

VALIDATION OF AN IMPROVED ISOLATION AND
DETECTION METHOD FOR *CAMPYLOBACTER*
JEJUNI IN VARIOUS FOODS

By

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CHAPTER I

INTRODUCTION

Campylobacter is the most common cause of enteric infection worldwide manifested as acute infective diarrhea in humans. Its isolation rate is higher than *Salmonella* and the economic impact is \$6 billion annually in the United States alone. The economic impact accrues from public health bills and lack of productivity due to the illness. Studies reported show that 80% of all *Campylobacter* related illness is transmitted through food because *Campylobacter* exist naturally in animals and avian species used for food production. *Campylobacter* are microaerophilic, fastidious and generally difficult to isolate from food. In addition, they change morphology from spiral to coccoid form on exposure to environmental stress, which hampers effective isolation from food. Although recognized authorities such as the Food and Drug Administration (FDA) have published protocols outlining the methods for isolation of *Campylobacter* from food, there is no generally accepted standard method. In addition, the Food and Drug Administration has set no limits for *Campylobacter* yet the infective dose is reported to be as low as 500-800 cells. The Food and Drug Administration Bacteriological Analytical Manual (FDA/BAM) method for the isolation of *Campylobacter* from food has been reported to be cumbersome, tedious and sometimes inconsistent.

The objective of this research was to validate an improved method, the Food and Agricultural Products Center method (FAPC) for isolation of *Campylobacter* from food and compare it to the FDA method.

CHAPTER II

REVIEW OF LITERATURE

History of *Campylobacter*

Campylobacter species have been causing illness for several centuries although they were only recognized as human pathogens in the 1970's (Butzler 2004). Until the development of a selective medium by Skirrow (1977), *Campylobacter* were known to veterinarians mainly as animal pathogens responsible for a wide variety of disorders in sheep, cattle and pigs (Moore 2001). More sophisticated techniques for detection of *Campylobacter* have enabled the identification of its true potential as zoonoses, capable of being transmitted to humans, through minimally processed foods and by a wide range of domestic animals (Moore 2001).

The genus "*Campylobacter*" was first described at the beginning of the last century as causing abortion when they were observed in fetal tissues of aborted sheep (McFadyean and Stockman 1913), and they were placed at the time in the genus *Vibrio*. The genus name *Campylobacter* a Greek word for "curved rod" was eventually proposed by Sebald and Veron (1963) to include microaerophilic bacteria that were different from *Vibrio cholera* and other species of *vibrio* in a number of respects. In 1886, Theodore Escherich described spiral bacteria in the colons of children who died of what he called

'*Cholera infantum*' (Butzler 2004). Between 1909 and 1944, veterinarians described the bacterium frequently isolated from bovines and ovines with several names including '*Vibrio fetus*,' and finally in 1944 it was described as 'winter dysentery' in calves caused by infection similar to vibrio and it was called *Vibrio jejuni*. The isolation of *Campylobacter* from feces was accomplished in 1968 and published in 1972 (Butzler 2004). The invasive ability of *C. jejuni* was later shown by using poultry, and subsequently, antigenic typing was performed using agglutination and complement fixation tests with antisera raised from reference strains of *C. jejuni* and *C. coli* and this showed the relationship between isolates from animals, man and poultry (Butzler 1974). Although Butzler and Skirrow (1979) described *Campylobacter* enteritis in man, it was not until the mid-1980s that *C. jejuni* was recognized as the most frequent cause of bacterial enterocolitis in man (Butzler 2004).

The genus *Campylobacter* was first proposed in 1963 and included two species, *Campylobacter fetus* and *Campylobacter bubulus* (On and others 1998) but to date, this classification has been revised due to the ecological diversity of the *Campylobacter* and their clinical importance (On 1996). The taxa has become more complex and has been evolving rapidly over the years. Although most of the known species are well defined, the number of taxa that constitute a group has increased with at least one new species or subspecies being described every year since 1988 (Penner 1988; On 1996). The continuous emergence of new species has made it difficult to construct a scheme that can identify groups accurately (On 1996). The family *Campylobacteriaceae* has 18 species and subspecies that exhibit wide ecological diversity (Nachamkin and others 2000). Due to such diversity and clinical importance, extensive reviews have been done and as a

result, reclassification and re-grouping has been done based on DNA base composition and phenotypic characteristics. *Campylobacter* are considered to be the most common cause of human enteritis in the USA (Tauxe 1992) and other developed countries. Most of the enteric *Campylobacter* infections are attributed to *Campylobacter jejuni* subsp *jejuni* and its close relative *Campylobacter coli* but the role of other species may be undermined as a result of inappropriate isolation and detection methods (Corry and others 1995; On 1996). The infective dose was found to be as low as 5-800 cells with the attack rate increasing with increasing dose (Black and others 1988).

