UNIVERSIDADE TÉCNICA DE LISBOA

Faculdade de Medicina Veterinária

"EFEITO DE STRESSE ÁCIDO NA SOBREVIVÊNCIA DE *CAMPYLOBACTER JEJUNI*"

"ACID STRESS RESPONSE IN *CAMPYLOBACTER JEJUNI*"

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DISSERTAÇÃO DE MESTRADO INTREGADO EM MEDICINA VETERINÁRIA

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To my dear mother, family and friends

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"ACID STRESS RESPONSE IN *CAMPYLOBACTER JEJUNI*"

ABSTRACT

Considering that the acid tolerance is an important virulence factor of foodborne pathogens and the expressive increasing of incidence of *Campylobacter jejuni* in recent years as etiologic agent of human campylobacteriosis, this work aimed to evaluate the ability of *C. jejuni* to survive under acid stress. Strains of different origins (reference, turkey isolates) and tolerance to particular stresses (acid and temperature) were tested for survival in liquid defined media with pH values of 5.0 and 7.0 adjusted with 1M HCl. Experimental conditions were performed at 2 different temperatures: 4 ºC and 37 ºC. *C. jejuni* cells were found to be very sensitive to acid stress at 37 ºC, whereas they were more resistant at 4 ºC. A strain effect was observed at 37 ºC. Temperature of 37 ºC combined with acid stress allowed a rapid decrease in the *C. jejuni* population, whereas low temperature (4ºC) considerably decreased the effect of acid stress. The survival curves were either described by the Weibull or traditional first-order model and goodness of fit of these models was investigated. Regression coefficients (R^2), root mean square error (RMSE) and correlation plots suggestted that Weibull model produced a better fit to the data than the traditional model.

Fluorescence Ratio Imaging Microscopy (FRIM) was used to determine intracellular pH (pH_i) as an indicator of the physiological state of *C. jejuni* cells at the single cell level after treatment with hydrochloride acid (1M HCl, pH 4.0) in liquid defined medium. For all the tested strains pH_i of healthy cells was found to be above 7.0. After exposure to HCl in liquid medium an immediate decline in pH_i to 5.5 (detection limit) was observed in the majority of cells (75%) of one strain within 15 minutes. The FRIM results revealed that the subpopulations with $pH_i>5.5$ increased for this strain, especially subpopulations with $5.5<pH_i<6.0$ and $6.0<pH_i<6.5$ following 200 min. of exposure to HCl. This indicates that some strains of *C. jejuni* may employ certain recovery strategies to extrude protons and to increase pH_i.

Keywords: *Campylobacter jejuni*; acid stress; survival; temperature; intracellular pH; Fluorescence Ratio Imaging Microscopy.

"EFEITO DE STRESSE ÁCIDO NA SOBREVIVÊNCIA DE *CAMPYLOBACTER JEJUNI*"

RESUMO

Considerando que a tolerância ao ácido é um importante factor de virulência de agentes patogénicos veiculados por alimentos, condicionando a sobrevivência dos microrganismos, e o expressivo aumento, nos últimos anos, da incidência de *Campylobacter jejuni* como agente etiológico de campilobacteriose humana, este trabalho teve como objectivo avaliar a capacidade do patogénico *C. jejuni* sobreviver em condições de stresse ácido. Estirpes de diferentes origens (referência, isolados de peru) sujeitas a diferentes factores de stresse (acidez e temperatura) foram testadas avaliando-se a sua sobrevivência em meio líquido (pH 5.0 e 7.0). As condições experimentais foram efectuadas a duas temperaturas diferentes: 4 ºC e 37 ºC. Células de *C. jejuni* mostraram-se muitos sensíveis a stresse ácido a 37 ºC, enquanto a 4 ºC foram mais resistentes. O efeito estirpe foi evidenciado a 37 ºC. A temperatura 37ºC em combinação com um pH 5.0 (stresse ácido) causou rápido decréscimo da população de *C. jejuni*, enquanto que a 4ºC o efeito do stresse ácido diminuiu consideravelmente. As curvas de sobrevivência foram descritas pelos modelos Weibull ou pelo modelo clássico de inactivação de primeira ordem, tendo-se concluído através dos coeficientes de regressão (R^2), raízes quadradas do erro médio (RMSE) e gráficos de correlação que o modelo Weibull se adequa melhor aos dados apresentados que o modelo tradicionalmente utilizado.

O racio da emissão de fluorescência medida pela técnica de microscopia óptica invertida foi utilizado para determinar o pH intracelular (pH_i) como indicador do estado fisiológico das células de *C. jejuni*, a um nível celular único, após aplicação de um tratamento com ácido clorídrico (1M HCl, pH 4.0) em meio de cultura líquido. Para todas as estirpes testadas, o pH_i de células saudáveis encontrava-se acima de 7.0. Após exposição a HCl em meio líquido, foi detectado para uma das estirpes, um decréscimo imediato do pH_i para 5.5 (limite de detecção) na maioria das células observadas (75%) ao fim de 15 minutos. Os resultados da medição de fluorescência revelaram um aumento de subpopulações com $pH_i>5.5$ para essa estirpe, nomeadamente subpopulações com $5.5<pH_i<6.0$ e $6.0<pH_i<6.5$ em 200 min. de exposição a HCl. Estes resultados indicam que algumas estirpes de *C. jejuni* poderão utilizar determinadas estratégias de recuperação para exteriorizar protões e elevar o pH_i.

Palavras-chave: *Campylobacter jejuni*; stress ácido; sobrevivência; temperatura; pH intracelular; microscopia de fluorescência.

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ABBREVIATIONS

AR	Acid resistance
ASC	Acidified sodium chloride
ASP	Acid shock protein
ATR	Adaptive tolerance response
BLAST	Basic Local Alignment Search Tool
CF	Carboxyfluorescein
CFA	Cyclopropane fatty acid
CFDA	Carboxyfluorescein diacetate
CFDA-SE	Carboxyfluorescein diacetate succinimidyl ester
CF-SE	Carboxyfluorescein succinimidyl ester
EFSA	European Food Safety Authority
ETC	Electron transport chain
FRIM	Fluorescence ratio imaging microscopy
GADH	Gluconate dehydrogenase
H^+-ATPase	Membrane-bound proton-translocating ATPase
HACCP	Hazard Analysis Critical Control Point
HCl	Hydrochloride acid
HSP	Heat shock protein
LOS	Lipooligosaccharide
OMP	Outer membrane proteins
pH_{ex}	Extracellular pH
pH_i	Intracellular pH
PPSS	Peptone physiological salt solution
$R_{488/435}$	Excitation ratio ($_{488nm\ /\ 435\ nm}$)
RNAP	RNA polymerase
RTE	Ready-to-eat
SOD	Superoxide dismutase
TCA	Tricarboxylic acid
TSP	Trisodium phosphate
UVB	Ultraviolet B radiation
VBNC	Viable but nonculturable
WHO	World Health Organization

1 INTRODUCTION

Campylobacter is recognized as the most frequent agent of foodborne bacterial diarrhoea in humans worldwide. Human cases are mostly caused by *Campylobacter jejuni*, a Gram-negative, spiral, microaerophilic bacterium that exists as a commensal organism in the intestinal tract of many wild and domestic birds and mammals. Recent studies led in Europe demonstrate that the number of declared cases of human campylobacteriosis in Europe is increasing. The incidence rate is 46.1 cases/100,000 people and is higher than that of salmonellosis (34.6/100,000) (European Food Safety Authority [EFSA], 2007).

Campylobacters are much more fragile than *Salmonella* or Gram-positive bacteria, like *Enterococcus*. While most food-borne pathogens are considered to be relatively robust organisms, as a consequence of the necessity to survive under diverse conditions both inside and outside the host, *Campylobacter* species have uniquely fastidious growth requirements and an unusual sensivity to environmental stress (Mead, 2004). Adding to this, *Campylobacter* spp. appear to lack many of the occurring survival mechanisms and adaptive responses that can be correlated with resistance to stress in other foodborne pathogens (Park, 2002). Despite this, *C. jejuni* has the potential for remarkable survival under conditions nonpermissive to growth (Chan, Le Tran, Kanenaka & Kathariou, 2001) being able to persist in the food chain and survive to be regarded as the greatest causative agent of bacterial foodborne illnesss in humans (Humphrey, O'Brien & Madsen, 2007). This is often referred to as the *Campylobacter* conundrum (Jones, 2001) and underlines the fact that the multiple survival mechanisms in the environment of this important human pathogen are poorly characterized.

The nature and the intensity of the stress response may vary between pathogenic species (e.g., *C. jejuni* and *C. coli*) (Chaveerach, ter Huurne, Lipman & van Knapen, 2003; Chaveerach, Lipman & Knapen, 2004; Shaheen, Miller & Oyarzabal, 2006) and, moreover, stress response varies widely between strains of *C. jejuni* (Chan *et al.*, 2001; Cools *et al.*, 2003). Pathogenic campylobacters are known to exhibit a stress response to sublethal environmental stresses (Moen *et al.*, 2005; Gaynor, Wells, MacKichan & Falkow, 2005; Georgsson, Þorkelsson, Geirsdóttir, Reiersen & Stern, 2006; Tangwatcharin, Chanthachum, Khopaibool & Griffiths, 2006; Mihaljevic *et al.*, 2007; Garénaux *et al.*, 2008). These stress responses may enable survival of foodborne pathogens under more severe conditions, such as a variety of environmental or processing parameters (Murphy, Carroll & Jordan, 2003b; Purnell, Mattick

& Humphrey, 2003; Shaheen *et al.*, 2006), and/or enhance virulence. Therefore, understanding the effects of stress on tolerance of *Campylobacter* is important in order to assess and minimize the risk of food-borne illness (Chung, Bang & Drake, 2006).

C. jejuni encounters a wide range of temperature and acid conditions in its contamination cycle and must therefore respond to these conditions. While travelling through the gastrointestinal tract they must endure extreme low pH in the stomach as well as volatile fatty acids present in the intestine and faeces. Even upon exiting a host, enteric organisms confront acid stress in the form of industrial waste, or in decaying organic matter (Bearson, Bearson & Foster, 1997). Organic acids are also used in food processing procedures to control contaminating pathogens on meat surfaces and in animal feeds, as they are found in fermented foods, and in a variety of minimally processed foods. In response to environmental encounters with acid, different pathogenic bacteria have evolved complex, inducible acid survival strategies (Chung *et al.*, 2006). Thus, the ability of *C. jejuni* to survive acid stress is of obvious relevance to its transmission cycle and ultimately to food safety and public health. Low temperature is also widely applied in the food chain, and it is vital that more become known about how this pathogen responds to these conditions with regard to survival and subsequent behaviour, as this has the greatest overall relevance to food safety.

Currently, however, survival of this pathogen under acid conditions remains poorly understood. Understanding these mechanisms of survival is crucial to the design of intervention strategies to reduce *C. jejuni* in the food chain and to reduce the burden of *C. jejuni*-associated disease (Moen *et al.*, 2005). Inactivation and challenge studies are important to know how these organisms will respond to conditions that may be present in the host and the environment, as well as when foods are prepared and/or stored. Additionally, as a species, *C. jejuni* exhibits high genetic heterogeneity (Park, 2005; Wieland *et al.*, 2006; Zorman, Heyndrickx, Uzunovic-Kamberovic & Mozina, 2006). For effective control of resistant pathogens in the food chain, it is necessary that genetics and physiology of resistant strains are studied as well as the mechanisms of survival and resistance development in stressful environment.

The aim of this work was to investigate *Campylobacter jejuni* behaviour under acid stress. The first part of the experimental work focuses on nongrowth survival conditions and emphasizes the interaction between pH and temperature in the experimental design. Various other approaches can be used to investigate the resistance of enteric bacteria to pH stress. A

number of fluorescence techniques have been introduced for assessment of viability and activity of microorganisms, which has contributed to a better understanding of the mechanisms involved in selective survival of microorganisms under different stress conditions (Breeuwer & Abee, 2000; McFeters, Yu, Pyle & Stewart, 1995). The purpose of the present study was also to investigate potential strain variations on *C. jejuni*'s ability of regulating intracellular pH under acid stress conditions on a single cell level applying the principles of Fluorescence Ratio Imaging Microscopy (FRIM).

Strains were selected based on previous data to investigate strain variations in response to acid. The experimental work was divided in two parts:

- Inactivation studies of *C. jejuni* strains from different origins under acid stress conditions, at different temperatures.

- Intracellular pH regulation in *C. jejuni* under acid stress conditions.

The first part of the thesis gives a brief introduction to the organism *Campylobacter jejuni*, their survival potential in food and the environment. In the later chapters of the review more emphasis is given to acid stress response in Enterobacteria and *Campylobacter jejuni*.

2 *CAMPYLOBACTER JEJUNI*

2.1 Pathogenesis

Campylobacters are found in a wide range of animal systems with some causing infections of the reproductive tract of certain domestic species which can lead to either abortion and/or infertility. Others are mainly involved in periodontal diseases. Campylobacters are principally known, however, as zoonotic pathogens. Depending on the country, either *Campylobacter* or *Salmonella* is the most frequently isolated bacterial pathogen from cases of diarrhoea (Tauxe, 2002).

Campylobacteriosis in humans is caused by thermotolerant *Campylobacter* spp. which grow in a temperature range of 30-46ºC. The species most commonly associated with human infection are *Campylobacter jejuni* followed by *Campylobacter coli*, and *Campylobacter lari*, although in the developing world *Campylobacter upsaliensis* is also important (Coker, Isokpehi, Thomas, Amisu & Obi, 2002).

The incubation period averages from two to five days. Patients may have mild to severe symptoms; the common clinical symptoms include watery, often bloody diarrhoea, abdominal pain, fever, headache and nausea (Skirrow & Blaser, 2000). Usually, infections are self-limiting and last only a few days but, in a fraction of patients, serious sequelae occur such as reactive arthritis and Guillain-Barré syndrome (Hannu *et al.*, 2002; Hughes & Cornblath, 2005), a polio-like form of paralysis that can result in respiratory and severe neurological dysfunction and even death. Disease outcome is likely to be dependent on virulence of the infecting strain and host immune status.

2.2 General Characteristics

2.2.1 Taxonomy

C. jejuni belongs to the epsilon class of proteobacteria in the order Campylobacteriales. The genus *Campylobacter* is included in the family Campylobacteriaceae and comprises 18 species and six subspecies (Euzéby, 2008). Table 2.1 shows the current members of *Campylobacter* in the family Campylobacteriaceae.

Table 2.1 Current listing of *Campylobacter* spp. (adapted from Humphrey *et al.* 2007).

Family member	Known source(s)	Disease associations	
		Human	Veterinary
C. coli (includes former *C. hyoilei*)	Pigs, poultry, cattle, sheep, birds	Gastroenteritis, septicaemia	Gastroenteritis, porcine proliferative enteritis
C. concisus	Man	Periodontal disease, gastroenteritis	None at present
C. curvus	Man	Periodontal disease, gastroenteritis	None at present
C. fetus subsp. *fetus*	Cattle, sheep	Septicaemia, gastroenteritis, abortion, meningitis	Bovine and ovine spontaneous abortion
C. fetus subsp. *venerealis*	Cattle	Septicaemia	Bovine infectious infertility
C. gracilis	Man	Periodontal disease, empyema, abscesses	None at present
C. helveticus	Cats, dogs	None at present	Feline and canine gastroenteritis
C. hyointestinalis subsp. *hyointestinalis*	Pigs, cattle, hamsters, deer	Gastroenteritis	Porcine and bovine enteritis
C. hyointestinalis subsp. *lawsonii*	Pigs	None at present	Unknown
C. jejuni subsp. *doylei*	Man	Gastroenteritis, gastritis, septicaemia	None at present
C. jejuni subsp. *jejuni*	Poultry, pigs, cattle, sheep, dogs, cats, water, birds, mink, rabbits, insects	Gastroenteritis, septicaemia, meningitis, abortion, proctitis, Guillain–Barré syndrome (GBS)	Gastroenteritis, avian hepatitis
C. lari	Birds (including poultry), water, dogs, cats, monkeys, horses, seals	Gastroenteritis, septicaemia	Avian gastroenteritis
C. mucosalis	Pigs	None at present	Porcine necrotic enteritis and ileitis
C. rectus	Man	Periodontal disease	None at present
C. showae	Man	Periodontal disease	None at present
C. sputorum bv. Sputorum	Man, cattle, pigs	Abscesses, gastroenteritis	None at present
C. sputorum bv. Faecalis	Sheep, bulls	None at present	None at present
C. upsaliensis	Dogs, cats	Gastroenteritis, septicaemia, abscesses	Canine and feline gastroenteritis
C. insulaenigrae	Seals, porpoises	None at present	None at present
C. lanienae	Cattle, pigs and humans	None at present	None at present
C. hominis	Humans	Gastroenteritis in immunocompromised	
C. canadensis	Whooping cranes	None at present	None at present

Within the species *C. jejuni* two subspecies, ssp. *doylei* (*Cjd*) and ssp. *jejuni*, (*Cjj*) can be distinguished on the basis of nitrate reduction, cephalotin susceptibility or growth at 42°C (Allos, 2001). Nearly all of *C. jejuni* strains isolated are *Cjj*; nevertheless, although *Cjd* strains are isolated infrequently, they differ from *Cjj* in two key aspects: they are obtained primarily from human clinical samples and are associated often with bacteremia, in addition to gastroenteritis. Despite the unusual clinical symptomatology, *Cjd* is isolated infrequently and few strains exist (compared to *Cjj*) for this subspecies (Parker, Miller, Horn & Lastovica, 2007). In this work where *C. jejuni* is mentioned it refers to *C. jejuni* ssp. *jejuni*.

2.2.2 Morphology

C. jejuni is a Gram negative organism which is 0.5 to 5 μm long and 0.2 to 0.8 μm wide (Vandamme, 2000). *C. jejuni* has a single polar flagellum on one or both of its ends (Park, 2002), which gives it its unique, "rapid darting" motility characteristics. It is usually spiral in shape when it is in its normal state (Vandamme, 2000; Park, 2002); however, exposed to oxygen, cells become slightly elongated and less coiled, with decreased motility (Boysen, Knøchel & Rosenquist, 2006); older cultures tend to take on a spherical or coccoid shape mostly due to starvation (Vandamme, 2000). *Campylobacter* spp. are non-forming-spores bacteria. Sometimes when daughter cells remain joined, long spiral forms may be seen.

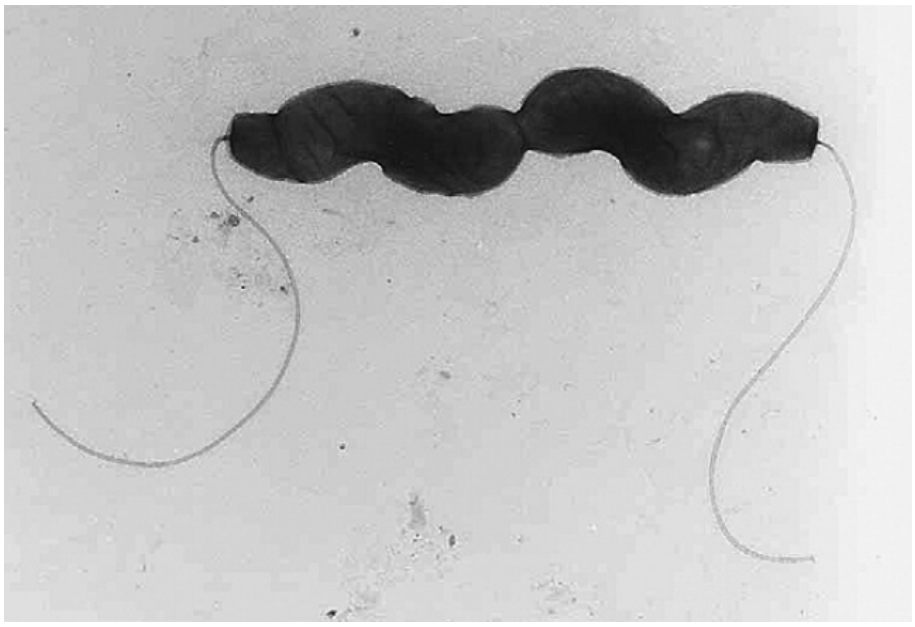


Figure 2.1 Photomicrograph of *Campylobacter jejuni* in the process of dividing. Institute of Food Research, Norwich, UK. (Humphrey *et al.*, 2007).

2.2.3 Identification

Confirmation of *Campylobacter* spp. is mostly based on colony morphology, microscopic appearance and the following phenotypic characteristics: motility, production of oxidase and catalase, and hippurate hydrolysis reaction (Nachamkin, 1995). Characteristic *Campylobacter* colonies are greyish and slightly pink with a metallic sheen on blood-containing agars. *C. jejuni* can be distinguished from other *Campylobacter* spp. on the basis of the hydrolysis of hippurate, as this is the only hippurate-positive species (Vandamme, 2000). However, hippurate hydrolysis negative *C. jejuni* do exist (Totten *et al.*, 1987) making it impossible to differentiate from *C. coli* using purely biochemical tests (Fields & Swerdlow, 1999). Most isolates of *C. jejuni* produce catalase; however, atypical strains are known to be catalase negative so that it is not considered a common feature of all isolates of the species (Hernandez, Owen, Costas & Lastovica, 1991). Additional major phenotypic characteristics that assist in species identification of *C. jejuni* include nitrate reduction to nitrite, absence of urease, nalidixic acid susceptibility, cephalothin resistance and the inability to utilize carbohydrates. Like other *Campylobacter* spp., they give negative results for both the methyl-red and Voges-Proskauer tests, and are unable to hydrolase gelatin.

Biochemical speciation may be supplemented or replaced by molecular methods. A variety of polymerase chain reaction (PCR)-based methods for identifying thermophilic *Campylobacter* spp. have been developed (Best, Fox, Frost & Bolton, 2005).

2.2.4 Growth

Campylobacter spp. are relatively slow-growing, fastidious bacteria that require specialised culture conditions. Most *Campylobacter* spp. are microaerophilic, growing best under reduced oxygen tension, but a few species show a range of oxygen tolerance and some are almost anaerobic. The possession of several oxygen-sensitive enzymes may be one important factor. Oxygen can present more generalized problems owing to the toxic effects of the products of its stepwise one-electron reduction, which results in the formation of the superoxide radical, hydrogen peroxide and the product of their interaction, the highly reactive hydroxyl radical (Kelly, Park, Bovill & Mackey, 2001). *C. jejuni* possesses well known major defence mechanisms against such oxidative stress, including proteins such as superoxide dismutase, catalase and ferritin, yet this organism is still oxygen sensitive (Kelly *et al*., 2001). Campylobacters studied so far have shown high sensitivity to oxidative stress (Boysen *et al.*, 2006; Garénaux *et al*., 2008). Despite their possession of protective enzymes, oxygen radicals

can be easily generated spontaneously in growth media by, for example, exposure to light (Kelly *et al*., 2001). Blood components (usually 5-10% blood) and charcoal are used on a nutritional basal media to remove such toxic oxygen derivates. Optimal growth is obtained in an atmosphere containing approximately 5% oxygen and approximately 10% CO_2 (Humphrey *et al*., 2007), and the growth of some species is enhanced by the presence of hydrogen (Goodman & Hoffman, 1983). Several methods are available to achieve the optimal gas mixture, by appropriate gas-generating envelopes such as Campygen (Oxoid Basingstoke UK), or airtight jars with valves for evacuation and filling of gas, for example the Oxoid anaerobic jars.

Campylobacter jejuni has a restricted temperature growth range and whilst they grow optimally at 42-45 ºC, this organism does not grow at 30 ºC and below or at 47ºC and above (Doyle & Roman, 1981). This in addition to its microarerophilic nature, place severe restrictions on its ability to multiply outside of an animal host and, consequently, unlike most other bacterial foodborne pathogens, these bacteria are not normally capable of multiplication in food during either processing or storage and in the environment (Park, 2002).

The early work of Doyle and Roman (1981) also determined that the best growth at 42 ºC for three strains used occurred in broth adjusted from pH 6.0 to pH 8.0, with the optimum pH ranging from 6.5-7.5. Remarkably, of the strains evaluated, there was one that was able to grow in a medium having an initial pH as low as 4.9 and as high as 9.5. This underlines the importance of strain-to-strain variation in *C. jejuni*'s response. On the other hand, it is not know for certain that the strains they used would also be called *C. jejuni* today.

2.2.5 Metabolism

Understanding aspects of metabolism can shed light on how bacteria grow and persist in their hosts. The ability of a pathogen to alter its metabolism in response to different conditions can also significantly affect its ability to traverse diverse environments. *C. jejuni* is unable to metabolize externally supplied carbohydrates as the key glycolitic enzyme phosphofructokinase is absent (Kelly, 2005); instead they obtain energy from amino acids, or tricarboxylic acid (TCA) intermediates (Velayudhan, Jones, Barrow, David & Kelly, 2004). This organism has a complete citric acid cycle, a complex and highly branched respiratory chain which allows both aerobic and anaerobic respiration with a variety of electron acceptors, and a large complement of regulatory functions. The *C. jejuni* NCTC 11168

genome (Parkhill *et al.*, 2000) predicts orthologues of several membrane-bound oxidoredutases (e.g. hydrogenase and dehydrogenases for succinate, formate, lactate, sulphite and malate) that may catalyse reactions that donate electrons to the menaquinone pool, initiating electron transport and energy conservation through a highly branched electron transport chain (ETC) (Kelly, 2005) These properties enable it to colonize and survive in a number of environments in addition to the mammalian or avian gut (Kelly *et al*., 2001).

2.3 Epidemiology

2.3.1 Incidence

Campylobacteriosis has been highlighted as the most frequently reported zoonotic disease in humans within the EU (EFSA, 2006). In most European countries, the number of reported cases of Campylobacteriosis increased during the 1990s, with a total of 200,122 cases of Campylobacteriosis in humans being reported in 22 Member States (MS) and two non-MS in 2005 (EFSA, 2006). The overall incidence of Campylobacteriosis was 51.6 per 100,000 inhabitants, with a remarkably wide range of variation in the incidence among the reporting countries, as Poland reported 0.1 cases per 100,000 inhabitants, France 3.3 and Czech Republic 302.7 (EFSA, 2006). No data were available from Greece, Italy and Portugal. In 2006, a total of 175,561 confirmed cases of Campylobacteriosis were reported from 21 MS (EFSA, 2007). The EU incidence was 46.1 per 100,000 population ranging from 0.3 – 220.2. There was a drop in the incidence compared to 2005, which is primarily explained by decreases in the number of reported cases in Czech Republic and Germany (EFSA, 2007). Campylobacteriosis is also one of the most common intestinal disorders in non-European countries. In 2001, the incidence was 125 cases per 100,000 inhabitants in Australia and 14 in the United States (Vally *et al.*, 2005). The highest national rate of reported Campylobacteriosis in the developed world is in New Zealand, exceeding 400 cases per 100,000 inhabitants (Baker *et al.*, 2006). However, these figures take no account of differences in healthcare systems or laboratory practices among the countries.

