. jejuni* poultry strain, POCC

Antibiotics used	Concentration of antibiotics (in µg)	Non-stressed	Acid-adapted *C. jejuni* POCC exposed to different secondary stresses		
			Starvation + microaerobic incubation	Acid + microaerobic incubation	Acid + aerobic incubation
Ampicillin	10 µg	S	S	S	S
Chloramphenicol	30 µg	S	S	S	S
Clindamycin	2 µg	S	S	S	S
Ciprofloxacin	5 µg	**S**	**I**	**S**	**R**
Erythromycin	15 µg	S	S	S	S
Gentamicin	10 µg	**S**	**S**	**S**	**R**
Kanamycin	30 µg	**R**	**S**	**S**	**S**
Nalidixic acid	30 µg	**R**	**R**	**S**	**R**
Streptomycin	10 µg	**I**	**S**	**S**	**R**
Tetracycline	30 µg	R	R	R	R
Vancomycin	30 µg	R	R	R	R

Table 4.4: Antibiotic profiles of *C. jejuni* poultry isolate, POCC (S-sensitive, I-intermediate or R- resistant) after acid-adaptation and exposure to secondary stresses. The profiles were compared with the control group with no stress exposure.

Antibiotic disc diffusion assay results for *C. jejuni* poultry strain, RECC

Antibiotics used	Concentration of antibiotics (in µg)	Non-stressed	Acid-adapted *C. jejuni* RECC exposed to different secondary stresses		
			Starvation + microaerobic incubation	Acid + microaerobic incubation	Acid + aerobic incubation
Ampicillin	10 µg	**R**	**R**	**R**	**S**
Chloramphenicol	30 µg	**S**	**S**	**I**	**I**
Clindamycin	2 µg	**S**	**S**	**R**	**R**
Ciprofloxacin	5 µg	**R**	**R**	**R**	**I**
Erythromycin	15 µg	**S**	**S**	**R**	**S**
Gentamicin	10 µg	**S**	**S**	**R**	**R**
Kanamycin	30 µg	S	S	S	S
Nalidixic acid	30 µg	**R**	**R**	**R**	**S**
Streptomycin	10 µg	S	S	S	S
Tetracycline	30 µg	R	R	R	R
Vancomycin	30 µg	R	R	R	R

Table 4.5: Antibiotic profiles of *C. jejuni* poultry isolate, RECC (S-sensitive, I-intermediate or R- resistant) after acid-adaptation and exposure to secondary stresses. The profiles were compared with the control group with no stress exposure.

DISCUSSION

Foodborne bacteria encounter a variety of stresses in the processing environments, in foods and inside their hosts. These stresses have been found to induce adaptive responses in these bacteria along with changes in the cell which affects their innate antimicrobial susceptibility (Poole, 2012). Sublethal food preservation stresses such as salt (>4.5%), reduced pH (<5.0) and high temperature (45^{o}C) were found to produce significantly different antibiotic sensitivity patterns for foodborne pathogens such as *E. coli*, *Salmonella Typhimurium* and *Staphylococcus*. Some of these stresses were found to induce permanent changes in antibiotic sensitivity profiles even after removal of the stresses (McMahon *et al.*, 2007). However, **research on the effects of stress-adaptation and subsequent exposure to secondary stresses on the antibiotic sensitivity profiles of foodborne bacteria is limited**. Various types of secondary stresses produce different types of responses in the bacteria including changes in their antimicrobial susceptibilities. In the present study, we observed that all the antibiotics tested except vancomycin showed a difference in their sensitivity profiles which was based on the isolates and acid-adaptation and subsequent exposure to secondary stresses. The difference in the antibiotic sensitivity patterns of the isolates might be due to the difference in the source of their isolation.

Acid-adapted *C. jejuni* human strain (81-176) was found to be resistant to nalidixic acid when it was exposed to the secondary stress of starvation. Starvation is known to activate a stringent response which produces a transcriptional switching resulting in reduced expression of genes affecting growth and increased expression of genes required

for the survival of the bacteria (Chatterji and Ojha, 2001). Stringent response is also known to produce increased levels of alarmone guanosine 5'-(tri)diphosphate 3'-diphosphate [(p)ppGpp] which is known to affect the cell physiology in many ways including antimicrobial susceptibility (Potrykus and Cashel, 2008). Increase in ppGpp under starvation conditions has shown to increase the resistance of *E.coli* toward antibiotics like penicillin, trimethoprim, gentamicin and polymixin B (Greenway, 1999). The stringent response also decreases the production of prooxidant molecules such as 4-hydroxy-2-alkyl-quinoliness (HAQs) and increases the antioxidant defenses which in turn increases oxidative killing by bactericidal antibiotics such as aminoglycosides, B-lactam, cationic and fluoroquinolone groups of antibiotics (Nguyen *et al.*, 2011). In our study, however, when the acid-adapted *C. jejuni* poultry strain, PRCC, was exposed to starvation stress it was found to be more sensitive to nalidixic acid. Similarly acid-adapted *C. jejuni* poultry strain, POCC, was found to be more sensitive to kanamycin when exposed to starvation. It might be assumed that some changes may be happening in these strains during acid-adaptation which might be changing the resistance pattern when they are subsequently exposed to starvation. The *C. jejuni* poultry strain, RECC, however did not show any difference in the sensitivity to the various antibiotics compared to its control.