Characteristics of *Campylobacter jejuni*

Campylobacter jejune which belongs to the genus *Campylobacter*; are Gram negative, slender spirally curved rods with tapering ends, non-spore formers with a corkscrew-like motility. They possess polar flagella at both ends of the cell, which imparts a high degree of motility. Through the corkscrew-like motility, they are able to remain motile in highly viscous environment and as a result, are able to colonize the intestinal mucosa (Takata and others 1992). Morphologically, *Campylobacter* are slim, s-shaped rods; 0.5-8.0 μm long and 0.2-0.5 μm wide. They are catalase and oxidase positive, and urease negative. They are hippurate hydrolysis positive (Griffith and Park 1990). *Campylobacter jejuni* are known to lack the adaptive response correlated with stress and are therefore highly fastidious, requiring unique growth requirements or complex growth media (Park 2000), and are unable to ferment or oxidize carbohydrates (Vliet and Ketley 2001). They obtain energy from amino acids or tricarboxylic acid cycle intermediates and the only respiratory quinones that have been detected are

menaquinones with menaquinone-5 and menaquinone-6 being the major components (Vandamme 2000).

Their small genome of 1.6-1.7 Mbp of AT-rich DNA explains the requirement for complex media for growth and their inability to ferment carbohydrates and to degrade complex substances (Griffith and Park 1990). The small genome may also explain the lack of lipase or lecithinase activity and lack of growth below pH 4.9. They are microaerophilic and are unable to grow in the presence of air but grow optimally in 5% oxygen. They are generally sensitive to oxygen and its reduction products, with exposure to oxygen leading to the formation of reactive oxygen intermediates such as peroxide radicals, which if not neutralized, lead to damage of cell nucleic acids, proteins and membranes (Park 2002). They grow optimally at 42 °C but do not grow at temperatures below 30 °C. *Campylobacter jejuni* is sensitive to osmotic stress; does not grow in concentrations of 2% sodium chloride (Doyle and Roman 1982) and is incapable of growth below pH 4.9.

Clinical manifestations of Campylobacteriosis

Campylobacter jejuni infection is characterized by acute self-limited gastrointestinal illness, manifested by diarrhea, fever and abdominal cramps in industrialized nations while in developing nations, it is generally mild, non-inflammatory, watery diarrhea (Ketley 1997). The incubation period and onset of symptoms range from 1-7 days, although often the source and timing of infection is difficult to establish. The diarrhea can last 2-3 days but abdominal pain and discomfort may persist after diarrhea has stopped. In one study, approximately half of the patients with laboratory-confirmed

campylobacteriosis reported a history of bloody diarrhea (Blaser and others 1983; Ketley 1997). In less frequent cases, the infection may produce bacteremia, septic arthritis, and other intestinal symptoms (Altekruse 1999). One of the most serious complications of *Campylobacter* infection is the paralytic condition Guillain-Barré Syndrome which has an incidence of 1/1000 infection (Butzler 2004).

Death from *C. jejuni* infections are rare and occur primarily in infants, the elderly and patients with underlying illnesses such as HIV-AIDS. Most cases of *Campylobacter* infection in industrialized nations appear to be sporadic with a consistent seasonality (Tauxe 1992; ACMSF 1993).

In developing countries, the infection appears to have different clinical and epidemiological characteristics to that of industrialized countries with no pattern of seasonality. In developing countries, there is also a higher incidence of carriage without symptoms (Taylor 1992), which is attributed to a higher rate of exposure and infection in early life resulting in immunity. Although the infection can cause severe illness lasting more than 7 days, it is usually self-limiting and complications are uncommon (Skirrow and Blaser 1992).