2.3.2 Sources and transmission of infection

Most (85-95%) human infections involve *C. jejuni*, with *C. coli* accounting for the majority of the remainder (Friedman, Neimann, Wegener & Tauxe, 2000); however, *C. coli* strains comprise almost 20% of the human clinical isolates (Gürtler, Alter, Kasimir & Fehlhaber, 2005). Many meat animals such as swine, sheep and cattle are regularly colonized with

Campylobacter spp. (Stanley & Jones, 2003; Nielsen, 2002). *C. jejuni* is more commonly detected among poultry and cattle than *C. coli*; however, this is not the case with swine where *C. coli* is more common (Payot, Dridi, Laroche, Federighi & Magras, 2004; Boes *et al.*, 2005). *C. jejuni* and *C. coli* can cause severe disease in infected people but are carried in the intestinal tracts of all types of domestic livestock and many wild animals, almost always without any harmful effects (asymptomatic carriers). This carriage does have major consequences for human health in terms of food-borne disease. The differences in pathogen behaviour in man and in animals are not yet fully understood but are likely to be due to differential bacterial gene expression in different hosts (Humphrey *et al.*, 2007).

Campylobacter jejuni has a very low infectious dose (500 cells) (Robinson, 1981) and therefore it is of concern as a foodborne pathogen. Although outbreaks of infection account for a small fraction of *Campylobacter* infections in humans (Frost, Gillespie & O'Brien, 2002), consumption of unpasteurized milk is the most frequently reported cause of outbreaks of infection (Gillespie, Adak, O'Brien & Bolton, 2003). In most cases when human infection by *Campylobacter* is concerned, the cases are classified as sporadic making it extremely difficult to track the source. Sources of sporadic infection include sausages or red meat (especially in Scandinavian countries), contaminated water, contact with pets (especially birds and cats), and international travel (Neimann, Engberg, Molbak & Wegener, 2003).

2.3.2.1 Poultry

As a result of contamination at preharvest and harvest levels, foods of animal origin, in particular poultry have been identified as the major source of infection of this pathogen (Corry & Atabay, 2001).

Campylobacters in birds are isolated in the large intestine, ceacum and cloaca (Corry & Atabay, 2001). In broiler chicks, intestinal colonization is usually detected after day seven. Once colonized, the birds remain asymptomatic until they reach maturity. Previous surveillance studies have reported 100% colonization of flocks (Jacobs-Reitsma, 1997) however, the exact route of transmission of *Campylobacter* to these poultry flocks remains unclear. The external environment around the broiler house is thought to be the most important source of campylobacters. A study has reported that flies may be an important source of *Campylobacter* infection of broiler flocks in summer (Hald *et al.*, 2004).

Contaminated water, flock thinning and carry over from a previous flock have all been suggested to contribute to infection of poultry (Humphrey *et al.*, 2007).

Various on-farm strategies have been advanced to reduce the incidence rates of poultry contamination: introduction of competing microbial populations into newly hatched chicks, chlorination of poultry drinking water, vaccination and treatment with bacteriophagues or selective breeding of poultry for resistance to pathogens (Wagenaar, Mevius & Havelaar, 2006). Sound management practices incorporating good husbandry and hygiene practices also play a part in limiting the occurrence of *C. jejuni* in poultry flocks (Kazwala, Jiwa & Nkya, 1993). Farms that use these practices tend to have lower rates of intestinal colonization with *Campylobacter* spp. (Humphrey, Henley & Lanning, 1993).

Mass raising of poultry in closed houses, spread of the bacteria during catching and transport to the slaughterhouses and a slaughter technology which give rise to gross faecal contamination, all result in high number of *Campylobacter* on the finished product (Humphrey *et al.*, 2007). During the slaughter process campylobacters are easily spread from the intestinal content to carcasses and may spread to poultry flocks free of *Campylobacter* during processing. In a recent study of Klein, Reich, Beckmann and Atanassova (2007), out of 99 samples examined, collected at a German poultry slaughterhouse, 51 (51.5 %) were positive for *Campylobacter*, with bacterial counts ranging from $\log_{10}$ 6.5 cfu sample^{-1} for carcasses to $\log_{10}$ 3.6 cfu ml^{-1} for scalding water. Strains found in cloacal swabs before processing could also be isolated from carcasses at different processing steps. The highest concentration of *Campylobacter* is therefore, found on meat directly after processing. In all subsequent steps in the food chain (for example transportation to retail refrigerator storage), the concentration may stabilise but is more likely to decrease, due to die-off of the bacteria (Wagenaar *et al.*, 2006).

There is no doubt that poultry is a major source of campylobacters (Jørgensen *et al.*, 2002). Although all commercial poultry species can carry campylobacters, the risk is greater from chicken because of the high levels of consumption (Humphrey *et al.*, 2007). Two additional pieces of evidence support the thesis that poultry is an important source of human *Campylobacter* infection (Humphrey *et al.*, 2007). The first comes from Belgium and occurred when Belgian poultry and eggs were withdrawn in May/June 1999 because of contamination with dioxins (Vellinga & Van Loock, 2002). There was a coincident 40% reduction in human *Campylobacter* cases. The second piece of evidence comes from Iceland.

In common with many other Nordic countries chicken was sold frozen in Iceland prior to 1996. However, increased consumer demand for poultry and market driven pressures led to the sale of chilled chicken after 1996. Following this, human *Campylobacter* infections increased and peaked in 1999, at a rate of 116/100,000. At this time 62% broiler carcass rinses were positive for campylobacters. A number of preventative measures were introduced, including improving biosecurity on farms, freezing of animals from flocks testing positive and public education. In 2000 human infection dropped to 33/100,000 and only 15% of broiler carcass rinses were *Campylobacter*-positive (Stern *et al.*, 2003). No specific measure was identified as contributing to the fall in cases but the combination of measures was effective. Today there is already sufficient data which support that freezing of carcasses lead to a significant reduction of *Campylobacter* counts after storage of frozen carcasses (Georgsson *et al.*, 2006).

2.3.2.2 Other animals

Other meat animals such as pigs and cattle have also been found to have the gastrointestinal tracts frequently colonized by campylobacters (Boes *et al.*, 2005). Several data suggest that *Campylobacter* of bovine origin contributes significantly to human illness (Nielsen *et al.* 2000). It is believed that these animals acquire the organisms by contact with a contaminated environment. Humphrey and Beckett (1987) demonstrated a link between the consumption of water from natural sources and the presence of campylobacters in dairy cows. Some instances of shellfish contamination due to harvesting of these shellfish in *Campylobacter* contaminated waters has also been reported (Wilson & Moore, 1996).

2.3.2.3 Food

Campylobacter infections are usually a result of consumption of inadequately cooked foods of animal origin. There has been a strong association between *Campylobacter* infection and handling and consumption of raw or undercooked poultry (Friedman *et al.*, 2000). In general, the frequency of contamination of red meat products at retail is lower than that seen in poultry (Humphrey, Mason & Martin, 1995). The slower rate of slaughter in red meat abattoirs will be a factor in this. The most important reason for the differences between red and white meat, however, is that carcasses of the former will be subjected to an extended chilling prior to entry into the food chain. Of the many stresses experienced by these pathogens in food production, desiccation appears to be the most damaging and campylobacters survive poorly on dry

surfaces. This means that when red meat carcasses are chilled the numbers of campylobacters present on exposed surfaces will be markedly reduced by drying (Humphrey *et al.*, 2007).

Another common related source is foods that have been infected by cross contamination in kitchens during food preparation (Cogan, Bloomfield & Humphrey, 1999). Given the high numbers of the pathogen on the meat surface in comparison with low levels of internal contamination, it has been concluded that cross-contamination during preparation of contaminated chicken is a more important pathway for consumers' exposure to *Campylobacter* than the consumption of undercooked meat (Luber & Bartelt, 2007). There have been reports of the incidence of *Campylobacter* in raw milk in the past (Gillespie *et al.*, 2003) possibly due to faecal contamination and more rarely due to udder infections with *C. jejuni* (Orr *et al.* 1995).

Although limited, *Campylobacter* has been found on some fresh fruits and vegetables (Buck, Walcott & Beuchat, 2003). There have been some reported incidences of *Campylobacter* in some modified atmospheric packaged ready-to-eat (RTE) foods (Phillips, 1998).

C. jejuni can enter the water supply, possibly by excretion of various animals or through waste from animal operations or sewage being dumped into the water system to name a few. It can associate with protozoans, such as freshwater amoebae, and possibly form biofilms. Figure 2.2 summarizes the sources and outcomes of *Campylobacter jejuni* infection.

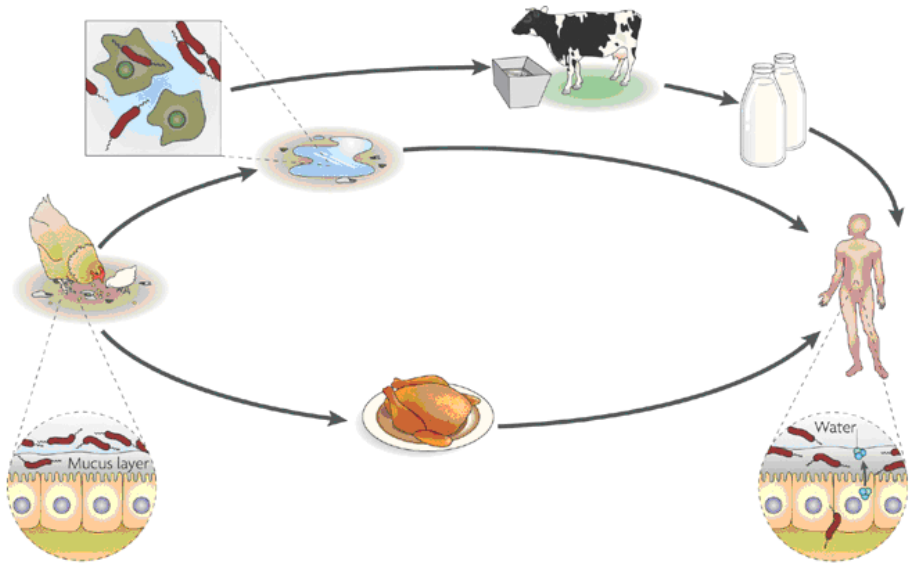


Figure 2.2 The sources and outcomes of *Campylobacter jejuni* infection (Young, Davis & DiRita, 2007).

3 LITERATURE REVIEW OF STRESS RESPONSE AND RESISTANCE

3.1 Campylobacters in food supply

3.1.1 Food processing control measures

In general, foods are made safe through inactivation, e.g. heat treatment, and/or through inhibition of growth to prevent multiplication of pathogens to harmful levels. However, since campylobacters remain a concern even at low levels, their presence in foods at the point of consumption must be prevented, so growth inhibition is not relevant as a method of control (Humphrey *et al.*, 2007). Compared to other pathogenic bacteria campylobacters are relatively heat sensitive, so commercial heat processes set within HACCP framework should guarantee control of these pathogens. Modelling pathogen behaviours in the food chain as a means of identifying the most effective and cost-effective control measures is of irrefutable value. Such approach is possible with campylobacters. However, these pathogens are inherently variable and a large body of data, using many strains examined under a multiplicity of conditions, is needed before modelling can be used with the necessary confidence (Humphrey *et al.*, 2007).

3.1.1.1 Control during poultry processing

Avoidance of fecal contamination during evisceration is an important control measure. Owing to the high concentration of *Campylobacter* in the intestines, in particular, the ceaca, the outside surfaces of chicken carcasses also become contaminated during processing. Carcasses from *Campylobacter*-negative broilers can be contaminated by machinery when they are processed after a positive flock. Critical control points and good manufacturing practices identified to prevent contamination of carcasses during processing (e.g. scalding, defeathering) include temperature controls (washer and product), chemical interventions, water replacements, counter-flow technology in the scald tank and chiller, equipment maintenance, chlorinated water sprays for equipment and working surface, the use of chlorine in process water and removal of unnecessary carcass contact surfaces (Mead, Hudson & Hinton, 1995).

Studies indicate that reduction in bacterial numbers obtained when carcasses are scalded may be beneficial in terms of reducing the likelihood of cross contamination during subsequent processing or in the domestic kitchen but it does not render the product '*Campylobacter*-free'

as it can be observed in Figure 3.1 (Purnell *et al.*, 2003). Significant variation in *Campylobacter* survival can be observed after the same temperature/time treatment is applied.

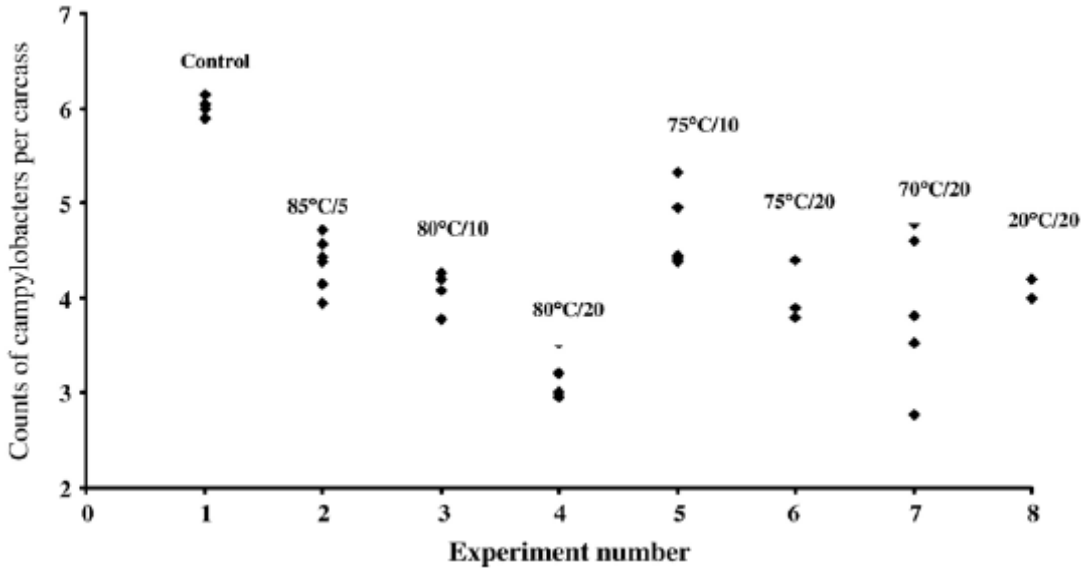


Figure 3.1 Mean $\log_{10}$ counts of *Campylobacter* from broiler carcasses before and after various water treatments. Counts are from a single 300 ml rinse of which serial dilutions were made and plated on selective agar. Each point represents an individual carcass. Treatments are given as temperature of exposure in °C followed by the length of exposure in seconds. Data from Purnell *et al.* (2003).

Table 3.1 gives details of the death rates of *C. jejuni* isolates under conditions relevant to food production. Data are taken from an International Commission on Microbiological Specifications for Foods (ICMSF) publication (Anonymous, 1996) and should be used as a guide only. Data do not take into account either strain-to-strain variation or the recovery and challenge methods used.

Table 3.1 Death rates in Log_{10} units of *Campylobacter jejuni* in food production-relevant environment[a]

Environment	Temperature	Death rate ($\log_{10}$)
Skim milk	−20	−4.4 to −7.5 over 5 days
Ground chicken	−18	−1.5 to −1.6 over 5 days
Raw milk	5	−2.8 over 2 days
Ground chicken	4	−0.5 to −2.0 over 7 days
Chicken scald water	52	−1.0 over 9 minutes
Ground beef	56	−1.6 to −1.04 per minute
Lamb cubes	60	−5 to −3.8 per minute
Ground cod with 0.5% NaCl	10	−1.6 over 5 days
Ground cod with 2.5% NaCl	10	−2.8 over 5 days
Egg white at pH 9.3	42	−7.1 over one day
Yoghurt at pH 4.4–5.4	NS[b]	−7.4 per hour
Ground turkey and 0.32 kGy	−30	−1.0

[a] from Anonymous (1996)
[b] Not stated.

3.1.1.2 Carcass decontamination methods other than heat

Chemical treatment of carcasses is not permitted in the EU but chlorine can be used in other countries such as the United States and Brazil. Oyarzabal, Hawk, Bilgili, Warf and Kemp (2004) found that the post-chill application of acidified sodium chlorite (ASC) to chicken carcasses caused a significant reduction in *Campylobacter* numbers and in the number of contaminated carcasses. A recent study also suggests that introduction of antimicrobial treatments with ASC, trisodium phosphate (TSP) and ASC followed by TSP into poultry processing systems could provide an added measure of safety (Özdemir, Gücükoğlu & Koluman, 2006).

There are several organic acids that have proven to be effective in poultry processing such as, acetic acid, lactic acid, citric acid and succinic acid. In some studies, addition of 0.1% acetic acid to scald water was found to reduce the level of *C. jejuni* by 1.5 $\log_{10}$ CFU/ml (Okrend, Jonhston & Moran, 1986). Several studies have shown the sensitivity of *Campylobacter* spp. to organic acids, such as formic, acetic, ascorbic, and lactic acids (Chaveerach, Keuzenkamp, Urlings, Lipman & van Knapen, 2002; Chaveerach *et al*., 2003).

Rapid freezing of chicken carcasses may also offer additional control measures (Bhaduri & Cottrell, 2004; Sandberg, Hofshagen, Ostensvik, Skjerve & Innocent, 2005). Georgsson *et al.* (2006) recently studied the effects of freezing, method of thawing, and duration of frozen storage on levels of *Campylobacter* spp. Their findings warrant consideration of the public health benefits related to freezing contaminated poultry prior to commercial distribution to reduce *Campylobacter* exposure levels associated with contaminated carcasses. Although *C. jejuni* can still be isolated from frozen meats and poultry products (Bhaduri & Cottrell, 2004; Sandberg *et al*., 2005; Georgsson *et al.*, 2006), freezing significantly reduces their survival (Chan *et al.*, 2001; Moorhead & Dykes, 2002; Zhao, Ezeike, Doyle, Hung & Howell, 2003; Georgsson *et al.*, 2006). These data confirm that frozen poultry poses a significant lower risk to health than fresh meat. Whilst several factors, including ice nucleation and dehydration, have been implicated in the freeze-induced injury of bacterial cells, more recently oxidative stress has been shown to be a mechanism that contributes to freeze-thaw induced killing of campylobacters, since it has been predicted that an oxidative burst occurs upon thawing (Park, Grant, Davies & Dawes, 1998). In addition, a superoxide dismutase (SOD)-deficient mutant has demonstrated sensitivity to freezing and thawing (Stead & Park, 2000).

3.1.2 Factors affecting survival of *C. jejuni*

Campylobacter spp. have been reported to survive in water, at low temperatures, for up to 4 months (Rollins & Colwell, 1986; Buswell *et al.*, 1998; Hazeleger, Wouters, Rombouts & Abee, 1998), during processing (Cools *et al.*, 2005) and in the environment in general (Park, 2002). Kärenlampi and Hänninen (2004) showed that *C. jejuni* can survive on fresh produce long enough to pose a risk to the consumer. Survival times will depend on the environmental stresses present, on the food matrix involved and the conditions under which foods are stored.

Campylobacters can respond to changes in pH, temperature and available oxygen although the consequences that this has for food safety have yet to be examined. Campylobacters will not survive pasteurisation treatments or proper cooking (Sorqvist, 1989). Also, *C. jejuni* shows an increased susceptibility to slow-air-drying on surfaces (Kusumaningrum, Riboldi, Hazeleger & Beumer, 2003), poorly survives Ultraviolet B (UVB) exposure (Obiri-Danso, Paul & Jones, 2001), hydrostatic pressure (Solomon & Hoover, 2004), as well as, low pH of the microenvironment (Waterman & Small, 1998). Campylobacters are also particularly sensitive to osmotic shock (Hamedy, Bori, Froeb-Borgwardt & Alter, 2005). Nutrient insufficiency has been shown to be the most powerful stress factor significantly affecting *C. jejuni* culturability and viability, as well as, adhesion and invasion properties (Mihaljevic *et al.*, 2007). When challenged in broth culture, campylobacters are more sensitive to heat and acid, for example, than *Salmonella* or *E. coli* (Humphrey *et al.*, 2007). This may not reflect what happens in food as the data on the heat treatment of chickens (Figure 3.1) suggest. Additionally, the methods used in many studies are not sufficiently sensitive to recover damaged cells and this may lead to an underestimation of resistance. Chicken juice has been proved to be a food-based model system suitable to study survival of *C. jejuni* at low temperatures (5 and 10ºC) and heat stress (48ºC) (Birk, Ingmer, Andersen, Jørgensen & Brøndsted, 2004). The liquid model with chicken juice was also found to be the best model system to study the freezing tolerance of *C. jejuni* when compared to chicken skin surface (Birk *et al.*, 2006).

Despite the reported sensitivity of this pathogen to the extra-intestinal environment, it seems clear that its infection potential is not compromised by such exposures. A myth has developed that thermophilic campylobacters are sensitive to conditions outside the host. This does not seem to be coincident with the fact that these bacteria are able to infect approximately 1% of the population of Western Europe largely as a result of the consumption of contaminated food (World Health Organization [WHO], 2000).

The increasing incidence of human enteric campylobacteriosis indicates that *Campylobacter* has developed mechanisms for survival such as entry into a viable but nonculturable state (Baffone *et al*., 2006), the transition from rod to coccoid shape (Moran & Upton, 1987), biofilm mode of growth (Joshua, Guthrie-Irons, Karlyshev & Wren, 2006) or extreme heterogeneity of the isolates, mainly for *C. jejuni* (Zorman *et al.*, 2006).

3.1.2.1 Viable but nonculturable state (VBNC)

In the adaptation to coccoid morphology, *Campylobacter* spp. may lose their ability to grow on media, as they can enter a viable but non-culturable stage (Baffone *et al*., 2006). This has been suggested to be a dormant state required for survival under conditions not supporting growth, e.g. during transmission or storage (Rollins & Colwell, 1986). However, the coccoid form is not necessarily associated with non-culturability. The role of these coccoid forms in the pathogenesis of *Campylobacter* is not known and the question whether or not VBNC *Campylobacter* is capable of causing infection is unclear. However, it has been reported that the coccoid forms retain virulence (Oliver, 2000). In a recent study, VBNC were unable to revert to the viable *Campylobacter* form and colonise chicken gut with normal caecal flora (Ziprin & Harvey, 2004).

The VBNC state in bacteria (reviewed by Oliver, 2005) remains the subject of much controversy, but it is clear that many of the techniques used to assess culturability are sub optimal. In this context, in the experimental work viability is not distinguished from culturability. A viable cell is a cell that will proliferate and produce a colony after serial dilutions and plating.

Chaveerash *et al.* (2003) studied the culturability of ten strains of *C. jejuni* and *C. coli* after the bacteria were exposed to acid conditions for various periods of time. *Campylobacter* cells could not survive 2 h under acid conditions (formic acid at pH 4). The ten *Campylobacter* strains could not be recovered, even when enrichment media were used. Viable cells, however, could be detected by a double-staining technique, demonstrating that the treated bacteria changed into a viable but nonculturable (VBNC) form (Figure 3.2).

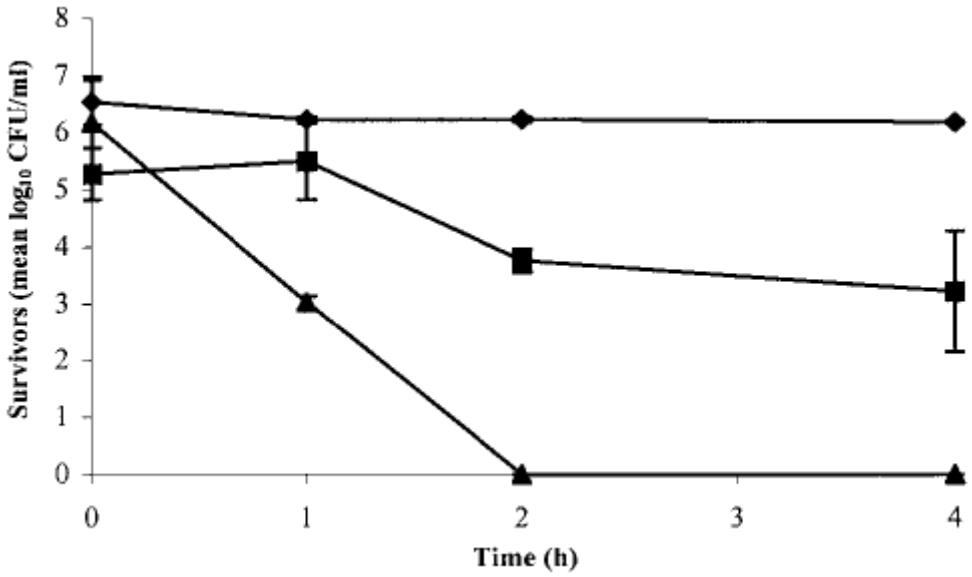


Figure 3.2 Numbers of total (♦), viable (■), and culturable (▲) cells of *C. jejuni* C350 over time. The bacteria were incubated with acidified Mueller-Hinton broth (formic acid at pH 4), and the double-staining (5-cyano-2,3-ditolyl tetrazolium chloride [CTC]–4',6'-diamidino-2-phenylindole [DAPI]) and culture technique was used (Chaveraash *et al.*, 2003).

3.2 Stress responses in food and the environment

3.2.1 Factors affecting stress response

Despite their importance as human pathogens little is known about how campylobacters cope with hostile conditions in the transmission chain from animals to humans and how these bacteria persist in foods or non-food environments. There is similarly scant information about the molecular mechanisms that enable *Campylobacter* strains to survive environmental stress conditions relevant to food production (Humphrey *et al.*, 2007).

3.2.1.1 Genetic Variability

C. jejuni, along with other members of the order Campylobacteriales (e.g., *Helicobacter pylori*, *Wolinella succinogenes*) has a small genome (about 1700 kilobase pairs) and can establish long-term associations with their hosts, sometimes with pathogenic consequences. The small size of the genome is perhaps reflected in a parasitical life-style as these related organisms appear to be host adapted and can establish and maintain their niches without generating a response in the host that is sufficient for clearance (Young *et al.*, 2007).

The sequence of the first *C. jejuni* genome revolutionized the *Campylobacter* research field in 2000 (Parkhill *et al.*, 2000), and the relatively small size of the genome has facilitated

additional genome sequencing (Poly & Guerry, 2008). Today a total of 8 *C. jejuni* genomes are available, either on websites (http://msc.tigr.org/campy/index.shtml) or published (Parkhill *et al.*, 2000; Fouts *et al*., 2005; Hofreuter *et al.*, 2006; Poly *et al.*, 2007).

Analysis of several sequenced microbial genomes has revealed a close relationship between the size of the genome and the proportion of the genome devoted to regulatory genes (Neidhardt, 2002). The genome of *C. jejuni* encodes 1654 proteins of which only about 3% have putative regulatory or signal transduction functions (Parkhill *et al.*, 2000; http://www.sanger. ac.uk/Projects/C_ jejuni). The GC content ranges from 29 to 35 % (Fouts *et al.*, 2005). This organism is regarded as having a restricted capacity for genetic regulation and adaptation to environment.

However, *C. jejuni* displays extensive genetic variation, which has arisen from intragenomic mechanisms, as well as genetic exchange between strains (Young *et al*., 2007). Sequencing the genome has revealed the presence of hypervariable sequences that consist of homopolymeric tracts[1], at which high frequency insertion and deletion frameshift mutations occur (Parkhill *et al.*, 2000; Park, 2002). Genome sequence data has also indicated that the high frequency of variation within these sequences may be partly due to the lack of clear homologues of many *E. coli* DNA-repair genes (Parkhill *et al.*, 2000). Most of the hypervariable sequences that have been found are in regions that encode proteins that are involved in the biosynthesis or modification of surface-accessible carbohydrate structures, such as the capsule, lipooligosaccharide (LOS)[2] and flagellum (Parkhill *et al.*, 2000).