Exposure to oxygen or an oxidative stress was found to induce the genes of the multidrug efflux system, MaxXY-OprM of *Pseudomonas aeruginosa* that are known to be activated upon exposure to various antimicrobials (Fraud and Poole, 2010). *C. jejuni* also possess several drug efflux pumps of different families, but many of them are yet to be functionally characterized. The main drug efflux pump of *C. jejuni* CMeABC is an RND

(Resistance Nodulation Division) type of efflux transporter which is known to confer resistance to several antibiotics (Lin *et al.,* 2002). This drug efflux pump was also found to be involved in bile resistance as well as colonization of *C. jejuni* in the chicken gut (Lin *et al.,* 2003). In this research, the poultry *C. jejuni* strains were found to be resistant to ampicillin, gentamicin, ciprofloxacin and clindamycin when compared to their non-stressed controls. Hence, it might be assumed that similar to *P.aeruginosa*, the efflux systems of *C. jejuni* may be playing an important role in the antibiotic resistance when these bacteria are exposed to a secondary stress of acid and exposure to oxygen.

The exposure of acid-adapted *C. jejuni* poultry strains to a sublethal pH of 4.5 was found to increase the sensitivity to antibiotics such as nalidixic acid, kanamycin and ampicillin. However, under this condition the retail isolate RECC was found to be more resistant to erythromycin. These changes might be due to sudden mutations in the genome as stress-induced mutagenesis is found to increase when bacteria are exposed to stresses like starvation, exposure to oxygen, low pH, temperature extremes and exposure to antibiotics (Foster, 2007).

CONCLUSION

The results of this research indicate that stresses commonly encountered in the food and poultry processing environments when given in a sequential pattern may give rise to more sensitive populations of *C. jejuni*. However some stresses are found to produce a reverse effect by increasing the resistance of *C. jejuni*, which may help them to survive further stresses such as passage through the gastrointestinal tract. This type of a stress-

adaptation could play a major role in the response of the bacteria towards antibiotics. Future research is needed to determine the types of stresses that could increase the susceptibility of *C. jejuni* towards antibiotics. Further studies could also be conducted to determine the mechanisms involved in the response of *C. jejuni* towards antibiotics under the influence of multiple stresses.

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CHAPTER 5

CONCLUSIONS

CONCLUSIONS

Stress-adaptation of foodborne bacteria is becoming a potential threat to the food industry. Foodborne bacteria are exposed to different types of stresses during production, transportation and storage of food as well as during handling, consumption and digestion of the food. Exposure of bacteria to mild stresses induces an adaptive response which provides protection when they are exposed to homologous or heterologous stresses. Stress adaptation results in complex changes in cell composition and regulation, which help the microorganisms to maintain the physiology of the cell resulting in survival and growth after exposure to various stresses. Stress-adaptation of foodborne bacteria thus poses a serious threat to the food industry and the consumers.

This research studied the effects of stress-adaptation, especially the acid-adaptation of *C. jejuni*, on the virulence and antibiotic profiles of *C. jeuni* after exposure to secondary stresses such as acid (pH 4.5), starvation, salt and exposure to oxygen. The virulence of stress-adapted *C. jejuni* was studied by determining the adhesion and invasion rates using the cell culture line INT 407 (human embryonic intestinal cells). Acid-adapted and non-acid-adapted *C. jejuni* strains exposed to different secondary stresses such as acid (pH 4.5), starvation and salt for a period of 2 h were used for the adhesion and invasion assays. Acid-adaptation was found to increase the adhesion and invasion rates of some *C. jejuni* strains when they were exposed to the secondary stresses. We found that the adhesion and invasion rates were dependent on the survivability of *C. jejuni* strains after exposure to secondary stresses. However, the survival rates as well as the adhesion and invasion

percentages were found to vary with the strain of *C. jejuni*, the time of acid-adaptation, the type of secondary stress and exposure time to the secondary stress.

The effects of acid-adaptation of *C. jejuni* on the antibiotic sensitivity profiles were studied in another study. Acid-adapted *C. jejuni* strains exposed to different secondary stresses such as acid (pH 4.5), starvation and exposure to oxygen for a period of 2 h were used to perform the Kirby Bauer antibiotic disc diffusion assays. The antibiotic profiles of acid-adapted *C. jejuni* exposed to secondary stresses were compared to those of non-stressed *C. jejuni*. Our results indicated that exposure to stresses in a sequential pattern could change the antibiotic sensitivity profiles of *C. jejuni*. Many of the stresses were found to change the antibiotic sensitivity profile of *C. jejuni* to sensitive compared to the control group and, in a few cases, *C. jejuni* was found to become resistant towards some of the antibiotics.