Pathogenesis and Virulence determinants of *Campylobacter jejuni*

Campylobacter in food and water enter the host via the stomach and colonize the distal ileum and colon. After colonization of the mucous membrane, they upset the absorptive capacity of the intestines, by damaging the cell function directly or by production of toxins causing an inflammatory response (Ketley 1997). Many pathogen-specific virulence determinants contribute to the pathogenesis of *C. jejuni* infection

through promotion of adhesion and invasion. These include chemotaxis, motility to penetrate the mucin barrier, flagella, which are required for motility, adhesion and colonization of the gut epithelium. Once colonization takes place, other virulence determinants such as iron acquisition, host cell invasion, toxin production, inflammation and active secretion, and epithelial disruption with leakage of serosal fluid take effect (Altekruse and others 1999). Palmer and others (1983) reported a waterborne outbreak of *Campylobacter* gastroenteritis at a boys' school in England. This epidemiological study led to the estimation that as few as 500 cells initiated the infection. However, studies in volunteers showed a variable range in the infecting dose, with many volunteers developing no illness (Black and others 1988). Earlier studies though, reported disease being caused by 500 cells in a single volunteer (Robinson 1981).

Chemotaxis and Motility

The pathogenic mechanism of *C. jejuni* is mediated by a number of virulence factors that include motility, adhesion and ability to invade the host cells as well as production of toxins, which are likely to be responsible for many of the acute manifestations of infection. In order to successfully colonize the host mucosal surface, *C. jejuni* must be able to detect and move against chemical gradient. *Campylobacter* are highly motile and through chemotaxis (response towards chemical stimuli), they detect and move toward the chemical attractant through corkscrew motility. Motility is required for reaching the attachment site, and penetration of the mucosal lining of the epithelial cells. The importance of chemotaxis was demonstrated in non-chemotactic mutants that failed to colonize the suckling mouse intestine (Takata and others 1992). Various

chemical attractants including mucin, L-serine, L-cysteine, L-glutamate and L-fucose; and chemorepellants such as bile acids have been reported for *C. jejuni* (Hugdahl and others 1988).

Motility in *C. jejuni* necessitates the presence of flagella, which is another virulence determinant. Flagella give *C. jejuni* the ability to move in a viscous environment such as mucus, enabling the colonization of the mucous membrane of the intestinal cell surface. Lee and others (1986) demonstrated that *C. jejuni* had a high affinity for mucus by challenging mice with *C. jejuni* via the oral-gastric route. They found heavy colonization of mucus rich crypts and proposed that *C. jejuni* does not require adhesion since it can successfully colonize the intestinal epithelia by colonizing the mucus. They demonstrated the colonization of the intestinal mucosa of adult mice by association with the intestinal mucus and the mucus filled crypts but saw no evidence of adhesion to epithelial cells of the gut mucosa. The flagellum of *C. jejuni* is made of unsheathed polymer of flagellin subunits that are encoded by *flaA* and *flaB* genes (Nuijten and others 1990b) and undergo antigenic and phase variation. The importance of *flaA* and *flaB* genes was demonstrated in mutational studies where the mutants produced truncated flagella that led to non-motile phenotypes (Wassenaar 1997). According to Parkhill and others (2002), *C. jejuni* NCTC 11168 genome contains several open reading frames that have been assigned functions in the flagella biosynthesis, export and assembly. However, to date there is no experimental evidence proving their role in flagella function (van Vliet and Ketley 2001) even though several studies using aflagellated mutants have shown the importance of flagella in the colonization and pathogenesis of *C. jejuni* (Nachamkin and others 1993; Wassenaar and others 1993).

Adhesion and Invasion

Adhesion and invasion of the host cells are important virulence factors in the pathogenesis of *C. jejuni*. During infection, *C. jejuni* crosses the mucosal layer of the epithelial cells and adhere to these cells with subsequent invasion. The invasion of the cells causes damage and inflammation exhibited in *Campylobacter* infections but it is not yet clear whether inflammation has a direct role in epithelial damage and/or diarrhea (van Vliet and Ketley 2001). Studies in vitro and vivo have shown that *C. jejuni* is invasive although different strains exhibit different invasive abilities. Experiments conducted on isolates from two clinically different patients classified as either non-inflammatory or inflammatory, revealed that all isolates from the inflammatory diarrhea patients invaded Caco-2 (cancerous human intestinal tumor cell) monolayers while only some isolates from the non-inflammatory patient were invasive (Everest and others 1992). On the contrary, all isolates from invasive enteric pathogens such as *Salmonella* and *Shigella*, have invasive abilities (Everest and others, 1992). This study and others (Konkel and Joens 1989; Babakhani and Joens 1993) confirmed that invasion was an important factor in the manifestation of inflammatory disease during *Campylobacter* infection.