C. jejuni is also naturally competent, meaning that it can uptake DNA from the environment. Flocks are usually infected with multiple strains (Jacobs-Reitsman, 2000). This leads to recombination between strains, which allows the generation of even more genetic diversity (Young *et al*., 2007). The horizontal transfer of both plasmid and chromosomal DNA occurs both *in vitro* and during chick colonization, which indicates that natural transformation could have an important role in genome plasticity and in the spread of new factors such as antibiotic resistance, even in the absence of selective pressure (de Boer *et al.*, 2002; Wilson *et al.*, 2003; Avrain, Vernozy-Rozand & Kempf, 2004). This diversity is evidenced by significant strain-to-strain variability in virulence and tolerance to particular stresses (Park *et al.*, 2005). This implies that strains can have important differences in their genetic makeup and this is

[1] A stretch of DNA that contains multiple repetitions of a single nucleotide.
[2] Found in the outer leaflet of the outer membrane of some Gram-negative bacteria, it consists of lipid A linked to a polysaccharide, but lacks the O-specific polysaccharide of the LOS that is found in other Gram-negative bacteria.

confirmed by studies showing much more extensive differences in genetic content compared to other enteric bacteria (Humphrey *et al*., 2007).

Studies illustrate *C. jejuni* diversity and highlight the presence of additional material in some strains and, in turn, the potential for strain-specific mechanisms of stress tolerance (reviewed by Park, 2005). A considerable variability is observed among *C. jejuni* isolated from poultry at flock level (Wittwer *et al.*, 2005). A significant decrease of genetic variability among *C. jejuni* isolates from the start of the slaughter-line to the end of the slaughter process has been shown in turkeys (Alter & Fehlhaber, 2003) and in broilers (Newell & Wagenaar, 2000), thus suggesting that some specific stress-resistant strains survived the slaughter and chilling process (Wieland *et al.*, 2006). Survival of this pathogen in water has also been shown to vary markedly among different strains (Jones, Sutcliffe & Curry, 1991; Terzieva & McFeters, 1991). One study indicated substantial variability among strains in cold survival, with human isolates being significantly more capable of prolonged survival at 4 ºC than poultry-derived strains (Chan *et al.*, 2001). Robust and stress-resistant strains were also found among environmental strains (Hänninen, Perko-Mäkelä, Pitkälä & Rautelin, 2000). An extracellular component of one strain, CI 120, has also been shown to confer acid tolerance to other *C. jejuni* strains (Murphy, Carroll & Jordan, 2003a). The conferred acid tolerance may have important implications in the environment as one strain could give protection to all strains present (Murphy, Carroll & Jordan, 2006).

3.2.1.2 Response regulators

Gram-negative bacteria have regulators, which mediate responses to environmental change. In this context, campylobacters appear to lack many of the adaptive responses that can be correlated with resistance to stress in other foodborne pathogens (Table 3.2; Park 2000). These include the oxidative stress defence SoxRS and OxyR, the osmoprotectants BetAB, GbsAB, OtsAB and ProP, the RpoS-encoded sigma factor stationary phase responsive genes, the major cold-shock protein (CspA) the leucine-responsive regulator (Lrp) and the alternative sigma factor (RpoH) that regulates the heat-shock response in *E. coli* (Murphy *et al*., 2006). *C. jejuni* contains several of the heat-shock proteins (HSPs), including GroELS, DnaJ, DnaK and Lon, but it also contains two negative regulators of the heat-shock response, HspR and HrcA (Murphy *et al.*, 2006). They also possess several two-component regulatory systems (not generally found in other bacteria) that appear to be involved in stress defence (Murphy *et al.*, 2006).

Table 3.2 The distribution of key orthologues from pathways responsible for resistance to environmental stress in *C. jejuni* and model bacterial species (Park, 2000).

Protein	Function	Presence in:		
		C. jejuni	*E. coli*	*B. subtilis*
Oxidative stress				
SoxRS	Positive regulators of the response to superoxide stress	-	+	-
OxyR	Positive regulator of the response to peroxide stress	-	+	-
PerR	Negative regulator of the response to peroxide stress	+	+	+
SodB or SodF	Iron cofactored superoxide dismutase	+	+	+
SodA	Manganese cofactored superoxide dismutase	-	+	+
KatA or KatE	Hydroperoxidase (HP) II, catalase	+	+	+
KatG	HP I, catalase	-	+	-
AhpC	Alkyl hydroperoxide reductase	+	+	+
Osmoregulation				
ProP	Low-affinity uptake of proline/glycine betaine	+	+	+
ProU or OpuC	High-affinity osmoregulatory uptake of compatible solutes	-	+	+
OtsAB	Osmoregulatory trehalose synthesis	-	+	-
BetAB or GbsAB	Osmoregulatory choline– glycine betaine synthesis pathway	-	+	+
Stationary phase starvation				
CsrA	Carbon storage regulator	+	+	+
RpoS	General stress/stationary phase sigma factor in Gram negative bacteria	-	+	-
SigB	General stress sigma factor in Gram positive bacteria	-	-	+
Heat and cold shock				
RpoH	Alternative sigma factor regulating the heat shock response	-	+	-
HspR	Negative regulator of the heat shock response	+	-	-
HrcA	Negative regulator of the heat shock response	+	-	-
GroELS, DnaJ, DnaK and Lon	Heat shock proteins	+	+	+
CspA	Major cold shock protein	-	+	+
Quorum sensing				
LuxI	Homoserine lactone synthesis	-	-	-
LuxS	Autoinducer 2 synthesis protein	+	+	+
ComQX	Peptide pheromone synthesis	-	-	+
PhrC	CSF, extracellular signalling pentapeptide synthesis	-	-	+
Global regulation				
Lrp	Global regulator of metabolism	-	+	+
Crp/Fnr	Catabolite gene activator or anaerobic regulatory protein	+	+	+

3.2.1.3 Cellular stage of growth and bacterial resistance

Entry into the stationary phase and/or starvation is accompanied by profound structural and physiological changes that result in increased resistance to heat shock, oxidative, osmotic and acid stress (Hengge-Aronis, 1996). For many foodborne pathogens, this adaptive process has an important bearing on the ability of the organisms to survive the physical challenges encountered during food processing. The central regulator for many of these stationary phase induced changes in a number of Gram-negative bacteria is the RpoS sigma-factor, which, accordingly, is critical for the survival of the bacterial cell in stationary phase and following exposure to many types of environmental stress (Hengge-Aronis, 1996). The RpoS response confers resistance to a range of stresses, and exposure to one factor such as low pH, and high osmotic pressure, can confer increased resistance to other stresses such as heat (Hengge-Aronis, 1996). The genome of *C. jejuni* contains only three sigma factors (*rpoD*, *rpoN* and *fliA*) and is unusual in lacking the stationary associated sigma factor *rpoS* (σs) (Parkhill *et al*., 2000).

A sublethal stress induces an adaptive tolerance response and provides protection towards subsequent exposure to a lethal stress, a mechanism known as the ATR. For instance, both *E. coli* and *Salmonella* will survive low pH by employing multiple acid defence systems geared toward different physiological states (e.g. log vs stationary phase), different levels of stress or the availability of different extracellular cofactors. Both organisms induce an ATR when exposed to acid pH in exponential phase, but the more effective acid resistance systems are present in stationary phase (Hersh, Farooq, Barstad, Blankenhom & Slonczewski, 1996; Lin, Lee, Frey, Slonczewski & Foster, 1995; Lin *et al*., 1996), although this regulator was also recognized as playing a significant role during exponential growth under a range of conditions (Cheville *et al.*, 1996; Audia, Webb & Foster, 2001).

Induction of an ATR has been described for *C. jejuni* and differences in stress resistance have been detected in different physiological growth phases of *C. jejuni* (Murphy *et al*., 2003b; Shaheen *et al*., 2006). Early stationary phase *C. jejuni* CI 120 cells adapted at pH 5.5 increased tolerance to pH 4.5 (Figure 3.3), but no ATR was induced when cells were in mid-exponential and late stationary phase, where cells exposed to pH 5.5 for 5 hours exhibited similar or increased death rate when compared to nonexposed cells (Murphy *et al.*, 2003b; Murphy, Carroll & Jordan, 2005). The adapted *C. jejuni* strain CI 120 had a 3 $\log_{10}$ cfu/mL survival advantage over control cells at pH 4.5 at approximately 4.6 h (Murphy *et al.*, 2005).

However, Shaheen *et al.*, (2006) observed an acid tolerance response in *C. jejuni* 2002-439 in late stationary phase cells that conferred adapted cells no more than 1 $\log_{10}$ cfu/mL survival advantage over unadapted cells. This underlines the variability encountered in the physiological properties of different *C. jejuni*. It is possible that changes in acid resistance might also be due to different challenge conditions. Nevertheless, it has been suggested (Shaheen *et al.*, 2006) that stationary phase cells of *C. jejuni* may exhibit an acid tolerance response that can be described as moderate when compared to the exponential and stationary phase responses described for *Salmonella* (Audia *et al*, 2001), *E. coli* (Lin *et al.*, 1995, 1996; Hersh *et al.*, 1996) and *Listeria monocytogenes* (Phan-Thanh, Mahouin & Alige, 2000).

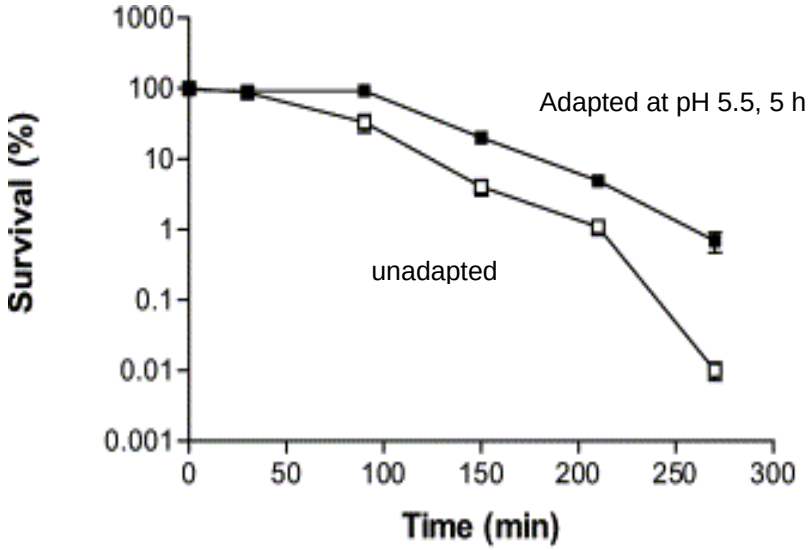


Figure 3.3 Early stationary phase cells of *C. jejuni* CI 120 challenged at pH 4.5 (Murphy *et al.*, 2003b). Cells were either unadapted (open squares) or adapted at pH 5.5 for 5 h (solid squares). Cultures were grown to the appropriate phase in Brucella broth (pH 7.0) at 42 ºC under microaerobic conditions. Experiments were undertaken in triplicate. Average values are shown with the standard error of the mean as error bars. Error bars are present but cannot be seen.

It is important to note that, in contrast to results found at low pH, stationary phase cells of *C. jejuni* NCTC 11351 have been reported to be more sensitive to heat and aeration than mid-exponential phase cells and this could physiologically confirm the lack of an RpoS mediated phenotypic stationary phase response (Kelly *et al.*, 2001). This organism does undergo selected physiological changes as the cells enter stationary phase, such as modulation of membrane composition, particularly the increase in cyclopropane fatty acids, which results in increased cell membrane integrity and pressure resistance, but not increased acid or heat resistance (Martıínez-Rodriguez & Mackey, 2005). Thus, the known RpoS-mediated acid tolerance response triggered in the stationary phase cells of *E. coli* and *Salmonella* does not appear to be present in *C. jejuni*. In *E. coli*, cyclopropane fatty acids (CFA) in the cell membrane have a significant effect in protecting against acid stress and their synthesis are

under regulation of *rpoS* (Grogan & Cronan, 1997). Cyclopropane fatty acids appeared to play little role in acid resistance in *C. jejuni* and since the organism lacks *rpoS*, the regulation of cyclopropane fatty acid synthesis must depend on different genetic regulatory mechanisms (Martínez-Rodriguez & Mackey, 2005).

A study also suggests that proteins produced by *C. jejuni* during growth may play an active role in the induction of acid stress response (Figure 3.4). Murphy *et al*. (2003a) determined the presence of a phase specific extracellular component that was secreted during growth and contributed to acid (and heat) stress tolerance during the growth phase. Cells resuspended in cell free spent medium showed a higher survival rate during challenge at pH 4.5 compared with cells in fresh medium especially in the mid-exponential phase.

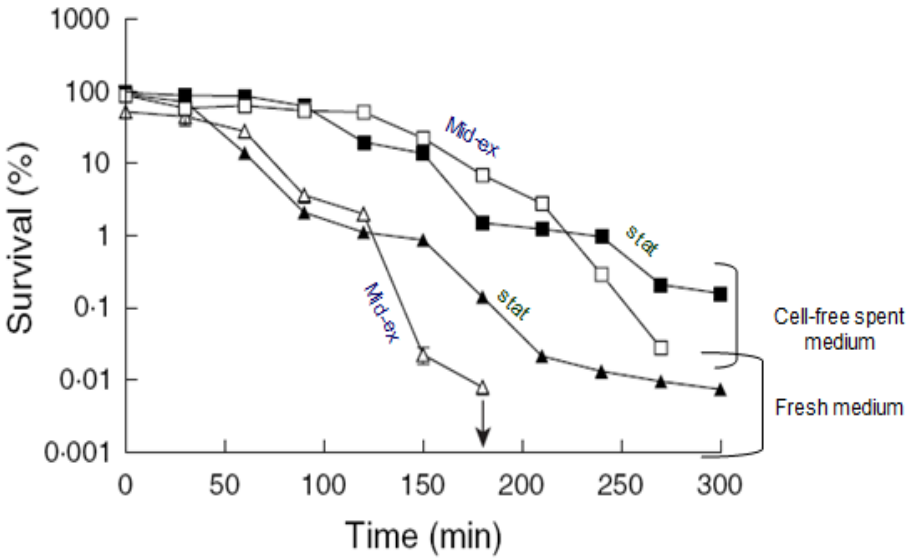


Figure 3.4 Survival of *C. jejuni* in cell-free spent medium (squares) and fresh medium (triangles) at pH 4.5 (Murphy *et al*., 2003a). *C. jejuni* CI 120 was grown to the desired growth phase in Brucella broth at 42 ºC under microaerophilic conditions. Experiments were undertaken in triplicate. Mid-ex = Mid exponential phase cells; stat = Stationary phase cells.

Cappelier, Rossero and Federighi (2000) also reported an increase in heat resistance in *C. jejuni* strain 79 as cells entered starvation in a surface water microcosm. Genetic differences between strains of *C. jejuni* have been described (Ahmed, Manning, Wassenaar, Cawthraw & Newell, 2002; Gaynor *et al.*, 2004) hence it is not surprising that strain differences in stress resistance occur in this organism. Analogous differences in stress resistance among strains of *Salmonella* or *E. coli* O157 appear to be related to polymorphisms in the *rpoS* gene (Jørgensen *et al.*, 2000; Robey *et al.*, 2001). Thus, the basis of the strain variation in *C. jejuni* stress resistance must be due to other causes (Martínez-Rodriguez & Mackey, 2005).

A recent study revealed that *C. jejuni* mounts a *relA/spoT*-dependent stringent response (Figure 3.5), a global stress response that alters gene expression pathways to allow survival under a multitude of adverse conditions (Gaynor *et al.*, 2005). The stringent response has been confirmed to play several important roles, such as capnophilic growth and aerotolerance. Interestingly, it was found to participate in the pathogen–host cell interaction such as adherence, invasion, and intraepithelial survival, indicating a correlation between stress response regulation and the pathogenic mechanism. This response has been demonstrated to play a role in *C. jejuni* survival during their 'non-traditional' stationary phase. However, as *C. jejuni* lacks *rpoS*, the precise mechanisms by which this occurs are likely to be distinct from those in *E. coli* (Figure 3.5) (Gaynor *et al.*, 2005).

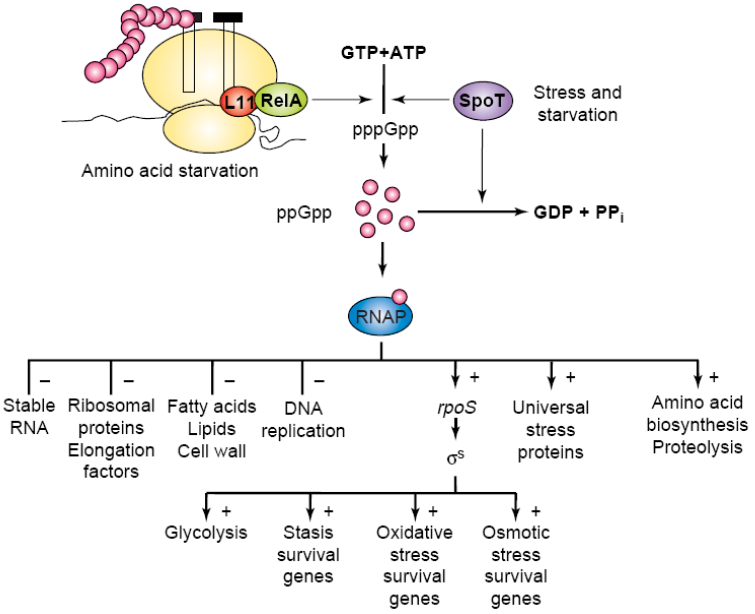


Figure 3.5 The stringent response in *E. coli* (Magnusson, Farewell & Nyström, 2005). Guanosine pentaphosphate (pppGpp) is produced from GTP and ATP by two parallel pathways in response to starvation and stress and is subsequently converted to guanosine tetra-phosphate (ppGpp). ppGpp binds RNA polymerase (RNAP) and redirects transcription from growth-related genes to genes involved in stress resistance and starvation survival. Nutrient deprivation has also been shown to induce a similar *spoT*-dependent (p)ppGpp synthesis in *C. jejuni* (Gaynor *et al.*, 2005).

3.2.1.4 Heterogeneity in cellular response to stress

In the absence of a recognizable phenotypic response to stationary phase, it has been suggested that it is possible that some, if not all strains, of campylobacters have developed an alternative strategy to promote survival that involves genetic variation (Park, 2002). *E. coli* mutants of increased fitness and which have a growth advantage in stationary phase have been associated with loss of function of both RpoS (Zambrano, Siegele, Almiron, Tormo & Kolter, 1993) and Lrp, the global regulator (Zinser & Kolter, 1999). *C. jejuni* lacks both RpoS and

Lrp (Parkhill *et al.*, 2000), thus, a similar process, which results in the evolution of fitter mutants, may operate in this species (Park, 2002).

Martínez-Rodriguez, Kelly, Park and Mackey (2004) have proposed that generation of variant emergent strains with altered phenotypes may play a role in the survival of *C. jejuni* in stationary phase. These authors reported that the secondary increase in viable count following the initial decline (Figure 3.6) might be due to the emergence of a new strain better able to survive in stationary phase. Prolonged aging of stationary phase cells of *C. jejuni* resulted in permanent phenotypic changes, such as a small increase in resistance to aeration, peroxide challenge and heat.

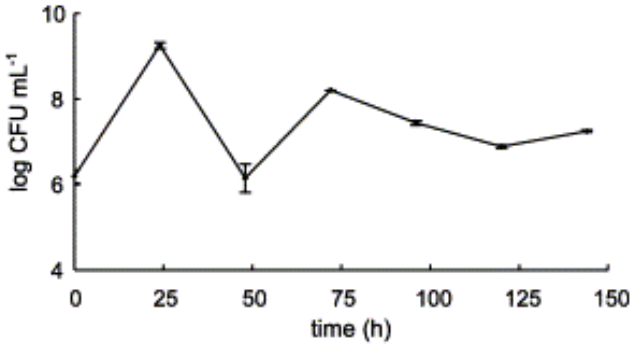


Figure 3.6 Stationary phase behaviour of *C. jejuni* NCTC 11351 (Adapted from Martínez-Rodriguez *et al.*, 2004).

3.2.2 Responses of *C. jejuni* to low pH

The ability of enteric pathogens to adapt and survive acid stress is fundamental to their survival and pathogenesis and therefore acid resistance is an important feature.

Gastric survival is a complex phenomenon that depends on a number of factors related to host, food and pathogen. For example, the level of gastric pH can greatly differ between hosts, food may act as a protective buffer against low pH (Waterman & Small, 1998), and previous exposure to sublethal acid stress enables many enterobacterial species to survive in otherwise lethal environments (Bearson *et al.*, 1997). Indeed, enteric pathogens that have developed efficient survival strategies to cope with acidic environments are known to have a low infective dose (ID) (Robinson, 1981). Thus, the low ID for *C. jejuni* suggests that this bacterium is well equipped to sense and respond to a sudden drop in pH (Reid, Pandey, Palyada, Naikare & Stintzi, 2008a). This implies that *C. jejuni* can tolerate exposure to acid in the stomach, subsequently being capable of colonizing both the large and small intestine, and causing infection (Babakhani, Bradley & Joens, 1993; Naikare, Palyada, Panciera, Marlow & Stintzi, 2006).

In fact, HCl does not have a significant effect on the survival of *C. jejuni* after 12 min of exposure at pH 4.0 in liquid medium and on a solid surface, although lactic acid at pH values of 4.0 inactivated *C. jejuni* significantly in the same conditions (Smigic *et al.*, 2008) (submitted for publication). HCl has little inhibitory effect on the survival of *C. jejuni* at pH values above 5.0 (Shaheen *et al.*, 2006). Unlike undissociated lipid-permeable weak acids, which can diffuse freely through the cell membrane and ionize in the cell to yield protons that acidify the interior of the cell, strong acids lower the external pH (pH_{ex}) but are not able to permeate through the cell membrane (Beales, 2004). These acids exert their antimicrobial effect by denaturing enzymes present on the cell surface and by lowering the cytoplasmic pH due to increased proton permeability when the pH gradient is very large (Beales, 2004). Gastric acid is a potent barrier against ingested pathogens and is primarily a pH-HCl-dependent mechanism. It is not currently known if an acid tolerance response expressed by *C. jejuni* at low pH with HCl will protect the cells against volatile fatty acids frequently found in the intestine, as it has been described for *Salmonella* serotype Typhimurium (Baik, Bearson, Dunbar & Foster, 1996).

Several studies have shown the sensitivity of *Campylobacter* spp. to acids, such as formic, acetic, ascorbic, and lactic acids (Chaveerach *et al.*, 2002, 2003). Due to the sensitivity of *C. jejuni* to acid, it has been suggested that ingesting *Campylobacter* spp. with buffers, such as milk or with water that aid rapid wash through, could reduce the oral infective dose (Blaser, Hardesty, Powers & Wang, 1980). The increased survival of acid-sensitive pathogens *in vitro*, when inoculated onto the surface of certain solid food sources, compared with their survival in an acidified broth environment demonstrates the ability of pathogens to survive extreme acid conditions when ingested with certain food sources (Waterman & Small, 1998; Björkroth, 2005).

Chicken is often sold as pieces marinated by chicken industry. Treatment with marination is based on low pH (often adjusted with acetic and lactic acids), high NaCl concentration, and on various spices into a marinated sauce. Perko-Mäkelä, Koljonen, Miettinen and Hänninen (2000) studied the survival of *C. jejuni* in plain marinade (pH 4.5, NaCl 5.9% (wt/wt) and in both marinated and non-marinated drumsticks and sliced breast meat strips during storage at 4 ºC. Surprisingly, marination was not shown to affect significantly the survival of *C. jejuni*. Björkroth (2005) has hypothesised that this may be due to the buffering capability of meat that quickly neutralizes the pH of the acidic marinade. This would result in dissociation of the lipophilic acids making their antimicrobial effect nonexistent.

Even though *C. jejuni* can mount an ATR to acid it has been shown to survive poorly in both cheese (Bachmann & Spahr, 1995) and yoghurt (Cuk, Annan-Prah, Janc & Zajc-Satler, 1987). In fact, rather than pose a risk of *Campylobacter* infection, the consumption of yoghurt has been shown to be protective in a study in Switzerland (Schorr *et al.*, 1994). The death rate of *C. jejuni* on strawberries (pH 3.4) has also been shown to be significantly higher than on other fresh produce (Kärenlampi & Hänninem, 2004).

Campylobacter jejuni genes involved in the response to acid conditions have recently been identified (Reid *et al.*, 2008a,b). Exposure to *in vitro* and *in vivo* (piglet stomach) acid shock (pH 4.5) caused down-regulation of genes involved in protein synthesis and the up-regulation of genes typically associated with numerous stress responses. This reflects the need to reshuffle energy toward the expression of components required for survival (Reid *et al.*, 2008a). These included heat shock genes (genes encoding the transcriptional regulator HrcA, chaperones DnaK, GroES, and GroEL, and cochaperones GrpE and ClpB), the stringent response genes, as well as genes involved in the response to oxidative (genes encoding the peroxide stress regulator PerR) and nitrosative stress. A role for the cochaperone ClpB in acid resistance was also confirmed *in vitro*. This is referred to as cross protection. Exposure to low pH environment is likely to cause acidification of the cytoplasm and protein misfolding. Heat shock proteins are part of a general stress response, and their expression is probably activated in response to unfolded or misfolded proteins. Phospholipid modification and hydrogenase activity may also be important for acid resistance *in vitro* (Reid *et al.*, 2008a). Some genes showed expression patterns that were markedly different *in vivo* and *in vitro*, which likely reflects the complexity of the *in vivo* environment (Reid *et al.*, 2008a). Interestingly, no heat shock genes were up-regulated under mildly acidic conditions and a *clpB* mutant was not impaired at steady-state acid stress (pH 5.5) (Reid *et al.*, 2008b). This proves that different mechanisms are required to protect *C. jejuni* cells against a lethal acid shock than those that are required for growth under mildly acidic conditions (Reid *et al.*, 2008b). The transcriptional profile at mildly acidic pH is characterized by the differential expression of respiratory pathways, by the induction of genes involved in phosphate transport, and by the repression of genes involved in energy generation and intermediary metabolism (Reid *et al.*, 2008b). While environmental stresses usually cause the induction of a large set of genes involved in coping with the particular stresses and/or repairing ensuing damages, the adaptation of *C. jejuni* to acidic conditions is characterized most notably, by the down-regulation of genes.

Several studies have shown that acid adaptations confer resistance to a wide range of stress conditions (Murphy *et al*., 2003b; Reid *et al*., 2008a,b). However, adaptation to other stresses does not typically induce significant acid tolerance. This implies that exposure to acid may be perceived by bacteria as a more general stress indicator whereas heat, salt and H_2O_2 may be more specific stress signals (Bearson *et al.*, 1997). Furthermore, the demonstration that acid shock induces cross-resistance to a variety of stresses (e.g. oxidative stress) suggests that cells undergoing acid shock in the stomach will be prepared to tolerate the environmental stresses subsequently confronted in the intestine (Bearson *et al.*, 1997).

This stress response in *C. jejuni* induces many different survival mechanisms and provides a first insight into mechanisms that lead to tolerance to acid conditions. Some of these mechanisms will be described more in detail in the next chapter.

3.2.3 Responses of *C. jejuni* to low temperature

Campylobacters are unable to grow below 30 ºC and, consequently, in moderate climates will not actually multiply during handling or storage at room temperature. Nevertheless, temperature impacts dramatically on the survival of campylobacters in food and, generally, survival is greater at temperatures below room temperatures (Park, 2002). In surface waters and water microcosms survival was shown to be limited to a few days at ambient temperatures of 20 ºC, but was noticeably enhanced (up to several weeks) at 4 ºC (Buswell *et al.*, 1998).

Low temperature is used at all stages of the poultry production process. Carcasses are submitted in the abattoir to low temperature regimes; whole chicken, chicken portions and cook–chill products are prepared and maintained under low temperature conditions; and the distribution and supply chain are, of course, maintained at low temperature. Very similar factors apply to other foods that can act as vehicles for infection by campylobacters such as raw cow's milk. Low temperatures are therefore central to the preparation, storage and distribution of food that may be contaminated with these organisms and a deeper understanding of how the bacteria respond to and survive in these conditions may help to inform interventions via the cold chain. Campylobacters, like other bacterial pathogens, can survive for extended periods at low temperature on key foods such as raw chicken and very high contamination levels have been reported (Luber & Bartelt, 2007). The means by which the bacteria do this are currently unknown. Data have shown that campylobacters numbers on chicken carcasses can remain largely unchanged for over 7 days at refrigeration temperatures

(Jørgensen *et al.*, 2002; Georgsson *et al.*, 2006). Substantial variability among strains in cold survival has been suggested, with human isolates being significantly more capable of prolonged survival at 4 ºC than poultry-derived strains (Chan *et al.*, 2001).

C. jejuni cells show considerable metabolic activity, including *de novo* protein synthesis, chemotaxis and aerotaxis at low temperature, even as low as 4 °C (Hazeleger *et al.*, 1998). Lázaro, Cárcamo, Audícana, Perales and Fernández-Asorga (1999) also demonstrated motility, oxygen consumption, protein synthesis and survival of *C. jejuni* at 4 ºC. *C. jejuni* strains have been demonstrated to exhibit a dramatic and sudden growth rate decline near the lower temperature limit (Hazeleger *et al.*, 1998). This suggests that *C. jejuni* does elicit a cold shock response that regulates gene expression at low temperatures (Murphy *et al.*, 2006). However, the major coldshock protein, CspA, which acts as an RNA chaperone to allow more efficient protein translation at cold temperatures (Qoronfleh, Debouck & Keller, 1992) and that is associated with the ability of bacteria to replicate at temperatures below the optimum growth temperature, is not present in the sequenced strain of *C. jejuni* (Parkhill *et al.*, 2000). This may explain why campylobacters cannot multiply below 30 ºC (Park, 2002).

In campylobacters the adaptive mechanisms underlying the responses to cold stress have not been examined in any detail although this is of considerable interest, given the involvement of chilled foods in infection (Murphy *et al.*, 2006). The analysis of transcript profiles at 4 and 10 ºC highlight that the most notable subgroup of genes that have their transcript abundance reduced upon cold shock encode proteins predicted to be involved in chemotaxis, flagellin biosynthesis, and flagellar motility (Stintzi & Whitworth, 2003). A number of genes involved in energy metabolism were upregulated at 5 ºC when compared with 25 ºC (Moen *et al.*, 2005). This indicates that *C. jejuni* has a greater need for energy at lower temperatures.

Campylobacters also show marked changes in the fatty acid composition of chilled cells compared to those held at higher temperatures. Data have shown that *C. jejuni* responds to chill in a manner different from *Salmonella* and *E. coli* (Mattick *et al.*, 2003). Unlike these bacteria it does not become heat sensitive when chilled. This may have important implications for food manufacturers and caterers, as the data demonstrate that campylobacters may be better able to survive certain processes than previously thought. It is important that food processors and caterers apply treatment regimes that take all relevant food-borne pathogens, their resistance to particular control measures and their probable maximum numbers into consideration (Humphrey *et al.*, 2007).

4 INTRACELLULAR pH IN ENTERIC BACTERIA

4.1 Role of intracellular pH (pH_i) for cellular functions

Bacteria have developed different ways to withstand stressful situations, such as a decrease in the extracellular pH (pH_{ex}). Most enteric bacteria are considered neutrophiles, i.e. they grow in the pH range of 4 to 9 with an optimum near neutrality. These bacteria maintain pH homeostasis, a very tight control with pH_i, which is kept close to 7.6 when the pH_{ex} is decreased and therefore generate large proton gradients ($\Delta pH=pH_i-pH_{ex}$) (Padan, Zilberstein & Schuldiner, 1981). Thus, bacteria that grow in the acid to-neutral pH range usually maintain pH_i at a more alkaline value than the pH of the environment (Booth, 1985), which is essential for the optimal activity of many important cellular processes, such as ATP and protein synthesis, DNA transcription and enzyme activity (Kobayashi, Saito & Kakegawa, 2000).

Since enzymatic activity is dependent on pH, changes in cytoplasmic pH disturb cellular metabolism, seriously challenging bacterial life (Kobayashi *et al.*, 2000). Several stress conditions can originate a decrease in pH_i (e.g. osmotic stress, acid stress).

Acidic conditions affect the capacity for nutrient acquisition and energy generation, cytoplasmic pH homeostasis and protection of proteins and DNA (Booth, Cash & O'Byrne, 2002). It has been reported that neutrophilic bacteria such as *Escheria coli* and *Listeria inoccua* maintain their pH_i close to the neutral despite variations in the pH of the environment, until the moment when differences between the external and internal pH become too high and pH homeostasis can not be maintained anymore which might result in cell death (Booth, 1985; Shabala, McMeekin, Budde & Siegumfeldt, 2006). Results recently obtained by Smigic *et al.* (2008) (submitted for publication) indicated that *C. jejuni* is as well a homeostatic bacterium. Therefore, pH_i is a physiological parameter that can be used to assess bacterial viability and subsequently their ability to cope with a multiplicity of stresses.

4.2 Mechanisms of pH_i regulation

The ability of a microorganism to maintain its pH_i at a value close to neutral is achieved by a combination of passive and active mechanisms (Siegumfeldt, 2000).

Mechanisms of active regulation of pH_i are numerous and involve active H^+ extrusion via ATP-driven pumps (Cotter, Gahan & Hill, 2000), proton/cation exchange systems (Foster,

1999), buffering of the cytoplasm (Foster, 1999), or decarboxylation processes (Cotter, Gahan & Hill, 2001). The literature does not suggest, however, which of these mechanisms is predominant in *C. jejuni*.

4.2.1 Proton translocating ATPase

H^+-ATPases play an important role in conferring acid tolerance to bacteria. As intracellular pH drops, certain bacteria up-regulate the F_1F_0 ATPase (Cotter *et al.*, 2000), which can pump proton out of the cell at the expense of ATP. However, this response was not detected in *C. jejuni*, as these genes either were unaffected or were down regulated in response to acid conditions (Reid *et al.*, 2008a,b).

4.2.2 Proton/cation exchange systems

The movement of protons across the membrane generates a membrane potential, preventing further proton removal. This membrane potential is, however, broken up by the movement of cations into the cell such as potassium ions (K^+/H^+ antiporter), which generates a transmembrane pH gradient and helps maintain the internal pH (Booth & Kroll, 1989).

The Na^+/H^+ antiporter of *E. coli* exhibits low activity at acid pH, is markedly more active above pH 7 and is required in the mildly alkaline pH range for reducing the sensivity of cells to high Na^+ concentrations (Padan *et al.*, 2004). Reid *et al.* (2008a,b), recently demonstrated the down-regulation of a putative Na^+/H^+ antiporter gene in *C. jejuni* under acid conditions, suggesting that decreased expression of this gene is an important component of this organism's response to acid stress.

4.2.3 Metabolic and energy generation processes

The growth of *E. coli* and *Shigella* under acidic or basic conditions leads to the preferential expression of metabolic systems that are compatible with the bacterium's environment. For instance, growth under acidic conditions leads to the up-regulation of genes encoding products that are involved in amino acid catabolism, which generates amines (decarboxylases) or ammonia (dehydratases) that can buffer the cellular environment and prevent and/or reverse the acidification of the cytoplasm (Reid *et al.*, 2008b).

It is therefore surprising that *C. jejuni* down-regulates a gene for the catabolism of serine, which generates ammonia, when grown on medium at pH 5.5 (Reid *et al.*, 2008b). On the other hand, methionine biosynthesis appears to play a role in *C. jejuni* acid stress response, as negative mutants for a gene encoding a key enzyme to this process were acid sensitive (Reid *et al.*, 2008b). The activity of this enzyme is known to generate homocysteine, pyruvate, and ammonia.

Respiring bacteria (e.g., *E. coli*) extrude protons via the respiratory chain. A proton motive force (PMF) generated by this chain is used for ATP synthesis, membrane transport, and cell motility (Harold, 1982). Many data support that the translocation of protons across the cytoplasmic membrane during electron transfer contributes significantly to the ability of *C. jejuni* to survive in mildly acidic conditions environments (Reid *et al.*, 2008b).

Exposure of *C. jejuni* at low pH led to the differential expression of genes that encode products that are involved in various electron transport pathways such as the NADH dehydrogenase (Reid *et al.*, 2008b). This complex can pump four H^+/electron pairs into the periplasm, which may help prevent and/or reverse cytoplasmic acidification. A negative mutant for the gene encoding succinate dehydrogenase was also shown to be impaired for growth at pH 5.5, which demonstrates that survival at mild pH relies on feeding electrons into the ETC by this dehydrogenase (Reid *et al.*, 2008b).

Acid shock has been shown to induce the expression of *C. jejuni* genes encoding products that are involved in a C_4-dicarboxylate antiporter, which is involved in fumarate (anaerobic) respiration (Reid *et al.*, 2008a). The reactions catalyzed by these transporters are electrogenic, resulting in the symport of succinate or fumarate and H^+ (Janausch, Zientz, Tran, Kroger & Unden, 2002). Thus, this enzymatic system might help *C. jejuni* resist cytoplasm acidification (Reid *et al.*, 2008a).

Genes that originate products that could serve to increase acid consumption by the TCA cycle have also been shown to be highly up-regulated in *C. jejuni*'s response to acid shock (Reid *et al.*, 2008a).

In response to acid shock, *C. jejuni* up-regulates the expression of hydrogenase (Reid *et al.*, 2008a) which may be important at low pH due to the ability of hydrogenases to extrude H^+ from the cytoplasm (Hayes *et al.*, 2006).

Exposure to acid stress has been found to induce the expression of genes encoding gluconate dehydrogenase (GADH), which likely plays an important role in the survival and adaptation to acid conditions (Reid *et al.*, 2008b). The ability of *C. jejuni* to use gluconate as an electron donor via GADH activity has also been shown to be an important metabolic characteristic that is required for full colonization of avian hosts (42 ºC) (Pajaniappan *et al.*, 2008).

4.2.4 Alkalinisation of the periplasm

The most striking example of alkalinisation of the periplasm as a mechanism of low pH survival is that of *Helicobacter pylori* (Rektorschek *et al.*, 2000). *H. pylori* colonises the stomach and can grow in this extreme acidic environment. The possession of urease (Andrutis *et al.*, 1995) and the urea transporter/channel, UreI (Rektorschek *et al.*, 2000) is a key determinant of acid tolerance in these bacteria. Urease splits urea to CO_2 and ammonia, which then diffuse into the periplasm causing a rise in the periplasmic pH to approximately 6 (Athmann *et al.*, 2000).

Despite being phylogenetically related, this mechanism has not been found in *C. jejuni* (Parkhil *et al.*, 2000). Results from a BLAST[3] (Birk, unpublished) showed that *C. jejuni* did not contain a system equivalent to the *H. pylori* UreI.

4.2.5 Passive regulation of pH_i

The passive regulation of pH_i is by large determined by the low permeability of the plasma membrane to protons (Booth, 1985). In prevention of large changes in pH_i as the pH of the environment varies, it is thought that the permeability of the cell membrane to protons plays an important role, with protons that are present in the environment being prevented from crossing the membrane and reducing the internal pH (Beales, 2004).

The modulation of membrane composition also might be expected to alter cell wall permeability to H^+. A negative *C. jejuni* mutant for the enzyme phospholipase A, which cleaves membrane phospholipids to yield lysophospholipids was impaired for growth at pH 5.5 (Reid *et al.*, 2008b). In *H. pylori*, the expression of active phospholipase A also leads to improved acid stress survival of the strain (Tannaes, Dekker, Bukholm, Bijlsma &

[3] The Basic Local Alignment Search Tool (BLAST) finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

Appelmelk, 2001). Thus this enzyme plays a role in the acid stress response of both bacteria (Reid *et al.*, 2008b). Fatty acid biosynthesis was also found to be important for *C. jejuni*'s adaptation to growth at mild pH (Reid *et al.*, 2008b).

At very acidic pH values, it has been proposed that alterations in the structure of the outer membrane may be a mechanism for enhancing microbial survival (Brown & Booth, 1991). From recent data (Reid *et al.*, 2008a) it does not appear that *C. jejuni* drastically remodels its outer membrane proteins (OMPs) composition in response to acid shock. However the authors do not discount a role of one or more OMPs in the acid shock response of this bacterium.

A cell exposed to low pH environments can also increase the cytoplasmic levels of proteins and glutamates and increase buffering capacity, which may prevent internal pH disruption (Booth & Kroll, 1989).

4.2.6 Phosphate acquisition

Phosphate uptake and polyphosphate (polyP) accumulation appear to play a role in the acid stress response of some bacteria (Mullan, Quinn & McGrath, 2002; Leaphart *et al*., 2006). Genes encoding products that are involved in this process were demonstrated to be up regulated at pH 5.5, supporting an intracellular buffering role for phosphate uptake and polyP accumulation in the acid stress response of *C. jejuni*. The formation of polyP during growth at pH 5.5 might alternatively provide a mechanism of pH homeostasis for *C. jejuni* by acting as an intracellular cation trap, sequestering H^+ ions.

4.2.7 Regulation by Fur

An important regulator of acid tolerance response is the ferric uptake regulator (Fur) that has been implicated in acid survival of both *S. enterica* Typhimurium and *E. coli* (Hall & Foster, 1996). Fur is a cytoplasmatic regulatory protein that can sense changes in iron concentration, which is its main function since it strongly regulates the expression of gene products required for iron scavenging. This protein appears to have acquired a second function namely the sensing of pH and control over expression of gene products that can assist cell survival at extremely acidic pH (Hall & Foster, 1996). It also governs the expression of several acid shock proteins (ASPs) as an activator in an iron-independent manner. Reid *et al.* (2008b) reported that a *fur* mutant was impaired in its ability to grow at pH 5.5, indicating that Fur plays a role in *C. jejuni*'s adaptation to mildly acidic conditions.

4.2.8 DNA repair systems and chaperones

Diminished protein stability is a likely consequence of cytoplasmic acidification and this may indirectly affect the integrity of DNA through failure of repair processes. DNA repair systems and chaperones appear to be important for the reparation of cellular material damaged by exposure to acid. There have been a number of reports of increased expression of both heat shock proteins DnaK and GroEL/ES classes of chaperones in response to growth at acid pH (Jan, Leverrier, Pichereuau & Boyaval, 2001). When exposed to acid, heat shock proteins were highly up regulated (Reid *et al.*, 2008a). Surprisingly these proteins were not found in *C. jejuni*'s ATR (Reid *et al.*, 2008b) as no heat shock genes were up-regulated under steady state acidic growth conditions (pH 5.5).

4.2.9 Adaptation to acid – acid resistance systems

The RpoS sigma factor sets the baseline for acid tolerance in the enteric bacteria as was already mentioned in chapter 3 (Audia *et al.*, 2001; Booth *et al.*, 2002). Low pH will increase the accumulation of *rpoS* (σ^S), through the modulation of σ^S proteolysis. It regulates one aspect of acid tolerance governing the expression of acid shock proteins (ASPs). The function of ASPs is presumed to include the prevention and/or repair of acid-induced damage to macromolecules (Bearson *et al.*, 1997).

Three acid resistance (AR) systems have been identified in the stationary phase of all *E. coli* (Audia *et al.*, 2001; Richard & Foster, 2003) that are under the regulation of σ^S (RpoS). The system induced depends on the type of medium and the growth conditions (Audia *et al.*, 2001). The three complex medium dependent acid resistance systems include an oxidative system (AR1) and two fermentative acid resistance systems involving a glutamate decarboxylase (AR2) and an arginine decarboxylase (AR3) (Lin *et al.*, 1995, 1996). The glutamate decarboxylase is the most extensively studied mechanism in *E. coli* (Figure 4.1). The hypothesis suggests that the consumption of intracellular protons during decarboxylation maintains the internal pH in a range conductive to cell survival. At an acidic pH (2.5) protons move into the cell and acidify the cytoplasm. Simultaneously glutamate is transported by GadC and decarboxylated by GadA/B into γ-aminobutyric acid (GABA) with consumption of one H^+ and release of CO_2. GABA is then transported out of the cell with the concomitant uptake of glutamate. It is predicted that H^+ remaining on the ionisable group of glutamate will be released in the periplasm prior to the passage through GadC (Richard & Foster, 2003).

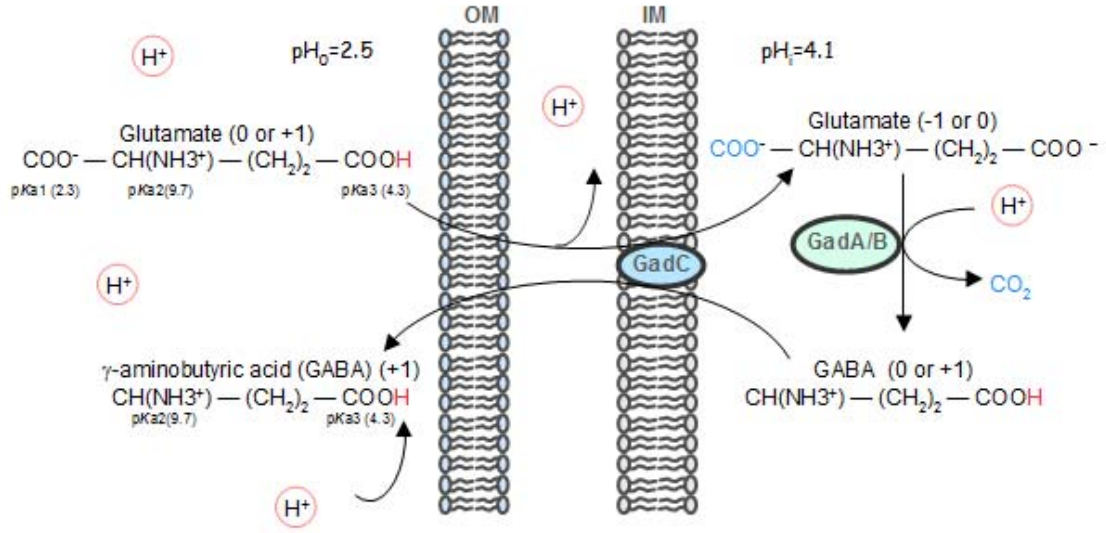


Figure 4.1 Proposed model for the acid resistance system (AR) 2 in *E. coli* (adapted from Richard & Foster, 2003)

Although no acid resistance systems have been identified so far in *C. jejuni*, an induction of an adaptive tolerance response has been described, as was already mentioned. The inducible acid tolerance response induces a number of proteins when a mild acidification occurs, and this prevents or reduces the detrimental effects of an extreme acid challenge (Audia *et al*, 2001). Some of the proteins that are induced by ATR in *C. jejuni* have already been described. For instance, H^+-ATPase was not induced by ATR (Reid *et al.*, 2008b). The mechanisms that seem to be involved include: proton extrusion that appears to rely on respiratory pathways of which the ETC plays a substantial role; phosphate acquisition as a buffering mechanism; and the shutdown of energy generation systems and intermediary metabolism which allows redirecting energy for acid survival (Reid *et al.*, 2008b).

This stress response in *C. jejuni* induces many pH_i regulation mechanisms and provides a first insight into mechanisms that lead to tolerance to acid conditions.

4.3 Technique for measuring intracellular pH

4.3.1 Fluorescent probe

The principle for determining pH_i utilises the pH dependency of several fluorescent compounds. Fluorescein and a number of fluorescein-derivatives have pH dependent emission intensities when excited at 488 nm. Carboxyfluorescein (Figure 4.2A) is a probe that covalently bounds to primary amines in the cytosol (Breeuwer, Drocourt, Rombouts & Abee, 1996). The fluorescence intensity is dependent on probe concentration as well as pH, and it would be ideal to have a pH-value independent of probe concentration in order to avoid artefacts. This can be accomplished by exciting fluorescein-derivatives at two different wavelengths, the isosbestic point and the excitation maximum (Figure 4.2B). At the isosbestic point, the fluorescence emission is independent of pH, and the emission merely reflects the concentration of probe. By dividing the emission from the excitation at the pH dependent wavelength (488 nm) with the emission from the isosbestic point (435 nm), a ratio value is obtained, which only reflects pH_i and not probe concentration (Siegumfeldt, 2000).

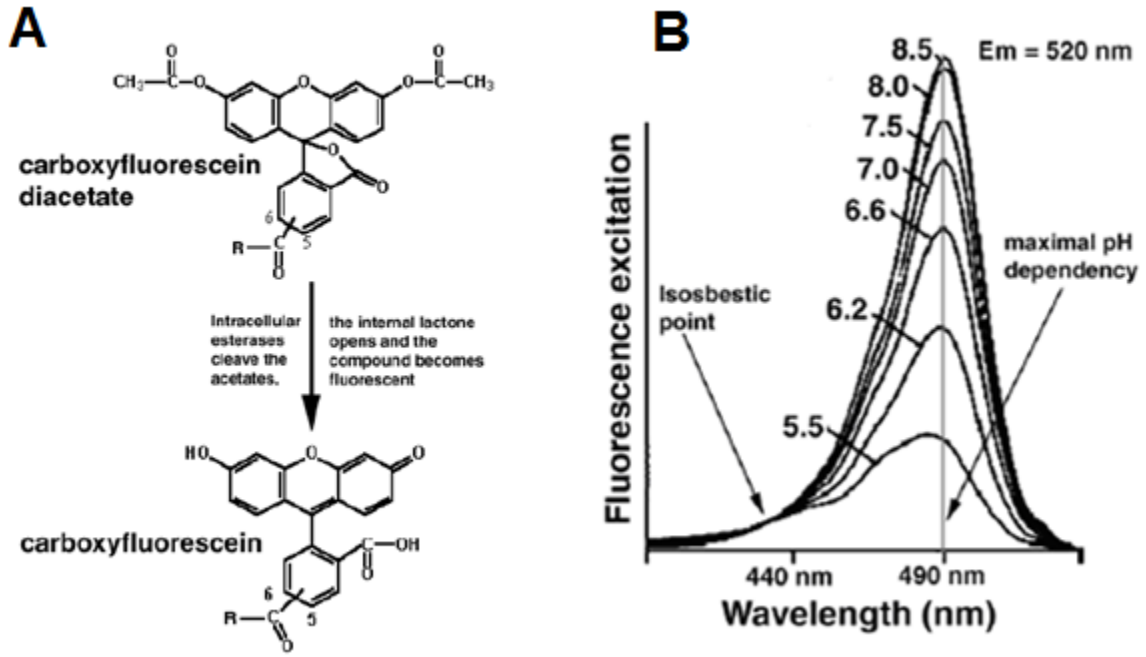


Figure 4.2 (A) The non-fluorescent precursor carboxyfluorescein diacetate is hydrolysed by intracellular esterases, and the resulting carboxyfluorescein is highly fluorescent (R = OH). By adding a succinimidyl group at (R), the compound can bind to intracellular amines. (B) A schematic excitation spectrum of a fluorescein derivative, showing the isosbestic point, which enables the acquisition of a concentration independent ratio $R_{490nm/435nm}$ (Siegumfeldt, 2000).

A problem that is associated with this technique is that the pH range, which can be validly determined using any one fluorescent probe, is limited. As shown in Figure 4.3, the range of pH that can be determined by carboxyfluorescein is at best from 5 to 9 (Siegumfeldt, 2000).

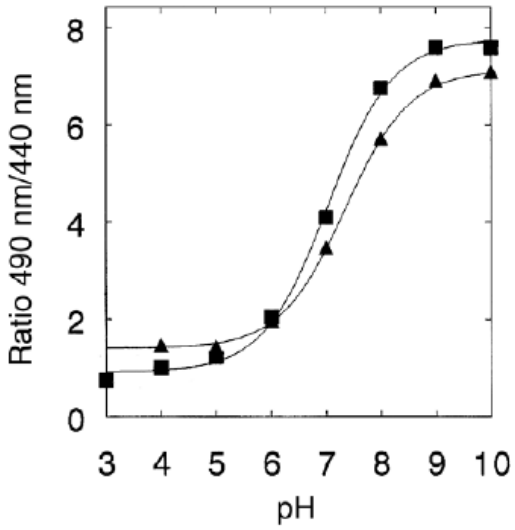


Figure 4.3 The correlation between pH and the ratio (490 nm to 440 nm) of carboxyfluorescein succinimidyl ester in stained, pH equilibrated cells of *Bacillus subtilis* (■), and *Lactococcus lactis* (▲) (modified from Breeuwer *et al.*, 1996).

Fluorescence ratio imaging microscopy (FRIM) is a technique where ratio determination of pH_i is used in conjunction with a microscope and an image analysis system (Figure 4.4A). This technique obtains information on a single cell level, and therefore this method can solve the problem of measuring pH_i in subpopulations simultaneously. This was the technique applied in the experiments of this work.

The images obtained give a very good overview about approximate pH_i values and heterogeneity of mixed populations, but in order to perform quantitative comparisons, it is preferable to calculate the pH_i of a number of cells and calculate average and standard deviations. This can be done by defining regions around representative cells and allowing the software to calculate the exact ratio value of individual cells (Siegumfeldt, 2000). The $R_{488nm/435nm}$ was calculated by dividing the intensity of individual pixels on the 488 nm image by the intensity of corresponding pixels at 435 nm image according to Equation 4.1.

$$Ratio = \frac{I_{488nm} - B_{488nm}}{I_{435nm} - B_{435nm}} \qquad \textbf{Equation 4.1}$$

The background fluorescence (B_{435nm} and B_{488nm}) are subtracted from the fluorescence intensity for each cell (I_{435nm} and I_{488nm}). The background fluorescence was measured individually for each cell as the fluorescence in an area next to the cell. This was done to correct for autofluorescence from the defined media used in the experiments.