Additionally, recent studies have revealed that *Campylobacter* can produce fimbriae. It is unclear if fimbriae are needed for adhesion and invasion however it is reported as a major event in pathogenesis of *C. jejuni* (Doig 1996b). Fimbriae/pilli are proteinaceous structures that extend from the cell surface which mediate adhesion of the bacterial cell to the host tissue through interaction with receptors located on the host cell during cell infection.

Iron Acquisition

Iron is essential for all living microorganisms. Organisms use iron storage systems to compensate for low iron environments. The ability to acquire iron from the host contributes to bacterial pathogenesis. *Campylobacter* like all other pathogens requires iron for essential metabolic roles during the establishment of infection but the amount of available free iron in mammalian cells is inadequate because iron is complexed into haem, transferrin (in serum) and lactoferrin (at mucosal surface). This iron limitation constitutes a non-specific host defense (Vliet and Ketley 2001).

Campylobacter jejuni do not produce siderophores but have evolved to use external siderophore (Field and others 1986) and have a transport system that scavenges siderophores in the intestinal tract of the host (Richardson and Park 1995). Siderophores are high-affinity iron compounds synthesized by a variety of pathogenic bacteria, which enable the bacteria to compete for iron when it is deficient or withheld by host iron binding proteins. Several systems for siderophore-mediated uptake of iron such as enterocholin transport and haemin uptake systems have been reported. *Campylobacter jejuni* produces the storage protein ferritin, which prevents oxidative damage by lowering the intracellular concentration of iron that may react to form various oxygen radicals (Wai and others 1996). Ferritin mediates the colonization of the host by *C. jejuni* and also protects the bacteria in conditions of high oxygen levels (Ketley 1997).

Toxin production

Although adhesion and invasion are significant factors in the virulence mechanism of *C. jejuni*, the levels of invasion detected in vitro have been low; less than

1% of the bacteria applied to the monolayer invaded the monolayer but with efficient killing of cells; suggesting that other critical factors such as toxins were involved in the virulence mechanism of *C. jejuni* (Blaser 1981). Toxins have been considered to be an important factor in the pathogenesis of *Campylobacter* and some reports (Wassenaar 1997) have suggested that *C. jejuni* produces both enterotoxins and cytotoxins.

Enterotoxins are secreted proteins with a capacity to bind to a cellular receptor, enter the cell and elevate the intracellular cyclic AMP (cAMP) levels. This activity results in the deregulation of intracellular adenylate cyclase regulatory system, which results in the production of watery type stools (Wassenaar 1997). Cytotoxins inhibit actin filament formation through the disruption of the actin cytoskeleton. They act by formation of pores in target membranes causing lytic activity of the erythrocytes, eliciting secondary reactions such as cytokine release, cytoskeleton dysfunction, and secretion of granule constituents and generation of lipid mediators. During infection, lysis of cells includes the killing of leukocytes, granulocytes or macrophages, which hampers immune response of the host (Wassenaar 1997). Cytotoxin activity results in the formation of pores in cells with a resultant inhibition of cellular protein synthesis and inhibition of actin filament formation.

Production of enterotoxins and cytotoxins by Campylobacter jejuni

Ruiz-Placios and others (1983) first described enterotoxin production by *C. jejuni*. The *jejuni* toxin (CJT) was found to cause intraluminal fluid secretion in the rat ileal loop test (RILT) model. In addition to Chinese hamster ovary (CHO) cell elongation, an increase in cAMP levels through stimulation of adenylate cyclase activity in the intestinal

mucosa was also observed in vitro (Walker and others 1986; Wassenaar 1997).

Campylobacter jejuni toxin shares functional and immunological properties with *Vibrio cholera* toxin (CT) and *Escherichia coli* heat-labile toxin (LT) (Walker and others 1986).