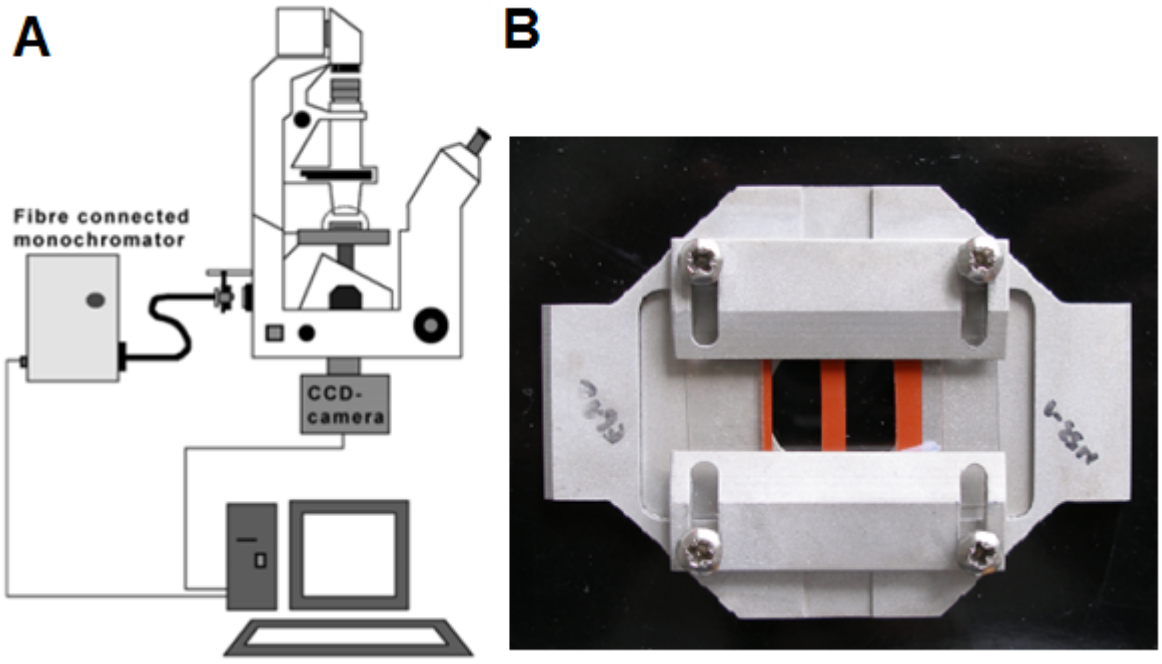


Figure 4.4 (A) schematic presentation of the microscopical set-up used in this work. The monochromator provides the two excitation wavelengths, and the CCD-camera acquires two images that are subsequently converted to a ratio image (Guldfeldt & Arneborg, 1998). (B) The chambers, where the cells were immobilised.

5 SURVIVAL OF *CAMPYLOBACTER JEJUNI* UNDER ACID STRESS CONDITIONS: ASSESSMENT OF STRAIN VARIATION AND TEMPERATURE EFFECT

5.1 Objective

Temperature and pH are two factors commonly employed by food processors to control or inactivate pathogens and undesirable microorganisms in foods. Furthermore, one of the reasons for susceptibility to *C. jejuni* may be suboptimal inactivation of bacteria in the stomach, which is the first in a series of barriers that protect the host from infection. It was already shown that *C. jejuni* sensivity to acid (HCl) varies with temperature (Doyle & Roman, 1981). However, these experiments were performed on one strain only. Few data are available on the survival of *C. jejuni* under acid stress conditions. If *C. jejuni* survival is actually affected by acid stress, a selection might occur during exposure to mildly acidic environments or inside the host. Therefore, only the most resistant strains would be able to survive and induce virulence. In addition, temperature has an effect on metabolism and the permeability of cellular membranes. Temperature has been demonstrated to be an important regulatory signal for *C. jejuni*, and temperature-regulated proteins have been shown to be necessary for the optimal colonization of chickens (Konkel, Garvis, Tipton, Anderson & Cieplak, 1997; Brás, Chatterjee, Wren, Newell & Ketley, 1999; Pajaniappan *et al.*, 2008). Thus, there might be a link between temperature, level of acid stress, and regulation of acid stress response. From these observations, this study was conducted to investigate:

1. How sensitive is *C. jejuni* to acid stress?
2. Do different strains have different levels of resistance?
3. What is the influence of temperature on *C. jejuni*'s acid stress resistance?

To investigate the temperature effect coupled with acid stress, two different temperatures were selected: 4 ºC, a temperature used for refrigeration and at which *C. jejuni* is known to survive well; and 37 ºC, which is in *C. jejuni's* optimal growth range temperatures and is the body temperature of humans (37 ºC).

There has been growing evidence that microbial survival curves may not always be log linear (Peleg & Cole, 2000). Different kinds of deviations from linearity have been observed such as sigmoid curves and curves with pronounced shoulders or tails. In consequence various models have been proposed to describe the nonlinear curves (Buzrul & Alpas, 2004). The Weibull

model has been used successfully in describing the nonlinear inactivation of different microorganisms under various experimental conditions, as stress resistance of a microbial population often follows a Weibull distribution (Mafart, Couvert, Gaillard & Leguerinel, 2002; van Boekel, 2002; Buzrul, Alpas & Bozoglu, 2005; Buzrul & Alpas, 2007).

This study also aimed to fit and compare models for acid inactivation kinetics of *C. jejuni* strains. Specifically, the "traditional" first-order model was compared with the Weibull model.

5.2 Materials and Methods

Bacterial strains

On the basis of previous studies performed by this laboratory (Department of Food Science, Faculty of Life Sciences, Copenhagen University, Denmark), a total of 3 different strains of *Campylobacter jejuni* were used in this study: one susceptible strain (327) and two less susceptible strains (305 and NCTC 11168) to other general stresses (e.g. oxidative) (Boysen *et al.*, 2006). The sequenced clinical human isolate *C. jejuni* NCTC 11168 was obtained from the National Collection of Type Cultures (Central Public Health Laboratory, London, UK). The turkey isolates (327 and 305) were received from the Department of Food Science, Faculty of Life Sciences, Copenhagen University, Denmark.

Preparation of the inocula and growth conditions

The experimental protocol was adapted from Birk *et al*. (2006). Strains were maintained at –80 ºC in brain heart infusion broth (BHIB) (Oxoid CM225, England) containing 15 % glycerol. A loopful of each strain was streaked onto blood agar base no. 2 plates (Oxoid CM271, England) with added 5 % horse blood. Cultures were prepared by growing the strains for 2 to 3 days. One loopful of each culture was subsequently streaked onto new blood agar base no. 2 plates, which were incubated for 24 h. During propagation and growth all plates were incubated at 42 ºC under a microaerophilic gas mixture (5 % O_2, 10 % CO_2, and 85 % N_2; Praxair, Inc., Danbury, Conn.) in sealed gas jars (Oxoid anaerobic jars). Cells were harvested with 2 ml of peptone physiological salt solution (8.5 g/l NaCl, Oxoid LP0005; 1 g/l Peptone, Oxoid LP0072) (PPSS) with a loop, and the inocula were adjusted in PPSS to an optical density at 600 nm = 0.1 (approximately 8 $\log_{10}$ CFU/ml). This was confirmed by plating serial dilutions and counting colonies. The average values were 8.44, 8.37 and 8.66 $\log_{10}$ units for strains NCTC 11168, 305 and 327, respectively.

Inoculation and survival studies

Ten μl of each previous bacterial suspension were suspended into flasks containing 20 ml of defined media[4] (Birk, 2008; unpublished) at pH 7.0 (approximately 10^5 CFU/ml; average values were 5.8, 5.74 and 5.44 $\log_{10}$ units for strains NCTC 11168, 305 and 327, respectively) and incubated at 37 ºC for 24 h at microaerophilic conditions. After incubation, the cells obtained were in late exponential phase of growth (Birk *et al*., 2006) and were used as inocula for survival inactivation studies. Twenty μl of each inocula (approximately 10^8 CFU/ml; average values were 8.61, 8.80 and 9.01 $\log_{10}$ units for strains NCTC 11168, 305 and 327, respectively) were subsequently inoculated onto 20 ml of defined media set at pH 5.0 and 7.0 (control) and incubated at 4 ºC and 37 ºC. The pH of the media was adjusted with 1M HCl or 1M NaOH.

Microbiological analyses

Counts of each *C. jejuni* strain were determined by the classic viable count method based on cell viability, defined as the ability to reproduce and grow, measured as CFU by plating serial dilutions and counting colonies. Determinations were done immediately after inoculation (Time 0) and at selected times during the survival experiments (4h, 8h, 20h, 44h, 68h, 92h). The average initial level (Time 0) was 5.08, 6.44 and 5.17 $\log_{10}$ units for strains NCTC 11168, 305 and 327, respectively. The number of *C. jejuni* cells in inoculated flasks was determined by making serial dilutions and spotting three times 10 μl of each dilution onto blood agar base nº.2 plates that were subsequently incubated at 42 ºC for 2-3 days under microaerophilic conditions. Triplicate determinations were done for each treatment studied. *C. jejuni* counts (CFU/ml) were log transformed ($\log_{10}$ CFU/ml), and the mean and standard error of the mean (SEM) calculated by Microsoft Excel 2003.

Statistical analysis

Statistical Package for Social Sciences (SPSS) 16.0 software was used for statistical analysis. A multifactorial analysis of variance allowed examination of the effect of the following three parameters on survival: (1) temperature, (2) strain and (3) pH. The confidence interval chosen to consider the effect of a parameter as significant was 5%.

[4] It is not possible to write the detailed list of components since it has not been published. Defined medium contained amino acids, vitamins, salts and buffers. Some components that are involved in TCA cycle were also included. No carbohydrates were added since *C jejuni* does not ferment sugars, obtaining energy from amino acids instead.

Modeling Inactivation kinetics

GInafit, a freeware tool to assess non-log-linear microbial survivor curves (Geeraerd, Valdramidis & Van Impe, 2005), was used for nonlinear regression analysis and to determine the parameters of the Weibull model. The goodness of the fit of the models was assessed by visual inspection and using regression coefficient (R^2) and root mean square error (RMSE). R^2 measures how well a model fit to the data and higher the R^2 value, the better is the adequacy of the model to describe the data. RMSE measures the average deviation between the observed and fitted values. Small RMSE value of a model indicates a better fit of data for that model.

In GInaFiT, it is the version as proposed by Mafart *et al*. (2002) which is used, based on a logarithm decimal form of Weibull defined as follows:

$$\log_{10}(N) = \log_{10}(N(0)) - \left(\frac{t}{\delta}\right)^p \qquad \textbf{Equation 5.1}$$

Herein, N represents the microbial cell density, expressed in for example, [cfu/mL], $N(0)$ the initial microbial cell density [cfu/mL], δ [time unit] corresponds to the first reduction time that leads to a 10-fold reduction of the surviving population. Parameter p characterises the shape of the curves: concave curves $p < 1$, convex curves $p > 1$ and linear curves $p = 1$.

5.3 Results

Influence of acid stress factor on *C. jejuni* survival

Survival curves obtained from three strains are presented in Figure 5.1. Two poultry-derived (305 and 327) and the sequenced clinical isolate NCTC 11168 were chosen for investigation of their acid tolerance coupled with temperature effect. Plate-count monitoring of the viability profile of different isolates over 92 hours at 4 ºC revealed inactivation of *C. jejuni* strains, albeit at different rates, at pH 5.0. Viability of certain strains (e.g. 305 and NCTC 11168) showed no appreciable decrease following 68 hours of incubation at 4 ºC (Figure 5.1), whereas viable counts of strain 327, which was more sensitive, had a 2.39 $\log_{10}$ decrease in population after the same period. Despite the slight decrease within the first 68 hours, strain 305 showed a 1.70 $\log_{10}$ decrease in population following the next 24 hours of exposure, compared to a 0.27 $\log_{10}$ decrease for strain NCTC 11168. At 4 ºC, strain 327 was clearly the most sensitive to acid followed by strain 305, the latest being only slightly more sensitive than NCTC 11168, which seemed to survive better at this temperature.

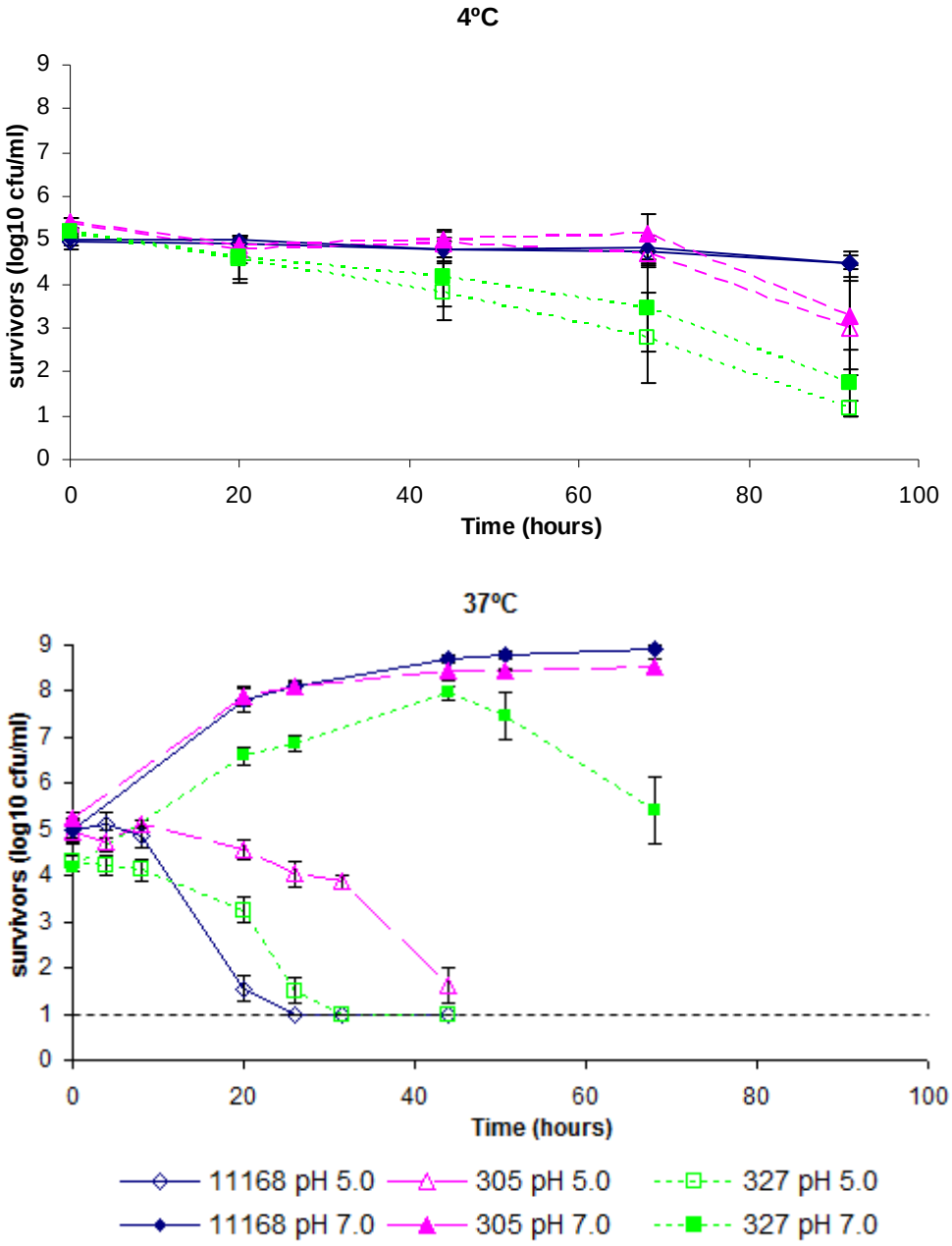


Figure 5.1 Survival of three *C. jejuni* strains exposed to pH 5.0 or pH 7.0 under microaerophilic conditions on defined media at different temperatures (NCTC 11168 reference strain, 305 and 327 poultry strains). Data are means ± SEM (standard errors of the mean) (n = 3). The horizontal dashed line represents the limit of detection.

At 4 ºC survival seemed to be slightly affected by acid. For strains 327 and 305, a slight acid effect was directly observed after 44 hours of exposure, whereas no acid effect was observed for strain NCTC 11168 during the time of experiment (Figure 5.1).

At 37 ºC considerable differences regarding acid tolerance were found among the three strains used (Figure 5.1). No important changes in cell counts were observed during the first hours (approximately 8 hours) for all strains. A slight decline in viable count was observed for strain 305, during the first 4 hours. The viability then increased in the next 4 hours before inactivation started to take place. The same was observed during the first 20 hours of exposure to acid at 4 ºC (Figure 5.1). The rise in viable count may reflect an increase in the number of culturable *C. jejuni* cells, possibly resulting from the adaptation to acid conditions. This response also occurred at 4 ºC at pH 7.0 which indicates a similar adaptation response of this strain to low temperature.

After the first hours, a rapid decrease of viable cells was observed at 37 ºC under acid stress but the viability of cells varied considerably among strains. For strain NCTC 11168, the major decrease occurred after 20 hours showing a 3.43 $\log_{10}$ CFU/ml reduction in cell counts comparing to a decline of 0.37 and 1.03 Log_{10} units for strains 305 and 327, respectively. Strain 305 was significantly more acid tolerant than other strains ($P < 0.0001$). Strain NCTC 11168 showed a higher decrease in population when compared with strain 327 but the difference in survival between these two strains was not significant ($P = 0.312$). These differences in viability among strains were reproducibly observed in independent experiments, suggesting that the phenotypes were a strain-specific property with a genetic basis.

At 37 ºC, there was a strong acid effect. A decrease of more than 4 $\log_{10}$ in population was observed for all strains within the first 44 hours of exposure, whereas no decrease in population was observed under control conditions (pH 7.0).

Survival after 20 hours was considered for statistical analysis (Table 5.1). The strain and acid effects observed at 37 ºC were statistically significant ($P < 0.0001$). No significant strain and acid effect were detected at 4 ºC ($P = 0.539$ and $P = 0.776$, respectively).

Table 5.1 Strain and acid effects on survival[a] The confidence interval chosen to consider the effect of a parameter as significant was 5%.

Temperature	Parameter	*P*
4 ºC	Strain	0.539
	pH	0.776
37 ºC	Strain	**< 0.0001**
	pH	**< 0.0001**

[a] Results of the multifactorial analysis of variance (ANOVA) (variable = survival after 20 hours; parameters = pH and strain). The values obtained from the three replicates were taken into account

After 44 hours at 4 ºC (Figure 5.1), the decline in population observed for strain 327 was significantly higher ($P < 0.05$) than that observed for other strains. Differences in survival between 305 and NCTC 11168 were not significant during the whole time of experiment (92 hours). Strain 327 was significantly more sensitive at 4 ºC at both pH levels (Figure 5.1) whilst other strains showed no appreciable differences in survival at this temperature.

After 92 hours at 4 ºC, still no acid effect was observed ($P = 0.639$). The mean decrease from the beginning to the end of the incubation period at 4 ºC, for each *C. jejuni* strain and pH value on defined media, can be more easily interpreted in Figure 5.2.

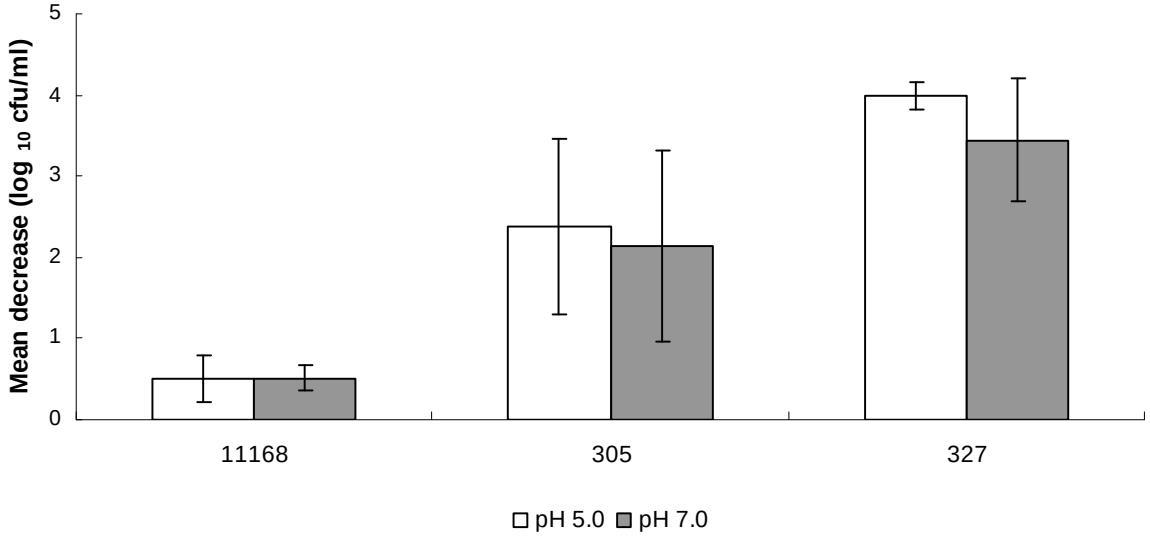


Figure 5.2 Mean decrease in numbers of three *Campylobacter jejuni* strains at pH 5.0 and pH 7.0 after incubation on defined media for 92 hours at 4 ºC. Data are mean values ± SEM (standard error of mean) (n = 3). Acid conditions did not significantly affect the decrease in population at 4 ºC ($P > 0.05$).

Influence of temperature on *C. jejuni*'s survival

Survival at pH 5.0 and pH 7.0 after 20 and 44 hours was also considered for statistical analysis. An Independent-Samples T-Test was performed to compare mean bacterial counts of each strain for two groups of temperatures: 4ºC and 37ºC.

Table 5.2 Statistical significance of temperature conditions 4ºC and 37ºC on *C. jejuni* survival in acid conditions (pH 5.0) at t=20 h and t=44 h. The confidence interval chosen to consider the effect of a parameter as significant was 5%.

Strain	Time	*P*
11168	20 h	**0.004**
	44 h	**0.002**
305	20 h	0.614
	44 h	**0.018**
327	20 h	0.180
	44 h	**0.050**

Table 5.3 Statistical significance of temperature conditions 4ºC and 37ºC on *C. jejuni* survival in acid conditions (pH 7.0) at t=20 h and t=44 h. The confidence interval chosen to consider the effect of a parameter as significant was 5%.

Strain	Time	*P*
11168	20 h	0.076
	44 h	0.060
305	20 h	0.692
	44 h	0.767
327	20 h	0.128
	44 h	0.061

At pH 7.0 there was no temperature effect, i.e, different temperature treatments did not affect significantly survival of three strains at both times considered ($P>0.05$). However, at pH 5.0, mean bacterial counts were significantly affected ($P<0.005$) for strain NCTC 11168 when temperature is changed from 4 ºC to 37 ºC. For strain 327 and 305 temperature effect was noticed at t=44 h. These differences can be more easily seen in Figure 5.3 that shows *C. jejuni* plate count numbers at pH 5.0 considering different times (t=20 and t=44 hours) and different temperature treatments.

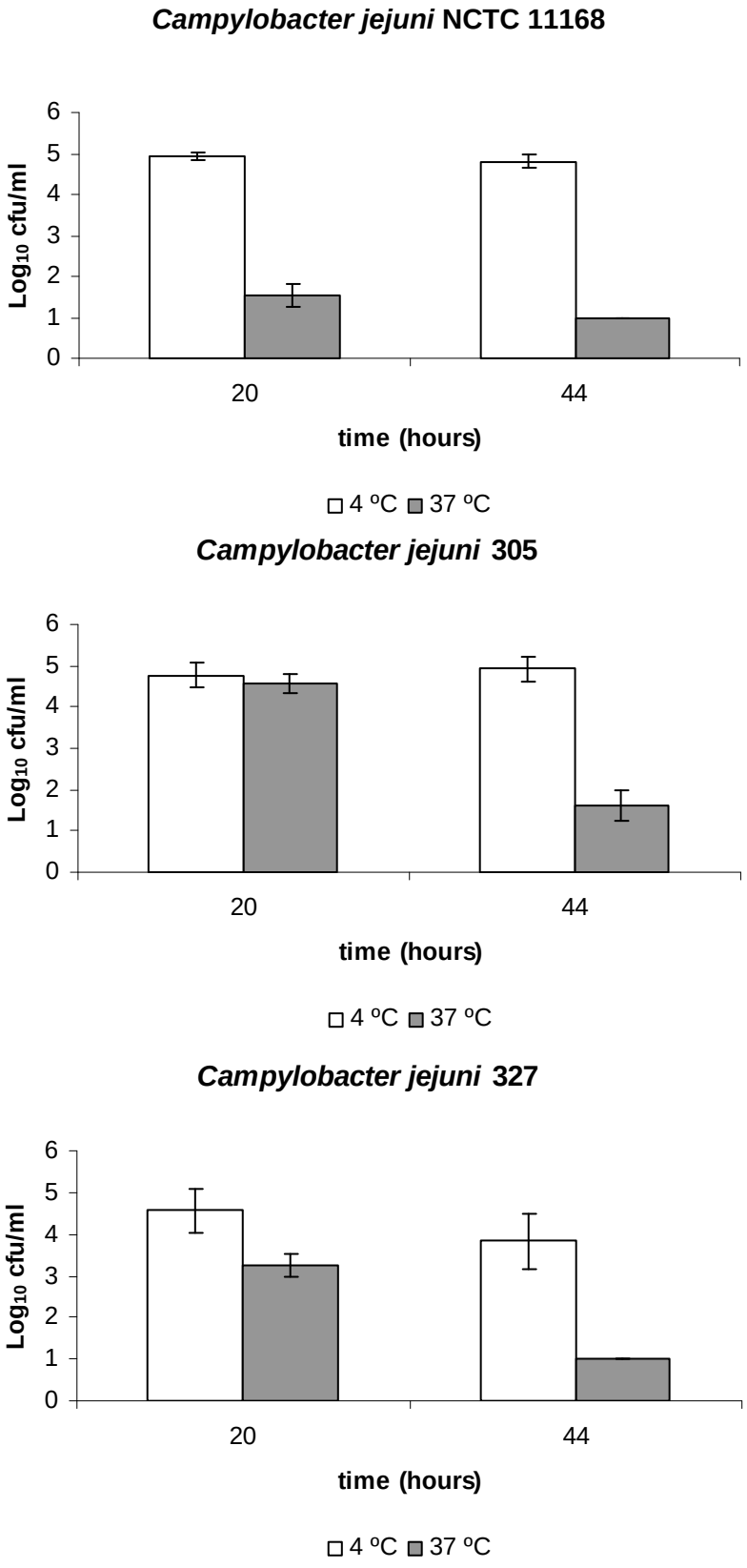


Figure 5.3 Average bacterial counts (log_{10} cfu/ml) at t= 20 h and t= 44 h at pH 5.0. Error bars represent standard error of the mean.

Modeling Inactivation kinetics at constant pH

Fit of the models

The numbers of viable bacteria in the culture medium adjusted to pH 5.0 at 4 ºC and 37 ºC were modelled to provide objective estimation of the parameters of the inactivation curves.

Figure 5.4A shows the fit of the Weibull model for *C. jejuni* strains at 37 ºC. Inspection indicates a good fit obtained by the Weibull model. It appears that the first-order kinetics is not appropriate in describing the data (Table 5.4). At 4 ºC all strains fitted better the Weibull model (Figure 5.4B).

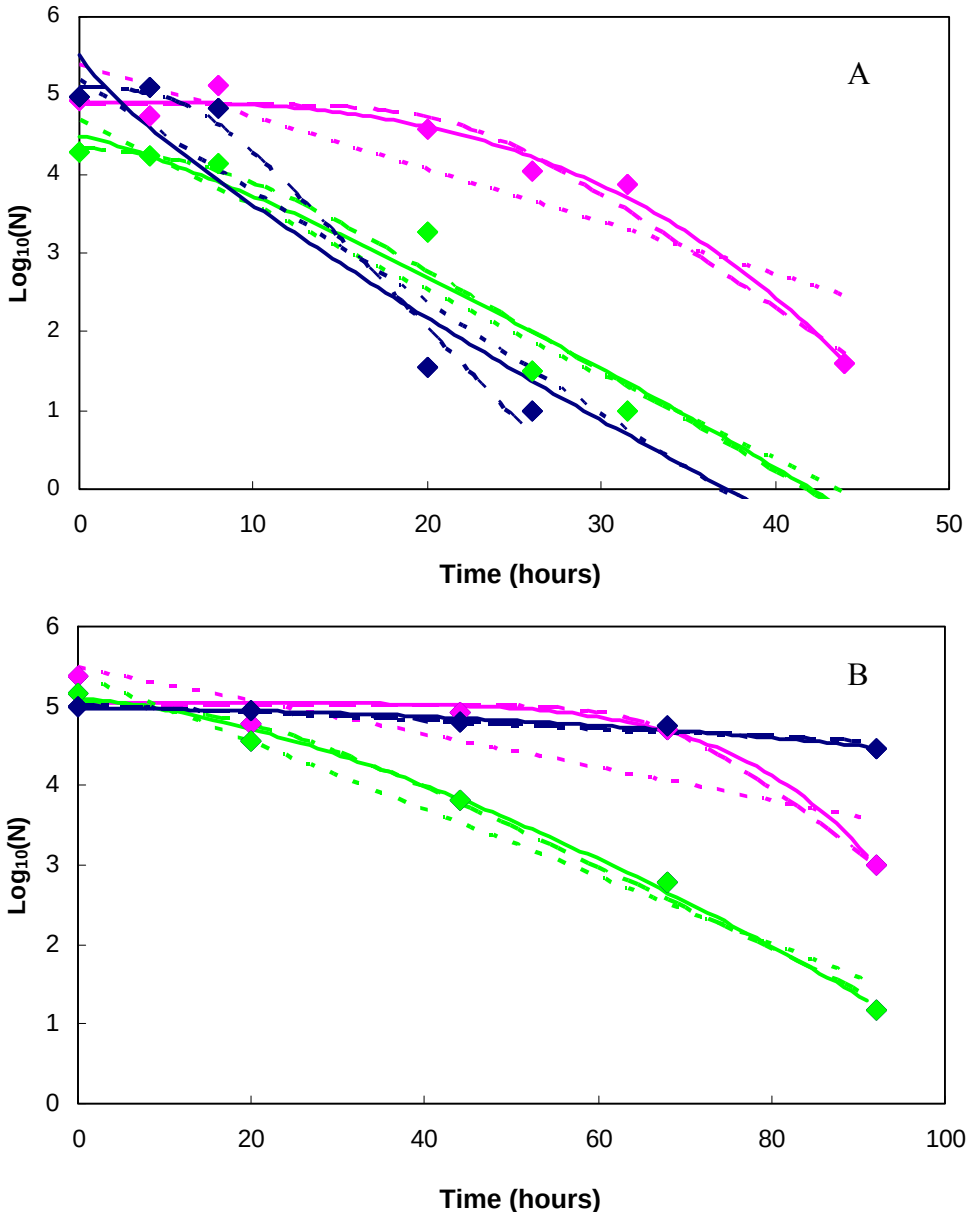


Figure 5.4 Survival curves of *C. jejuni* NCTC 11168 (dark blue line), 305 (pink line) and 327 (green line) during inactivation (pH 5.0) at a temperature of 37ºC (A) and 4ºC (B). Data were fitted with the Weibull ——— ; Log-linear ····· ; and Log-linear+shoulder – – – models to better illustrate the difference.

The goodness of fit of the Weibull and first-order models was compared by computing the R^2 and RMSE values (Table 5.4). Models Log-linear+shoulder and Weibull lead to similar RMSE values and the models seemed to describe more accurately the data when compared to first-order kinetics.

Table 5.4 Comparison of the first-order, Weibull and log-linear+shoulder models for the survival curves of *C. jejuni* at an inactivation temperature of 4ºC and 37ºC.

Bacterial strain		First-order kinetics		Weibull distribution		Log-linear+shoulder	
		R^2	RMSE	R^2	RMSE	R^2	RMSE
11168	37 ºC	0,92	0,83	0,93	0,87	0,97	0,46
	4 ºC	0,92	0,07	0,98	0,04	0,93	0,08
305	37 ºC	0,78	0,61	0,98	0,19	0,97	0,26
	4 ºC	0,71	0,56	0,94	0,31	0,94	0,31
327	37 ºC	0,95	0,45	0,97	0,46	0,96	0,41
	4 ºC	0,97	0,32	0,995	0,15	0,99	0,25

Parameters of the models

Parameters of the Weibull model (δ and p) plus k_{max} (log-linear decline) and S_1 (magnitude of shoulder time) are given in Table 5.5. The shape factors (p) of Weibull model indicate that survival curves fitted with this model were concave downward (convex) ($p > 1$) at 4ºC (Figure 5.4B). Although the Weibull model is of an empirical nature, a link can be made with microbial inactivation as follows. Downward concavity ($p > 1$) indicates that remaining members became increasingly damaged; whereas upward concavity ($p < 1$) indicates that remaining members have the ability to adapt to applied stress (van Boekel, 2002). Therefore, concave downward ($p > 1$) survival curve of *C. jejuni* strains at 4 ºC fitted with this model can be interpreted as an evidence of weak or sensitive members of the population that are destroyed at a relatively slow rate leaving behind survivors of increasingly lower resistance. Survival of strain NCTC 11168 at 37ºC under acid stress showed upward concavity ($p < 1$) which can be interpreted as members of the population being destroyed at a relatively fast rate leaving behind survivors increasingly more resistant (Buzrul *et al*., 2005).

Table 5.5 Parameters of the Weibull, Log-linear+shoulder and first-order models at an inactivation temperature of 37ºC and 4ºC.

Bacterial strain		First-order kinetics		Weibull distribution		Log-linear+shoulder	
		k_{max}	D	δ	p	k_{max}	S_1
11168	37 ºC	0,33	7.08	4,51	0,81	0,53	6,57
	4 ºC	0,01	189.84	134,26	1,94	0,01	28,12
305	37 ºC	0,15	15.06	29,41	2,96	0,34	22,37
	4 ºC	0,05	47.99	81,38	5,80	0,19	67,38
327	37 ºC	0,25	9.30	12,50	1,23	0,29	7,82
	4 ºC	0,10	23.66	37,87	1,52	0,12	19,54

In the Weibull model, δ [time unit] corresponds to the time needed to reduce the number of microorganisms by a factor 10 (analogous to the *D*-value[5] of firs-order kinetics). δ can play the same role as the parameter *D*, even though it has a different meaning. δ is not equivalent to the *D*-value, which is the same at any time during the process. For instance, calculation of time needed for six log reduction for first-order model is 6*D*. However, the time needed for a six log reduction for the Weibull model is not 6δ but it is δ_6, as a consequence of nonlinear behaviour (van Boeckel, 2002).

When δ and *D* values in Table 5.5 were compared, it can be seen that δ values are smaller than the *D* values for strain NCTC 11168. This indicates over-processing (if target is one log reduction) if first order model is used instead of Weibull model. For poultry strains δ values are greater than the *D* values indicating under-processing. In such case the calculated acid inactivation death time based on first order kinetics, at least theoretically, is shorter than that truly needed. Thus, using a non-optimal model may result in non safe predictions of inactivation. On the other hand, log linearity is easy from a data handling view point. These under and over-processing phenomena could explain the impressive safety record of the first-order model for many years (Corrandi, Normand & Peleg, 2005).

The strain variation observed in acid resistance, is evidenced by different values of δ with the time needed for one log reduction ranging from 4.51 to 29.41 at 37 ºC and from 37.87 to 134.26 at 4 ºC (Table 5.5). Strain variation in acid resistance was also evidenced by different

[5] The traditional log-linear approach can be described as follows: $\log_{10}(N) = \log_{10}(N(0)) - \frac{t}{D} = \log_{10}(N(0)) - \frac{k_{max} t}{\ln(10)}$

acid death rates which ranged from 0.29 to 0.53 (Table 5.5) at 37ºC and from 0.01 to 0.19 at 4ºC for the Log-linear+shoulder model.

Temperature effect on survival at low pH is also evidenced by differences in δ values (Table 5.5). In the Weibull model, δ is considerably larger at 4 ºC when compared to 37 ºC meaning that it takes longer to have a 10-fold reduction of the surviving population at 4 ºC. In other words, the time needed to induce one log reduction in population with a constant acid treatment of pH 5.0 is greater at 4 ºC when compared to 37 ºC. As expected, the values of $k_{\max}$ were larger at 37ºC than at 4ºC, indicating that the numbers of viable cells were reduced more rapidly upon exposure to pH 5.0 at 37ºC.

Stationary phase behaviour of *C. jejuni* 327

Viable counts of *C. jejuni* 327 were monitored on defined media (pH 7.0) during growth (37 ºC) and as cells entered the stationary phase. After maximum cell count had been achieved at about 44 hours, viable numbers estimated on defined medium remained roughly constant for about 6 hours and then began to decline (Figure 5.5A). After 68 hours the viable counts increased to leave a residual population at 92 hours. The observations made were unexpected; however, they were very consistent over the three trials. The typical behavior of *C. jejuni* 327 during stationary phase is shown in Figure 5.5A.

The data are means of three independent experiments in which the cultures behaved almost identically. It is important to note however that significant variations on this basic pattern were seen. In some cases, the decline in bacterial counts was lower at 68 hours (Figure 5.5B), while in other experiments the decline in numbers was higher than depicted in Figure 5.5A and Figure 5.5B (data not shown) underlining the inherent problems of reproducibly of this species.

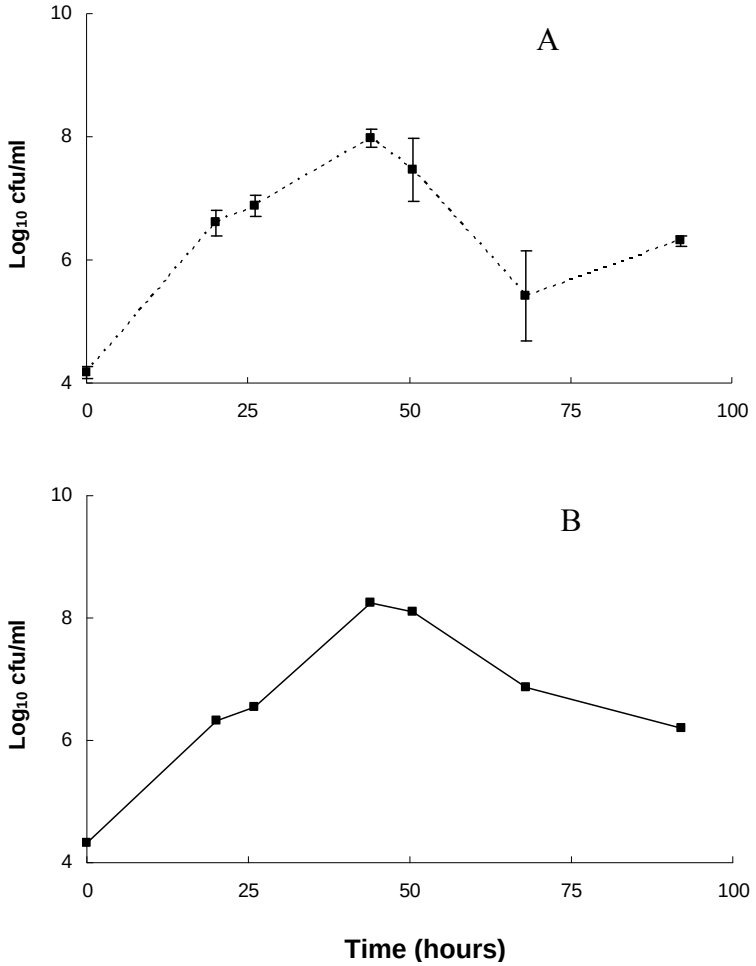


Figure 5.5 Stationary phase behavior of *C. jejnui* 327. The typical behavior is shown in panel (A), in which the data represented are the means of three independent experiments ± SEM (standard error of the mean). Some variations on this basic pattern were seen. In the experiment shown in panel (B), the decline in cell number at 68h was considerably lower.

5.4 Discussion

Although data obtained from *in vitro* survival studies cannot directly be extrapolated to foods or to host conditions, such information is useful in defining how *C. jejuni* will respond to different environments. Few data are available on survival of *C. jejuni* under acid stress conditions; thus, it is hard to comment on its legitimacy as an acid stress-sensitive pathogen.

The results of the present study clearly demonstrate that temperature influences the ability of three *C. jejuni* strains to survive under the acid conditions used (HCl, pH 5.0). From this study, it appears that *C. jejuni* acid stress sensivity depends on temperature. All strains survived better at 4 ºC and were found to be more sensitive to acid conditions at 37 ºC, in agreement with previous findings. Doyle and Roman (1981) reported that in a medium having the same pH, *C. jejuni* was inactivated more rapidly at 42 ºC than at 25 ºC and similarly, more rapidly at 25 ºC than at 4 ºC. *C. jejuni* has also been reported to survive better at 4 ºC than at

22 ºC or 30 ºC as a function of temperature, pH and sodium chloride (Kelana & Griffiths, 2003). At comparable pHs, *C. jejuni* cells died most rapidly at 30 ºC and most slowly at 4 ºC, which is in agreement with the results presented here. This is of particular importance because low temperatures used to limit bacterial growth on meat during storage also allow bacterial survival at higher rates. At 37 ºC, which is in its optimal range of growth temperatures, *C. jejuni* is severely affected by acid and survives less than 44 hours, whereas total population shows a reduction as low as 0.17 $\log_{10}$ at 4 ºC, for some strains. In Tryptic Soy Broth (TSB) acidified at pH 5.0 with HCl, the decrease in surviving cells between 4 and 8 hours at 42 ºC was reported to be approximately 2 $\log_{10}$ cfu/mL for *C. jejuni* ATCC 33560 (Shaheen *et al.*, 2006). In the present study, viable cell counts remained more or less constant between 4 and 8 hours of acid exposure at 37 ºC, for all strains used. Apart from strain variation, the different results obtained can be partly explained by different temperatures used. Since the present findings suggest that temperature is an important factor contributing to different survival patterns at pH 5.0, it is not surprising that at a higher temperature (42ºC) a higher decrease in numbers is observed compared to 37ºC.

Several studies have demonstrated that *C. jejuni* survival is considerably enhanced at low temperatures. In surface waters and microcosms, survival was shown to be limited to a few days at ambient temperatures of 20 ºC but was noticeably enhanced (up to several weeks) at 4 ºC (Terzieva & McFeters, 1991; Buswell *et al.*, 1998). A recent study performed with turkey meat has also demonstrated that after incubation at 25 ºC, population was severely decreased compared to the surviving population after incubation at 4 ºC (Hänel & Atanassova, 2007).

Concerning survival at 4 ºC, two hypotheses can be considered:

1. Acid might be less toxic at 4 ºC because of less active metabolism and lower permeability of the plasma membrane to protons.
2. It has already been shown that vital cellular processes like oxygen consumption, catalase activity, ATP generation and protein synthesis, were decreased at this temperature (Hazeleger *et al.*, 1998; Lázaro *et al.*, 1999).

Although *Campylobacter* spp. lack the major cold-shock response protein, CspA (Parkhill *et al.*, 2000), it has been suggested that this organism does elicit a cold shock response that regulates gene expression at low temperatures (Hazeleger *et al.*, 1998; Stintzi & Whitworth, 2003). Potential mechanisms for tolerance of and adaptation to cold temperatures include acquisition or biosynthesis of cryoprotectant molecules, maintenance of translation initiation

and efficiency, and alteration of the membrane lipid composition (Stintzi & Whitworth, 2003). This leads to the down regulation of as many genes as possible to save energy and the up regulation of genes involved in energy metabolism and modification of the cell wall components (Moen *et al.*, 2005). *C. jejuni* has a greater need for energy at lower temperatures. Temperature regulation can be critical for pathogenesis, and may prevent the bacteria from inappropriate expression of energetically costly proteins until they are in the environment in which the proteins are required (Pajaniappan *et al.*, 2008).

It appears from this study that the ability of *C. jejuni* to alter its metabolism in response to low temperatures can also significantly affect its ability to survive under acid conditions. The fact that no significant differences in decrease of population were observed at pH 5.0 and pH 7.0 suggests that pH values were probably not the most significant factor contributing to the survival pattern of *C. jejuni* organisms following incubation at 4 ºC.

Structural and metabolic changes involved in cold shock response may have a protective effect against acid stress. In fact, many of the changes verified in cold shock response are also present in acid shock response, such as regulation of energy systems and modification of membrane composition. It has also been shown that *C. jejuni* has considerable electron transport chain (ETC) activity even at temperatures below the minimal growth temperature (Hazeleger *et al.*, 1998). As it was previously discussed, the ETC plays a considerable role in the ability of *C. jejuni* to survive in acidic conditions (Reid *et al.*, 2008b). Thus, one of the major proton extrusion mechanisms employed by *C. jejuni* in acid stress response is able to function at low temperatures and this is of obvious importance to acid resistance at 4 ºC.

Conditions other than low metabolic activity and membrane changes in composition, may account for the prolonged survival in acid conditions at 4 ºC when compared to 37 ºC. Cross protection between the cold shock response and acid-stress responses might explain this increased resistance at low temperatures. A global response could be induced at low temperatures that could overlap the acid stress response, thus conferring cross-protection.

Another part of this study concerned the strain effect. It appears that some poultry strains (305) can be significantly more acid stress resistant than reference strains (NCTC 11168) and strains isolated from the same origin (327). If poultry is contaminated by diverse strains which vary in acid tolerance, applying acid agents may constitute a powerful selection for acid tolerance in poultry-derived strains that enter the pool of human clinical isolates. Strain 327 was significantly more sensitive at 4 ºC at both pH levels. Chan *et al.* (2001) also

demonstrated that the rate of CFU decline at 4 ºC varied markedly among different strains of *C. jejuni*. Refrigeration of carcasses and poultry products (which is often prolonged) may also select cold tolerant strains. Further studies conducted on a larger number of wild strains are needed to identify resistant strains.

The order of sensivity to acid treatment was not found to be the same at the different temperatures used. Although strain 327 was shown to be the most acid sensitive at 4 ºC, at 37 ºC viable counts decreased faster for strain NCTC 11168. Although the difference in survival was not found to be significant (P>0.05), this might be explained by the duration of the incubation period (2-3 days). Longer incubation periods may be needed to confirm these results. Viability estimates which were obtained here should be regarded as minimal estimates, since cells may remain viable substantially longer than can be cultured. Strain 305, which was the most acid tolerant strain at 37 ºC, did not show significant differences in survival when compared to strain NCTC 11168 at 4º C. Further investigation with a longer period of acid exposure at 4 ºC may be needed to detect more significant differences in survival in these two strains.

The Weibull model proved to fit well inactivation data of *C. jejuni* at a constant pH of 5.0. For further investigation of the Weibull and loglinear+shoulder models (temperature and pH dependence of the parameters) more temperatures and pH should be studied. However, this was not possible in this study since only two temperatures (4ºC and 37ºC) and one pH 5.0 were used. Another effect that would be signalled using the Weibull or loglinear+shoulder models is that microorganisms may change acid resistance depending on conditions. For instance, their acid inactivation behaviour may be quite different when they have been adapted to certain stress conditions in foods or when they have grown in laboratory conditions (van Boeckel, 2002).

In conclusion, at 37 ºC, the body temperature of humans, *C. jejuni* is sensitive to acid stress. However, temperature should be taken into account while applying acid agents to eliminate *C. jejuni* because it is more resistant to low temperatures. Exposure to acid conditions at 37 ºC has a stronger effect on *C. jejuni* survival than acid stress at low temperatures (4 ºC). This resistance might be related to overlapping mechanisms of survival induced after cold shock response that could confer acid stress protection. It would be interesting to perform transcriptomic and proteomic analyses to further characterize the defence mechanisms induced by *C. jejuni* under acid stress conditions at 37 ºC and 4 ºC.

The lack of stationary-phase response in *C. jejuni* was demonstrated here in a single strain, 327. Although the results are consistent with the absence of an *rpoS* homologue in this strain (Parkhill *et al.*, 2000), it is possible that other strains may behave differently given the genetic diversity of this species. The unusual pattern of fluctuating viable counts that occurs during the stationary phase of *C. jejuni* 327 was also reported by Kelly *et al.* (2001) in *C. jejuni* NCTC 11351. These authors suggested that the secondary increase in viable count following the initial decline could be due to the emergence of a new strain better able to survive in stationary phase. Martínez-Rodriguez *et al.* (2004) further demonstrated that this emergent strain showed permanent phenotypic changes that may play a role in the survival of *C. jejuni* in stationary phase. Interestingly, strain 327 was shown to be the most sensitive strain in the acid challenges. However inactivation studies were performed directly in late exponential phase cells. It is possible that this strain has evolved an alternative strategy to promote survival in stationary phase, which involves genetic variation (Park, 2002). These findings could also suggest that growth phase affects cellular stress responses (Audia *et al.*, 2001). Strain specific differences suggest a genetic basis. Genetic plasticity appears to be a characteristic feature of campylobacters that seems to explain the wide variation in stress responses encountered in this species.

6 RESPONSES OF *CAMPYLOBACTER JEJUNI* TO ACID STRESS MONITORED BY MEASUREMENTS OF INTRACELLULAR pH DETERMINED BY FLUORESCENCE RATIO-IMAGING MICROSCOPY

6.1 Objective

Acid shock, involves exposing bacteria to a sudden drop in pH (generally below the threshold for growth). This reflects the situation naturally encountered by the bacterium as it goes from a food or water source into the host gastrointestinal (GI) tract. Acid shock studies have been undertaken with various enteric bacteria, but they often involve the exposure of the cells to a moderate acid stress prior to acid shock, which induces the expression of acid tolerance proteins that may not otherwise be expressed (Foster, 1999). *C. jejuni*'s acid shock response has been shown to be distinct from adaptation to mildly acidic conditions (Reid *et al.*, 2008b).

As discussed earlier in the chapter 4, pH_i or ΔpH plays a central role in cell functions, and a correlation between a sudden drop in ΔpH, and loss of viability has been demonstrated (Nannen & Hutkins, 1991; Breeuwer *et al.*, 1996; Siegumfeldt, Rechinger & Jakobsen, 1999, 2000; Shabala, Budde, Ross, Siegumfeldt & McMeekin, 2002).

It was the aim of this study to investigate the lethal effect of inorganic acidulant (HCl, pH 4.0) on the pH_i regulation of *C. jejuni* at the single cell level by means of Fluorescence Ratio-Imaging Microscopy (FRIM) according to the methods of Smigic *et al.* (2008) (submitted for publication). Moreover, a relationship between pH_i and the viability studies previously investigated for strain NCTC 11168 at mildly acidic conditions (pH 5.0) was examined and discussed.

6.2 Materials and Methods

Bacterial Strains and growth conditions

A total of 3 different strains of *Campylobacter jejuni* were used in this study. The sequenced clinical human isolate *C. jejuni* NCTC 11168 was obtained from the National Collection of Type Cultures (Central Public Health Laboratory, London, UK). The turkey isolates (327 and 305) were received from the Department of Food Science, Faculty of Life Sciences, Copenhagen University, Denmark. A stock culture of *C. jejuni* strains was kept at – 80 ºC in brain heart infusion broth (BHIB) (Oxoid CM225, England) containing 15% glycerol. A loopful of each strain was streaked onto blood agar base no. 2 plates (Oxoid CM271,

England) with added 5% horse blood. Cultures were prepared by growing the strains for 2-3 days. Subsequently, one loopful of each culture was streaked onto new blood agar base no. 2 plates, which were again incubated for 24h. These cells were assumed to be in stationary phase of growth (Shaheen *et al*., 2006). During propagation and growth all plates were incubated at 42 ºC under a microaerophilic gas mixture (5% O_2, 10% CO_2, and 85% N_2; Praxair, Inc., Danbury, Conn.) in sealed gas jars (Oxoid, anaerobic jars). Cells were harvested with 2 ml of PPSS with a loop, and the inocula were adjusted in PPSS to an optical density at 600 nm = 0.1 which corresponds to approximately 8 $\log_{10}$ CFU/ml.

Solutions, fluorescent probe and liquid medium

For experiments in liquid medium, defined media[6] (Birk, 2008; unpublished) was prepared, and pH was adjusted to pH 4.0, pH 5.0 and pH 6.5 using 1M NaOH and 1M HCl.

For construction of calibration curves, defined media with adjusted pH values in the range from 5.5 to 8.0 were prepared adding appropriate volumes of 1M NaOH and 1M HCl.

The fluorescent probe 5(6)-carboxyfluorescein diacetate succinimidil ester (CFDA-SE, Molecular Probe Inc., Eugene, OR, USA) was dissolved in water-free dimethyl sulfoxide (DMSO, Merck) to a final concentration of 4.48 mM.

Microscopic slides

Microscopic slides were prepared according to Smigic *et al.* (2008) (submitted for publication) procedure. To aid the immobilization of *C. jejuni* cells from liquid medium, microscopic glass slides were soaked in ethanol/HCl solution (100 ml ethanol (70% v/v) mixed with 2.2 ml HCl (1M)) overnight. Afterwards, the slides were rinsed with milliQ water[7], and dried on air. Ten µl of bind silane (PlusOne, Amercham Biosciences, Piscataway, NJ, USA) was applied on the surface of the glass slide for 5 min. Glass slides were rinsed with milliQ water, and dried on air. Chambers were subsequently assembled with the prepared glass slides. The perfusion chamber thus formed (approximately 70 µl volume) is ready to be filled with the bacterial suspension in defined media of the desired pH value.

[6] It is not possible to write the detailed list of components since it has not been published. Defined medium contained amino acids, vitamins, salts and buffers. Some components that are involved in TCA cycle were also included. No carbohydrates were added since *C jejuni* does not ferment sugars, obtaining energy from amino acids instead.

[7] Water that has been distilled and fed through a special ion exchange cartridge, which increases its purity.

Fluorescence labelling of *C. jejuni* cells

The CFDA-SE labelling procedure used in this study was modified from Smigic *et al.* (2008) (submitted for publication). *C. jejuni* colonies grown on blood agar base no. 2 plates supplemented with horse blood were harvested with 2 ml of peptone physiological salt solution (PPSS) with a loop, and the inocula were adjusted to an optical density at 600 nm = 0.1 (approximately 8 $\log_{10}$ CFU/ml). One ml of this cell suspension was transferred to a sterile eppendorf tube and centrifuged at 12000 x *g* for 5 min. The supernatant was removed and the later step was repeated. The cell pellet was subsequently dissolved in 990µl of citrate phosphate buffer (pH 7.0), and 10 µl of 4.48 mM CFDA-SE were added. The cell suspension was incubated at 37 ºC for 30 minutes, under microaerophilic conditions. Stained cells were harvested by centrifugation at 12000 x *g* for 5 min and resuspended in 500 µl of defined media with set pH values (4.0, 5.0 and 6.5). The suspension of the stained cells was diluted in defined media at a 1:50 ratio. The perfusion chambers were immediately filled with the prepared bacterial suspensions and kept in the dark. The set glass slides were subsequently centrifuged at 3000 x *g* for 4 min to induce cell attachment. Due to the experimental set up, the cell suspension was ready for microscopy only after 15 min.

Fluorescence microscopy and data analysis

The set-up for fluorescence microscopy was the same as described by Guldfelt & Arneborg (1998), and consisted of a fibre-connected monochromator with a 75 W xenon lamp to provide excitation wavelengths of 488 nm (pH sensitive) and 435 nm (pH insensitive). The inverted epifluorescence microscope (Zeiss Axiovert 135 TV) was equipped with a Zeiss Fluar 100 x objective (numerical aperture 1.3), a band-pass emission filter and a dichromic mirror. The fluorescence emission was recorded using a cooled charge-coupled device camera. To minimize photobleaching of the stained cells, a 10% neutral-density filter was used in the excitation path.

Images were collected using the Methamorph 7 software program (Universal Imaging Corp., West Chester, Pa.) at specific wavelengths. Data analysis was performed using the Meta Vue v. 7.1 (Universal Imaging) software program.

Calibration Curves

The procedure used for construction of calibration curves was modified from Smigic *et al.* (2008) (submitted for publication). CFDA-SE stained *C. jejuni* cells were incubated with 1 ml of ethanol (70% v/v) at 37 ºC for 30 minutes assuming cell death and full loss of membrane

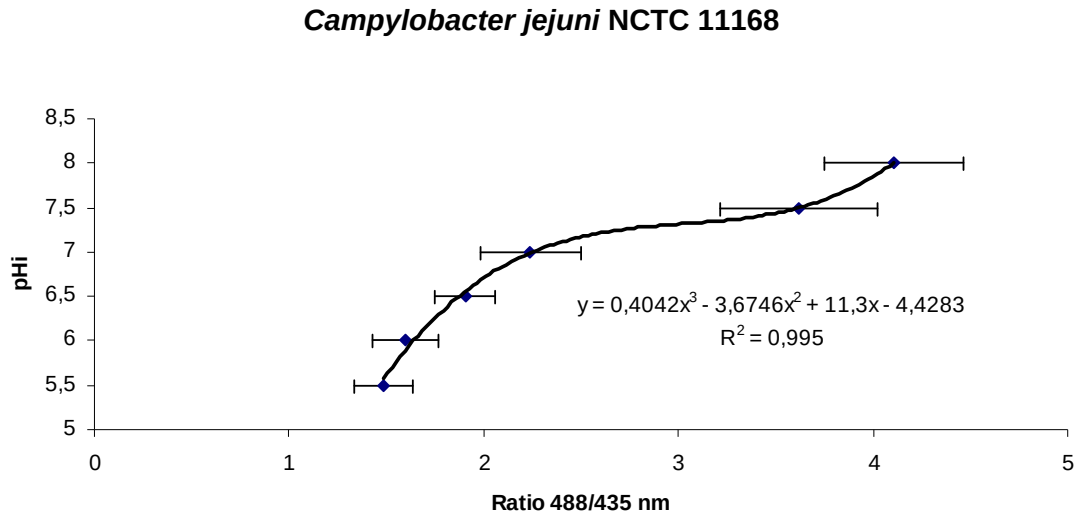
integrity. Subsequently, the cells were harvested by centrifugation at 12000 x *g* for 5 min, and resuspended in defined media with pH ranging from 5.5 till 8.0, with 0.5 intervals. The pH_i of a dead cell will be the same as the pH_{ex}. The ratios $R_{488/435}$ were determined as described above. With this probe, the three strains revealed different calibration responses, and therefore a calibration curve for each strain was made (Figure 6.1). Calibration curves for each *C. jejuni* strain were generated by plotting the ratio $R_{488/435}$ versus pH of equilibrated cells in the pH range from 5.5 to 8.0. A third degree polynomial model (Microsoft Excel, 2003) was fitted to the experimental data of each strain and used for pH_i calculations. Between 21 and 70 randomly selected individual bacterial cells were analysed in each experiment. CFDA-SE is a reliable probe within a relatively narrow pH range, between approx. 6 and 8 (Breeuwer *et al.*, 1996; Siegumfeldt *et al.*, 1999, 2000; Shabala *et al.*, 2002). At low pH values the pH sensitivity of the fluorochrome decreased, as demonstrated by the calibration curves. A value of pH_i 5.5 appeared to be the lower limit of the sensitivity for CFDA-SE.

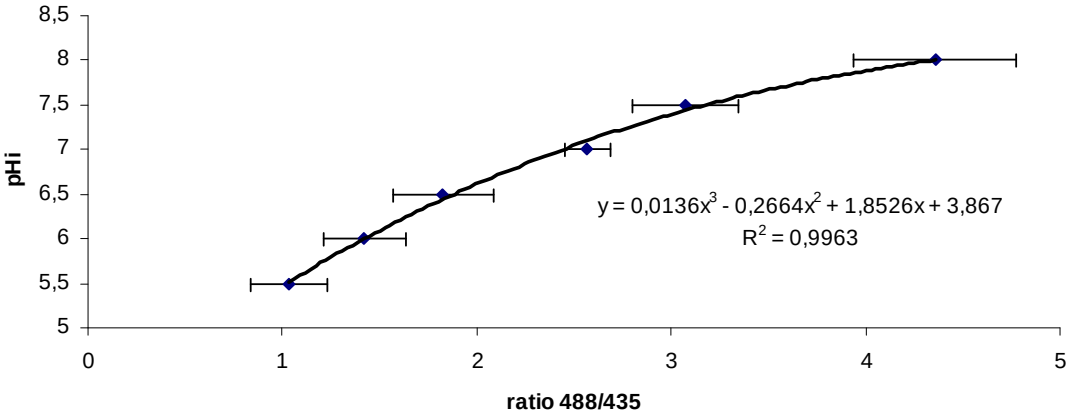
Experimental set up - pH_i

To determine the pH_i of healthy cells, defined media with pH value adjusted to pH 6.5 was used during a period of 15 minutes. For acid challenges, two different and independent set of studies were performed and the experimental work was divided in two parts (Part I and II).

In the first set of studies (Part I – section 6.3), the cells were stressed with defined media set at a lethal pH value of 4.0. The same cells were followed over a period of approximately 3.5 h and pH_i measurements were taken in 50 min intervals. For the three strains, between 30 and 98 cells were analysed. The study was carried out at aerobic conditions at room temperature (25 ºC). The stress response under these conditions was investigated for the three strains used and potential strain variation in acid stress response was evaluated based on pH_i measurements. Control experiments were performed in defined medium using HCl at a pH value of 6.5. For the three strains, between 61 and 105 cells were analysed.

In the second set of studies (Part II – section 6.4), cells were harvested as described above and stressed under pH 5.0 in defined medium. The media used were previously set at two different temperatures (4 ºC and 37 ºC) and pH_i was measured at regular intervals of 4h during a period of 24h. Chambers were incubated at 4 ºC and 37 ºC in a microaerophilic atmosphere and kept in the dark between each pH_i measurement. This experiment was performed with the reference strain NCTC 11168.





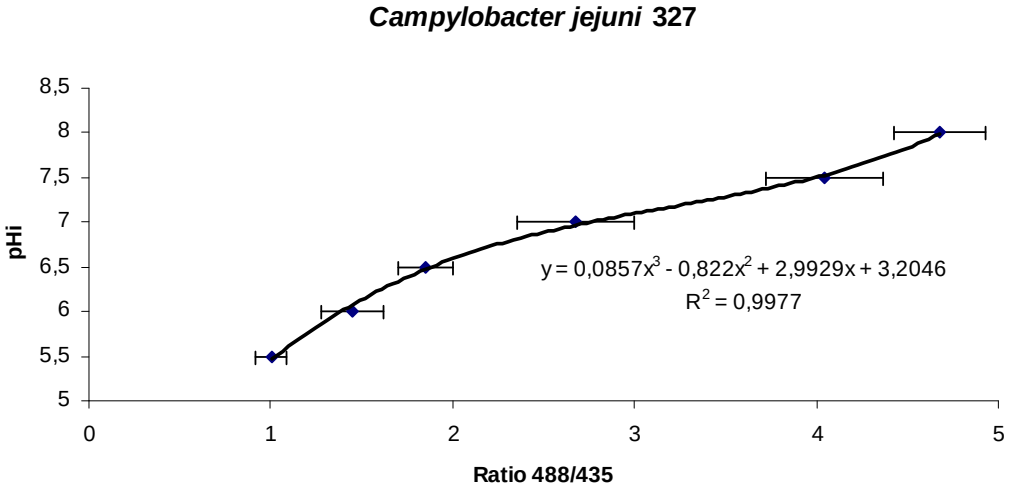


Figure 6.1 Calibration curves for pH_i determination of three *C. jejuni* strains. The pH_i was equilibrated to pH_{ex} by permeabilising the cells with ethanol and resuspending in defined media of known pH (5.5-8.0). The values are averages of 21-70 single cells with error bars (standard deviation). A third degree polynomial was fitted to the calibration points.

6.3 Part I - pH_i measurements in *C. jejuni* strains NCTC 11168, 305 and 327 under acid stress conditions (pH 4.0) at 25 ºC and aerobic conditions.

6.3.1 Results

pH_i of healthy cells

Because of the experimental set up, the first pH values could only be measured approximately 15 min after applying the cells into the perfusion chambers (see material and methods). The pH_i values for the time 0 (Figure 6.2) were established from the average pH_i obtained during 15 min of exposure to defined medium (pH 6.5). All three *C. jejuni* strains showed pH_i of healthy cells to be above 7.0.

Inactivation at pH 4.0

Change in the pH_i of three different *C. jejuni* strains during 215 min of liquid inorganic acid inactivation (defined media, pH 4.0 adjusted with 1M HCl) determined at the single cell level by FRIM is presented in Figure 6.2.

For strain NCTC 11168, a drop in pH_i values to the level of 5.5 (detection limit) was observed in most of the cells (75%) within the first 15 min of HCl treatment (pH 4.0). During the following 200 min, the subpopulation with $pH_i \leq 5.5$ remained the greatest one. At the same time an increase in subpopulation with $pH_i > 5.5$ was noticed. Among cells with $pH_i > 5.5$, two different subpopulations were recognized, one with $5.5 < pH_i < 6.0$ and the other with $6.0 < pH_i < 6.5$ (Figure 6.2).

The results obtained for the two poultry strains (305 and 327) indicated a drop in pH_i values to the level of $5.5 < pH_i < 6.0$ in most of the cells (90 %) within the first 15 min of exposure to acid. During the following 200 min, only two subpopulations were observed for both strains, one with $pH_i \leq 5.5$ and other with $5.5 < pH_i < 6.0$. For strain 327, the subpopulation with $5.5 < pH_i < 6.0$ remained the greatest one although a limited, but constant increase in subpopulation with $pH_i \leq 5.5$ was noticed. For strain 305, a considerable increase in subpopulation $pH_i \leq 5.5$ was observed together with a marked decrease in subpopulation $5.5 < pH_i < 6.0$ during the time of experiment. By the end of pH_i measurements, all cells (100 %) had $pH_i \leq 5.5$ for strain 305 while strain 327 approximately 20 % of the cells had $pH_i \leq 5.5$.

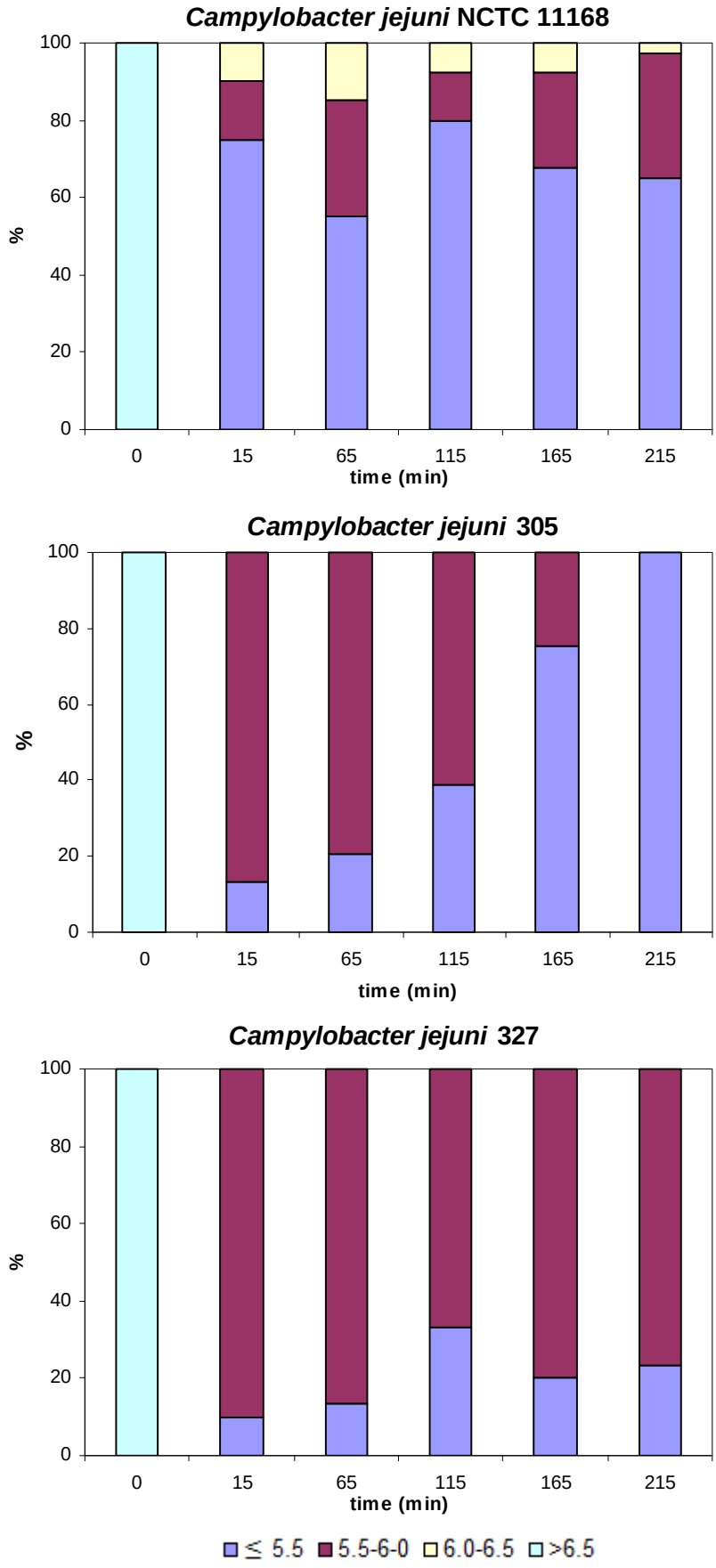


Figure 6.2 Percentage of cells on each pH_i subpopulation during 215 min of exposure to pH_{ex} 4.0 for *Campylobacter jejuni* NCTC 11168, *Campylobacter jejuni* 305 and *Campylobacter jejuni* 327.

Control experiment at pH 6.5

A control experiment with pH 6.5 was performed under the same conditions (Figure 6.3). All three strains were submitted to pH 6.5, which is in the optimal range of pH values for *C. jejuni* growth, at 25 ºC under a non-microaerophilic atmosphere.

For all strains three subpopulations were noticed after 15 min of exposure to these conditions, one with 6.5<pHi<7.0, the other with 7.0<pHi<7.5 and the third with pHi>7.5. The greatest subpopulation for all strains at 15 min was the one with 7.0<pHi<7.5 (70-90%). For strain NCTC 11168 (Figure 6.3) this subpopulation remained approximately constant during the following 200 min. At the same time, subpopulation with 6.5<pHi<7.0 became extinct after approximately 115 min and a constant increase of subpopulation pHi>7.5 was observed.

For both poultry isolates (Figure 6.3), the subpopulation with pHi>7.5 became extinct after approximately 115 min of exposure, while subpopulation 6.5<pHi<7.0 showed an increase that was accompanied by a decrease in subpopulation 7.0<pHi<7.5. For strain 327 (Figure 6.3) the observed alterations were more gradual than for strain 305, with approximately 57 %/43 % of each subpopulation, respectively (time 215 min). Strain 305 (Figure 6.3) showed a striking decrease in subpopulation 7.0<pHi<7.5 that became extinct at time 215 min, together with a marked increase in subpopulation 6.5<pHi<7.0. At approximately 165 min the emergence of a subpopulation with pHi≤6.5 was noticed for strain 305 and at time 215 min it was already the greatest subpopulation present (60%).

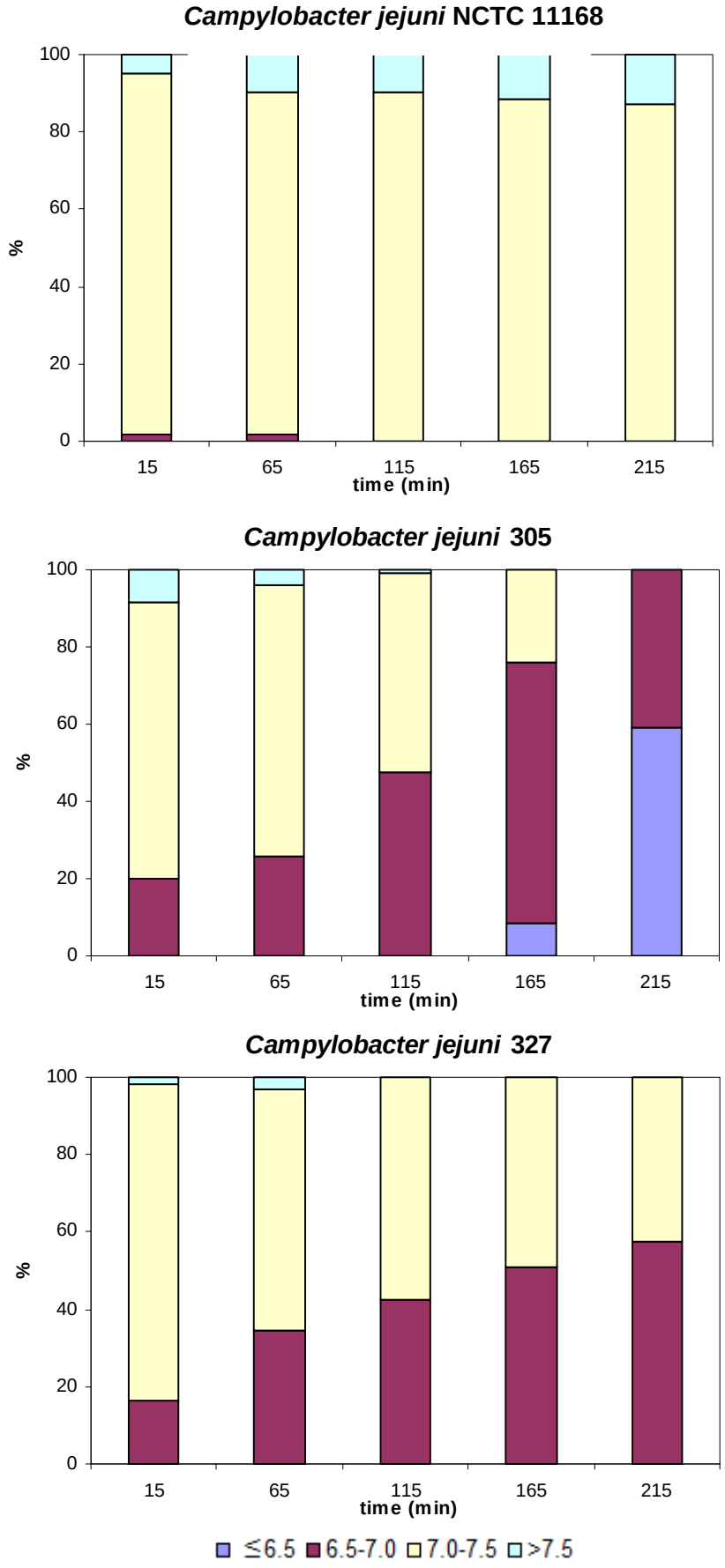


Figure 6.3 Percentage of cells on each pH_i subpopulation during 215 min of exposure to pH_{ex} 6.5 for *Campylobacter jejuni* NCTC 11168, *Campylobacter jejuni* 305 and *Campylobacter jejuni* 327.

Average pH_i

For comparison, average pH_i values in *C. jejuni* strains are shown in Figure 6.4. After initial drop, average pH_i values obtained in this study indicated no considerable pH_i change during following 200 min and values were in the range from 5.05 to 5.64. When strain NCTC 11168 was exposed to pH_{ex} 4.0, the resulting average pH_i values decreased below pH_i 5.5 within 15 min and therefore were beyond the detection limit.

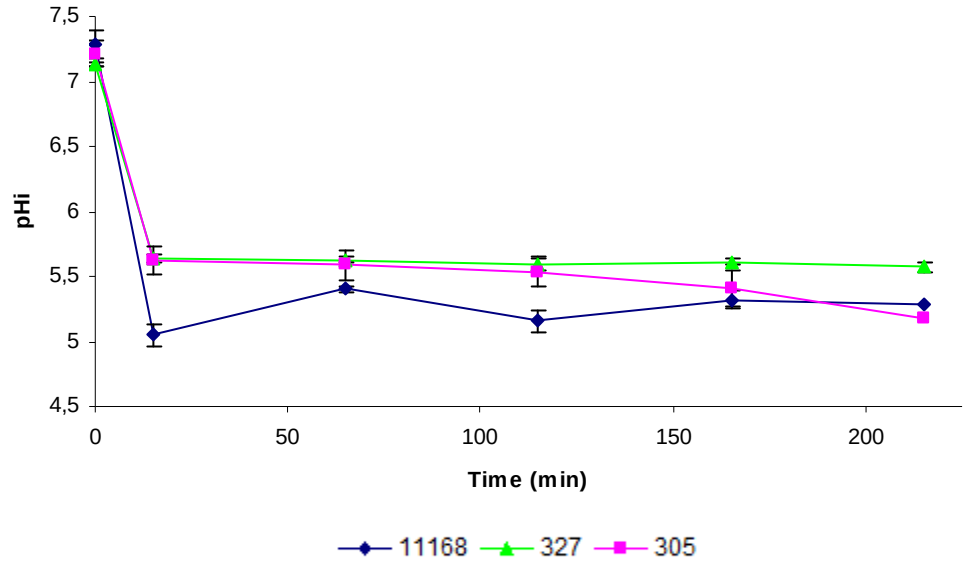


Figure 6.4 Average pH_i values for different *Campylobacter jejuni* strains during 215 min of inorganic acid inactivation in liquid defined medium (pH 4.0, adjusted with HCl 1M). The pH_i values are the mean based on 30-98 individual cells. Error bars represent the standard error of the mean.

The average pH_i values in *C. jejuni* in response to an external medium of pH_{ex} 6.5 are shown in Figure 6.5. After 200 min pH_i values ranged from 6.34 to 7.38. Strains NCTC 11168 and 327 maintained a relatively constant pH_i level at external pH_{ex} 6.5 for a long time, with pH gradient (ΔpH, the difference between pH_i and pH_{ex}) after 200 min being 0.88 and 0.47, respectively. pH_i response in strain 305 was different from those of other strains. pH_{ex} 6.5 caused a constant decline in pH_i of strain 305 and virtually no ΔpH was detectable after 200 min of exposure.

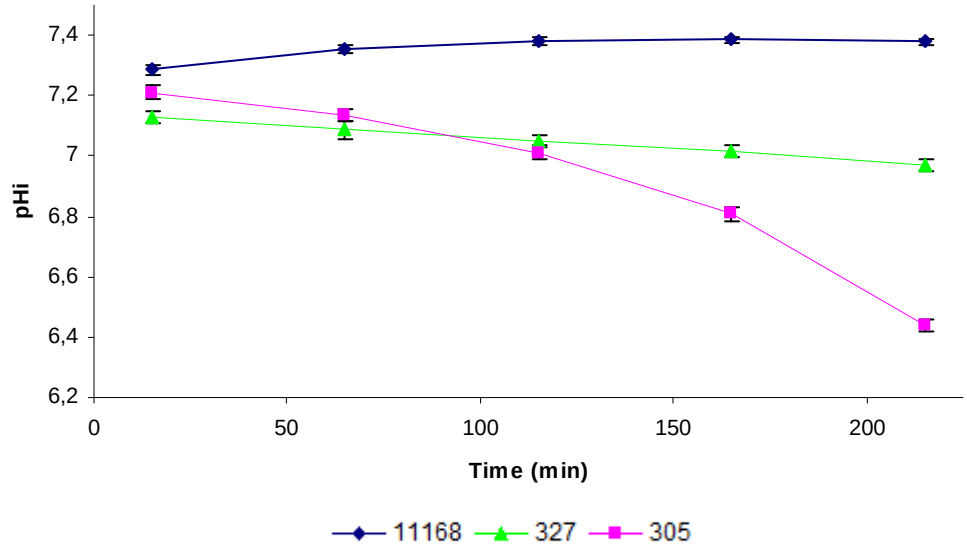


Figure 6.5 Average pH_i values for different *Campylobacter jejuni* strains during 215 min of exposure to liquid defined medium (pH 6.5, adjusted with HCl 1M). The pH_i values are the mean based on 61-105 individual cells. Error bars represent the standard error of the mean.

6.3.2 Discussion

The average pH_i values obtained during 15 min of exposure to defined medium (pH 6.5) provide information about pH_i values of healthy *C. jejuni* cells. For all three tested strains pH_i of healthy cells was found to be above 7.0. This is in agreement with the results of Smigic *et al.* (2008) (submitted for publication) that found that seven of eight *C. jejuni* strains had pH_i above 7.0 and that for only one strain this value was established at 6.8. The pH_i of stationary-phase cells of *L. inoccua* and *E. coli* was also reported to be close to neutral (Riondet, Cachon, Wache, Alcaraz & Divies, 1997; Shabala *et al.*, 2006). The pH_i of healthy *C. jejuni* cells obtained in the study of Tholozan, Cappelier, Tissier, Delattre and Federighi (1999) was between 6.63 and 6.73. The reason why the pH_i values for *C. jejuni* reported by Tholozan *et al.* (1999) differ from those obtained by Smigic *et al.* (2008) (submitted for publication), and by the present study might be found in the medium in which the cells were suspended. Smigic *et al.* (2008) (submitted for publication) used a nutritionally rich Bolton broth (pH 6.5) and in the present study a defined medium (pH 6.5) was used, while Tholozan *et al.* (1999) determined pH_i of *C. jejuni* cells suspended in less favourable microcosm water (filtrated surface water (pH 6.0)).

When cells of different *C. jejuni* strains were exposed to inorganic acid inactivation (defined medium, pH 4.0 adjusted with HCl) a drop in pH_i to the value of 5.5 (detection limit (Budde

& Jakobsen, 2000)) and 5.5<pH_i<6.0 was observed in the majority of cells within 15 min (Figure 6.2). Smigic *et al.* (2008) (submitted for publication) determined the effect of HCl (pH 4.0) during 12 min in liquid medium and demonstrated that *C. jejuni* cells maintained their pH_i values close to pH_i of healthy cells, indicating that *C. jejuni* is a homeostatic bacterium which maintains pH_i close to neutrality and generates large proton gradients when exposed to low pH environment of inorganic acidulant. The present results indicated that *C. jejuni* cells did not maintain their pH_i values close to pH_i of healthy cells with 15 min of exposure. Apart from strain specificity, the main reason for the disparity between this and the latter results could be due to a greater level of stress imposed by the conditions used in present study, which was carried out under room temperature (25 ºC) and in the presence of an oxygen atmosphere. It might also be explained by different growth conditions. Smigic *et al.* (2008) serially passaged the bacteria in a rich Bolton broth before plating and harvesting the cultures. Serial passage can result in genomic diversity and enhanced likelihood of selection of resistant cells. In addition, after the staining procedure, they resuspended the cells in Bolton Broth (pH 6.5) adjusted with HCl 5M and kept on ice in the dark prior to acid challenges and microscopic analysis. This might have conferred some level of protection and the pH_{ex} 6.5 used before acidification may have increased bacterial resistance to later acid treatment. *C. jejuni* has been shown to exhibit an adaptive acid tolerance response following exposure to mild acid, which is capable of protecting cells from normally lethal acid stress (Murphy *et al.*, 2003b; Shaheen *et al.*, 2006). Additionally, the present study used cells isolated from colonies grown on an agar plate at optimal conditions. It is likely that the heterogeneity in a colony with respect to pH and nutrient availability (Walker, Brocklehurst & Wimpenny, 1997) imposes various stresses on the cells, which may change their acid sensitivities (for example, by altering the membrane composition) (Correa, Rivas & Barneix, 1999). For instance, *Listeria monocytogenes* cells originating from broth cultures have been shown to be homogeneous in their sensitivities to nisin while cells grown on agar plates have a much more heterogeneous response towards the same treatment (Budde & Jakobsen, 2000).

Important differences among strains were observed during the acid challenge conditions. For strain NCTC 11168, the subpopulation with $pH_i \leq 5.5$ remained the greatest one (65-75%) during the whole time of experiment but subpopulations with pH_i>5.5 increased, specifically subpopulations with 5.5<pH_i<6.0 and 6.0<pHi<6.5. Thus, apparently some of the cells in the subpopulation with pHi≤5.5 (detected after 15 min) were able to increase the activity of proton extruding systems in order to return the cytoplasmic pH closer to healthy physiological values (Smigic *et al.*, 2008; submitted for publication). There are several possible

mechanisms by which a bacterium can regulate pH_i. The literature does not suggest which of these mechanisms is predominant in *C. jejuni*. It has been reported that *Enterococci* extrude protons via F-type proton-translocating ATPase and potassium ion accumulation (Kobayashi *et al.*, 2000), while respiring bacteria (e.g. *E. coli*) regulate the cytoplasmic proton concentration through the respiratory chain, but also via a potassium accumulation mediated through specific transport systems (Booth, 1985). The ECT is believed to play an important role in *C. jejuni*'s acid stress (Reid *et al.*, 2008a,b). In addition to proton extruding systems, decarboxylation of amino acids may also contribute to acid tolerance leading to biochemical consumption of protons (Cotter *et al.*, 2001; Rollan, Lorca & Font de Valdez, 2003). The defined media used in this study contained amino acids, and therefore consumption of amino acids might have been involved in pH_i regulation to some extent.

The increase in pH_i that was observed for approximately 10% of the cell population of strain NCTC 11168 might indicate that this strain has the ability to slowly recover and change cell viability. Whether this change reflects entry into the physiological state known as viable but nonculturable (VBNC) is not clear and therefore further investigation is required. Chitarra, Breeuwer, van den Bulk and Abee (2000) showed for *Clavibacter* sp. that when the pH_i drops to 5.5 or below, growth of this bacterium is inhibited. Smigic *et al.* (2008) (submitted for publication) reported that the decrease in pH_i to or below the FRIM detection limit ($pHi \leq 5.5$) does not necessarily designate a subpopulation of dead cells. The dead/life staining employed enabled these authors to conclude that some of the cells from this subpopulation still had an intact cell membrane (Nebe-von Caron, Stephens & Badley, 1998) and that pH_i of dead cells in the environment with $pH_{ex} \leq 5.5$ will be equal or below 5.5, but the reciprocal conclusion that all cells with pH_i 5.5 are dead is not plausible (Smigic *et al.*, 2008; submitted for publication).

Poultry strains showed an increase of the subpopulation with $pHi \leq 5.5$ in contrast with the apparent decrease in this subpopulation for strain NCTC 11168. However, the subpopulation with $pH_i \leq 5.5$ showed a limited increase during time for *C. jejuni* 327 and a marked increase for *C. jejuni* 305. Strain 327 was able to maintain the highest average pH_i values, but only strain NCTC 11168 appeared to be increasing pH_i. Considering average pH_i results one would not be able to recognise the repair of the cells, observed as the increase in the subpopulations with $pHi > 5.5$ as occurred for strain NCTC 11168 (Figure 6.2). When a population of cells is exposed to acid shock, the average pH_i is therefore a weighted average of various populations of cells with high and low pH_i. This could mean that individual cells have varying degrees of

acid resistance or that acid molecules do not target all cells in a population. Therefore obtained results emphasize the importance of determination of physiological parameters at the single cell level (Smigic *et al.*, 2008; submitted for publication). Moreover, average pH_i is biased by the detection limit of the method (FRIM), which masks the proportion of cells with $pH \leq 5.5$ that contribute to the average pH_i (Smigic *et al.*, 2008; submitted for publication).

Strain NCTC 11168 might therefore have an advantage over other strains since it seems to have a more heterogeneous population regarding stress response. In fact, the standard deviations in the pH_i values ranged from 0.45-0.65 pH units for strain NCTC 11168, which indicates that the population was heterogeneous. For strain 327 and 305 these values ranged from 0.14-0.22 and 0.10-0.14 pH unit, respectively. This indicates the presence of more homogeneous populations. Among poultry isolates, pH_i decreases faster for strain 305 than it decreases for strain 327. Strain 305 was therefore the most sensitive.

Interestingly, strain 305 was shown to be the most acid resistant strain at 37 ºC (pH 5.0) in the previous survival studies (see chapter 5). Studies involving *C. jejuni* are however, of notoriously difficult reproducibility. Aditionally, it is difficult to compare these results due to variations in the assay conditions used (that is, log vs. stationary phase cells, different challenge pH conditions, different atmosphere and temperatures used etc.).

In order to investigate pH_i regulation under control conditions, the same study was performed at pH 6.5 at 25 ºC, under aerobic atmosphere (Figure 6.3). Once again, the sequenced strain seemed to cope better with the imposed conditions. Strain NCTC 11168 kept pH_i within neutral values showing a slight but constant increase in pH_i over time. Conversely, poultry strains showed a decrease in pH_i. For strain 305 pH_i decreased faster and a new subpopulation with $pH_i \leq 6.5$ emerged at 165 min of exposure. For strain 305, pH dissipation occurred at a pH_{ex} of 6.5 to a value of 0 at approximately 215 min of exposure. By the end of the experiment this was the largest subpopulation with a total of 60 % of cells. It is not clear why a subpopulation of cells with $pH_i < pH_{ex}$ has emerged for this strain but the influence of methodological artefacts needs to be investigated to confirm this observation. Further experiments where pH_{ex} could be monitored during pH_i measurements are required. *C. jejuni* 305 did not seem to employ the same defending mechanisms to cope with the stress factors imposed. This demonstrates a greater sensitivity of this strain to experimental conditions.

These results prove that poultry strains, in particular 305, were under additional stress imposed by this experimental set up and that the observed initial response under pH 4.0 could not be attributed exclusively to acid stress. It is demonstrated by the control that acid stress conditions per se are not responsible for the observed inability of poultry strains to maintain pH_i homeostasis. One possible explanation is that the reference strain could have increased tolerance to oxidative stress. It would make sense that this strain has employed a strategy to cope with oxidative stress since it has been extensively passaged in laboratory for many years. Therefore, the improved ability of *C. jejuni* strain NCTC 11168 to tolerate oxygen could be an important physiological event governing its ability to maintain pH_i homeostasis. Murphy *et al.*, (2003b) have also demonstrated that the induction of an adaptive tolerance response under aerobic+acid conditions resulted in cross protection to acid stress. A recent study by Reid *et al.* (2008a) has demonstrated that the peroxide stress regulator gene (*perR*) is involved in the acid shock response in *C. jejuni* NCTC 11168.

The temperature used in the study (25 ºC) was also a stress factor imposed. *C. jejuni* is known to poorly survive at 25 ºC (Hänel & Atanassova, 2007; Garénaux *et al.*, 2008). Thus, the relative contribution of each stress factor used could only be assessed with further investigation, where a larger number of controls would be included.

In conclusion, a pH_{ex} of 4.0 was found to induce a collapse in pH_i homeostasis of *C. jejuni* strains under the conditions tested in this study within 15 min of exposure. Despite the lethal effect of pH_{ex} 4.0 used, some cells were able to increase their pH_i and therefore we can predict that under more favourable conditions eventually encountered by this microorganism, these cells may recover and pose a risk to human health. This is of great concern, especially for foodborne pathogens with low infective dose. The present work also demonstrates that the ability of *C. jejuni* to maintain a ΔpH under acid stress is strain specific and also depends on other stress conditions imposed. Oxygen and temperature are important factors to take into account that influence *C. jejuni* survival in the host, in the environment and during food processing. Further studies are required to investigate the weight of their contribution to pH_i regulation during acid stress conditions.

6.4 Part II - pH_i measurements in *C. jejuni* strain NCTC 11168 under pH 5.0 at 4 ºC and 37 ºC.

6.4.1 Results and Discussion

In a second experiment, ratio images were recorded for *C. jejuni* strain NCTC 11168 during 24 h of acid treatment (defined media pH 5.0 adjusted with 1 M HCl) determined at the single cell level by FRIM at both temperatures 4 ºC and 37 ºC. The study was conducted under the same conditions used in chapter 5.

The ratios in Figure 6.6 cannot be converted to pH_i values by using the equation described above (Figure 6.1) because of technical problems occurred with the microscope during the experiment. The ratios are undervalued and consequently pH_i will also be underrated. However, the relative response of the bacterial population can still be evaluated. An increase in ratios corresponds to an increase in pH_i values. Again, ratio images were taken at time 0, 15 min after the bacterial suspension was applied onto the chambers.

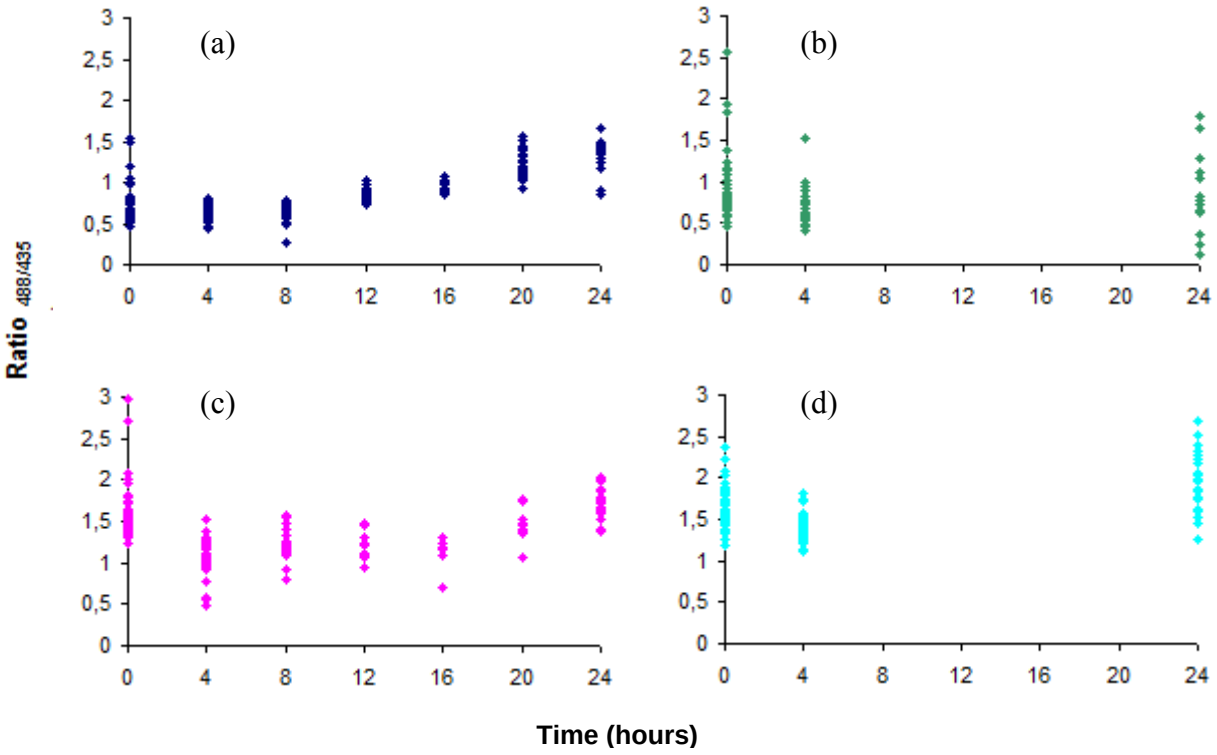


Figure 6.6 $Ratio_{488/435}$ plotted over a period of 24h of individual *C. jejuni* cells in defined media pH 5.0 (a, b) and pH 6.5 (c, d). Studies were performed at 4 ºC (b, d) and 37 ºC (a, c). Though it was not possible to follow the same cells over time, each symbol indicates a single cell.

$Ratio_{488/435}$ values decreased in *C. jejuni* NCTC 11168 during exposure to all four combinations of pH and temperature used, within the first 4 hours. After initial 4 hours the general response was an increase in ratio values (and therefore an increase in pH_i values). The increasing trend might reflect adaptation of bacterial cells to pH_{ex} 5.0. Shaheen *et al.* (2006) have demonstrated that the acid shock induced under pH 5.0 for 4h was not lethal for *C. jejuni* based on survivals on agar plates. They have also proved that exposure of late stationary phase cells of *C. jejuni* to this time/pH combination induced a significant acid tolerance response when compared to nonexposed cells (Shaheen *et al.*, 2006). The cells used in the present study were most probably in the stationary phase of growth. Adaptation to low pH might be expected to involve the down-regulation of genes encoding products that take up H^+ and the up-regulation of genes encoding proteins capable of H^+ extrusion (Reid *et al.*, 2008b) which would explain the adaptation response and the ability to raise the pH_i.

Presenting findings did not reveal apparent differences in pH_i response of *C. jejuni* to acid treatment regarding different temperatures. However, at pH 5.0 overall ratios (and pH_i) presented as average ratios (Table 6.1) were higher at temperature of 4 ºC (except for t=24 h). The same was noticed at pH 6.5. This might be explained by lower permeability of membrane to protons. Temperature has been shown to influence membrane composition (Stintzi, 2003).

Table 6.1 The average $Ratio_{488/435}$ values obtained on defined medium for *Campylobacter jejuni* strain NCTC 11168 during 24 hours of exposure to HCl at different pH values and different temperatures.

		Time						
***C. jejuni* strain**	**Conditions**	**0 hours**	**4 hours**	**8 hours**	**12 hours**	**16 hours**	**20 hours**	**24 hours**
NCTC 11168	pH 5.0 4 ºC	0.89±0.40[a]	0.69±0.21	n.d.[b]	n.d.	n.d.	n.d.	0.83±0.47
	pH 5.0 37 ºC	0.76±0.22	0.61±0.10	0.64±0.08	0.85±0.07	0.95±0.05	1.19±0.15	1.35±0.19
	pH 6.5 4 ºC	1.66±0.30	1.42±0.15	n.d.	n.d.	n.d.	n.d.	1.96±0.33
	pH 6.5 37 ºC	1.56±0.26	1.07±0.23	1.20±0.19	1.24±0.16	1.09±0.23	1.45±0.18	1.72±0.20

[a] value represents mean ± standard deviation
[b] n.d. – not determined

At 4 ºC after 24 h of exposure, the ratio (and pH_i) of some cells had raised slightly, while it was unchanged for other cells i.e., a larger variation in ratios (and pH_i) at t=24 hours, which can be seen in Figure 6.6 (b,d) as more disperse distribution of cells, and in Table 6.1 as an increased standard deviation. A more heterogeneous population is present at 4 ºC after 24 h. At refrigeration temperature the population appeared to increase heterogeneity in respect to

their pH_i regulation under acid stress. However, further studies are needed to confirm these data.

Survival of late exponential phase cells of strain NCTC 11168 at 37 ºC under pH 5.0 (see Chapter 5) was considered for comparison with the results obtained in the present study that was carried out with a stationary phase culture (Figure 6.7).

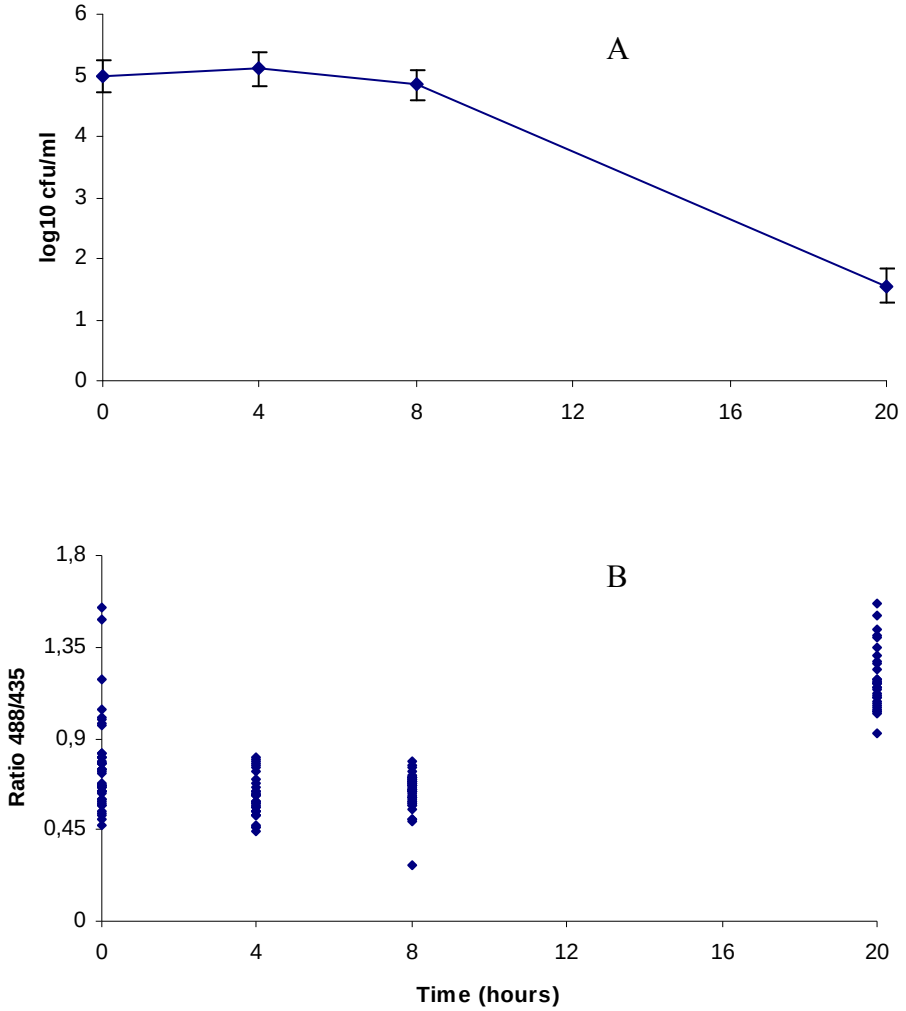


Figure 6.7 Survival **(**log CFU/ml) in late exponential phase cells (see chapter 5) (A) and $Ratio_{488/435}$ values of late stationary phase cells (B) for *Campylobacter jejuni* strain NCTC 11168 during 20 h of inorganic acid treatment (defined medium, pH 5.0 adjusted with 1 M HCl) at 37 ºC under microaerophilic incubation. In (A) error bars represent the standard error of the mean.

The increasing trend obtained in ratio (pH_i) measurements/viability in stationary phase cells (Figure 6.7B) was not observed in the culturability/viability study (Figure 6.7A). Figure 6.7A shows that 20h of HCl treatment (pH 5.0) at 37 ºC induced approximately a 3.5 log CFU/ml reduction in the number of culturable cells as determined on non-selective agar plates. Cells analysed in this study seemed to increase their intracellular pH showing that under HCl

treatment of pH 5.0 at 37 ºC cells are able to recover viability by maintaining a ΔpH (Figure 6.7B).

The most plausible explanation for such differences in viability is the fact that different physiological phase cells were used in these two independent experiments. In this context, data in Figure 6.7AB suggest that cells challenged in exponential phase could show increased susceptibility to acid stress at pH 5.0 while stationary phase cells seem to exhibit an adaptation response raising pH_i after the initial 4 hours. This is in agreement with a study by Murphy *et al.* (2003b) which demonstrated that mid-exponential phase *C. jejuni* cells adapted to pH 5.75 for 5 h became sensitized showing an increased death rate at acid challenge of pH 4.5. Conversely, an acid tolerance response was observed in *C. jejuni* cells in late stationary phase cells at pH 5.0 for 4 h (Shaheen *et al.*, 2006). The growth phase of the culture appears to define the kind of stress response expected at low pH (Audia *et al.*, 2001). This could have important implications since in the natural environment, optimal growth conditions are rarely encountered and *Campylobacter* will most likely be in stationary phase. Consequently, the ability to induce an ATR to acid in stationary phase may play a significant factor in the survival of this organism in the environment (Murphy *et al.*, 2003b).

To establish a comparison between culturability and pH_i values, studies would have to be conducted simultaneously, using the same challenged cultures (the same culture with cells in the same physiological state). For instance, if ratios had been measured in cells in Figure 6.7A, a subpopulation at t=20 hours would most probably be observed, represented by low ratios which would be in accordance with the decrease in the number of culturable cells. Due to the observed ability of some *C. jejuni* NCTC 11168 cells to raise their pH_i (see section 6.3) a second subpopulation of cells raising their pH_i could also be observed. Rechinger & Siegumfeldt (2001) have reported the analysis of pH_i of individual *Lactobacillus delbrueckii* subsp. *bulgaricus* cells by FRIM at an extracellular pH of 6.0. Their results revealed two populations of cells in which one was observed to correlate well with CFU. This two subpopulations behaviour has also been observed for *L. monocytogenes* at subinhibitory concentrations of bacteriocins (Hornbæk, Brockhoff, Siegumfeldt & Budde, 2005).

Several studies have found a high correlation between cell viability determined by CFU and cells maintaining their ΔpH (Siegumfeldt *et al.*, 1999; Budde & Jakobsen, 2000; Rechinger & Siegumfeldt, 2001; Shabala *et al.*, 2002; Hornbæk *et al.*, 2005). Smigic *et al.* (2008) (submitted for publication) found this relationship in *C. jejuni* only for the first 2 min of lactic

acid treatment (3 % v/v lactic acid, pH 4.0). These authors have concluded that pH_i and colony count may not be directly correlated in all phases of resuscitation and growth, but that they complement each other. Although this study has highlighted the ability of *C. jejuni* stationary phase cells to raise pH_i under pH 5.0, further research is still needed to examine how physiological state affects intracellular parameters, such as pH_i.

7 CONCLUSIONS

The purpose of this work has been to contribute to the understanding of acid stress response in *C. jejuni*. The main conclusions can be summarised as follows:

- There is a marked interaction between temperature and sensivity to acid stress. At 37ºC, the body temperature of humans, *C. jejuni* is far more sensitive to acid stress than at low temperatures (4ºC). In practical terms this means that bactericidal treatments with acids on skin or during food marination are more efficient at high temperatures. Additional observations of the kinetics at a pH level of 5.0 at additional temperatures between 4ºC and 37ºC would be necessary to describe the variation in inactivation as temperature increases above 4ºC.

- Despite being an acid sensitive organism, the results of this study indicated important variation in acid resistance, among strains of *C. jejuni*. Furthermore, significant differences in acid resistance were observed among strains originating from the same food source (poultry). The high level of acid tolerance of some *C. jejuni* strains raises doubts about the efficacy of the acid wash procedures proposed for poultry carcasses as a means of reducing *Campylobacter* contamination. Adding to this, one of the strains used was characterized by almost no stationary phase since die-off commenced shortly after the maximum numbers were reached, a behaviour which was distinctly different from the rest of the isolates. It is important to note that where *C. jejuni* is involved, having knowledge of one strain's behaviour is not the same as knowing the behaviour of all, given the high variability in stress response observed in this species. Such results highlight the importance of careful selection of strains in food safety research, and particularly when low temperatures or acid treatments are evaluated as anti-bacterial hurdles. The findings of this study should be useful in strain selection for the evaluation of antimicrobial alternatives, and for the completion of risk assessments.

- This study indicated that the Weibull and loglinear+shoulder models fit the inactivation data better during relatively slow acid inactivation than the first order models commonly used. In some cases use of simple log linear may produce imprecise or even unsafe predictions of inactivation behaviour at acid pH. However, it is known that models developed for one bacterium in a model system or in a food matrix should not be used to predict the inactivation kinetics in other conditions. The complexity of food and *in vivo* conditions potentially gives rise to interactions of several factors related to host, food and pathogen, and the interactions

can appreciably influence microbial behaviour. The identification and quantification of these interactive effects will be an extensive process. Nevertheless, experiences already indicate that models developed in model system could offer a rapid, convenient and reasonable first estimate of how an organism will behave in other conditions.

- Based on FRIM these studies have confirmed that rather than dying after exposure to a lethal pH of inorganic acid in a subpopulation, *C. jejuni* cells do have the ability to increase its pH_i. Sublethal injury induced by mild bactericidal treatments, might therefore result in subpopulations of cells of *C. jejuni* where only one subpopulation is affected by the treatment rather than weakening each individual cell of the population. FRIM provided rapid bacterial viability assessment by testing the ability to regain pH_i, after an apparently lethal treatment applied.

8 FUTURE PERSPECTIVES

The adaptive nature of *C. jejuni* must also be considered in the development of future experiments, as the response to environmental stresses can affect the survival of this pathogen. Future predictions of *C. jejuni* response to acid stress should take into account all microecological factors involved in the processing of a specific food or *in vivo* conditions.
Further investigation of genotypic and phenotypic heterogeneity of *C. jejuni* isolates will improve the understanding of the significance and role of stress responses for the survival of these organisms in the food chain.

Novel concepts for detection of viable cells in complex samples (e.g. foods) have been developed. Future application of rapid PCR methods for detection of viable stressed cells in food related environments (Takashi *et al*., 2008) can be used in analysis of *C. jejuni* stress responses. A PCR method that employs ethidium monoazide (EMA) can potentially be used as EMA can only enter cells with compromised membranes (dead cells) binding to DNA and preventing PCR amplification. This is particularly relevant for pathogens subjected to killing treatments such as disinfections or antibiotics that have specific growth requirements and may enter a VBNC state.

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