Enterotoxins are still poorly understood. For example the genome sequence of *C. jejuni* NCTC 11168 contains only the *cdt* genes coding the cyto lethal distending toxin (CDT), genes encoding other toxins have not been isolated (Wassenaar 1997). This could possibly be due to differences in strains and assays used or different cytotoxins are specific for certain cell lines (Wassenaar 1997). Johnson and Lior (1988) first described the cytolethal distending toxin (CDT) produced by *C. jejuni*. Its activity causes certain cell lines such as the human adeno carcinoma (Caco-2), Chinese hamster ovary (CHO), African green monkey kidney (Vero), human tumor epithelial (Hep-2, HeLa) cells to slowly distend with progression to death (Johnson and Lior 1988; Whitehouse and others 1998). Cyto lethal distending toxin causes progressive cell distention by inducing the cells to irreversibly prevent phosphorylation of CDC2, which is a catalytic subunit of the cyclin-dependent kinase and must be activated for cells to enter mitosis (Konkel 2001). Although CDT is known to affect the G₂/M transition of the cell phase, the mechanism is not yet clearly understood (Konkel 2001).

Lipopolysaccharides (LPS)

The surface polysaccharide structures that form the outer membrane of Gram negative bacteria contain lipopolysaccharides (LPS) which are important virulence factors involved in serum resistance, endotoxicity and adhesion and can influence interactions with host cells. In Gram negative bacteria, the LPS consist of a lipid moiety

(lipid A), a core polysaccharide and an O antigen. Lipopolysaccharides which also are referred to as endotoxins are a stimulant of the immune system and thus a virulence factor. Unlike other Gram negative pathogens, the lipopolysaccharides of *Campylobacter* species are antigenically different resulting in diverse serotypes (Walker and others 1986) which can impact antigenicity, serum sensitivity and adhesion. *Campylobacter jejuni* surface polysaccharide structures are sialyated which could be responsible for eliciting an immune response leading to Guillain-Barre' syndrome associated with immune disorders of the peripheral nervous system through ganglioside mimicry (Nachamkin and others 1998).

Secretion of proteins

Campylobacter jejuni secretes proteins that are collectively termed as *Campylobacter* invasion antigens (Cia proteins) and although only one secreted protein CiaB has been identified (Konkel and others 1999b), findings have revealed that Cia proteins promote the organism's uptake and thus contribute to the pathology of *C. jejuni*-mediated enteritis (Konkel and others 2001). *Campylobacter* invasion proteins are released via the flagella type III secretion apparatus that in turn, is involved in the secretion of bacteria effector proteins that form pores in eukaryotic cell membrane. This could explain the manifestation of bloody diarrhea in some patients that have been infected by *C. jejuni*.

Campylobacter heat shock proteins (HSPs)

Heat shock proteins are associated with thermal stress response of bacteria and in *C. jejuni*, like other pathogens, are important virulence factors. Several heat shock proteins have been identified in *C. jejuni* such as GroESL, *DnaJ*, *DnaK* and *ClpB* (Konkel and others 1998) but only *DnaJ* has been identified to have a role in pathogenesis. They further demonstrated that *DnaJ* negative mutants did not colonize chickens. This confirmed that *DnaJ* had a vital role in *C. jejuni* pathogenesis through colonization of host cells. *Campylobacter jejuni* exhibits various virulence determinants during its pathogenesis cycle. It has evolved to colonize various avian species and animals used in food production; it is widely distributed. This could explain why *C. jejuni* is still the major cause of gastroenteritis worldwide causing an enormous economic impact. Although much has been done to study the mechanisms of its pathogenesis, its detection in food and water still remains a challenge.

Factors influencing the growth of *C. jejuni*

Influence of atmosphere

In comparison to other foodborne pathogens, the growth conditions required for culturing *C. jejuni* are unique and unusual which poses difficulty with its isolation and detection from food matrixes. *Campylobacter* are highly susceptible and less tolerant to environmental stresses than are other foodborne pathogens. *Campylobacter jejuni* is microaerophilic, unable to grow in air but will grow optimally in a microaerophilic environment consisting of 5-10% oxygen, 3-10% carbon dioxide and 85% nitrogen.

Influence of Temperature:

Low temperature

The optimum growth temperature for *C. jejuni* is 42 °C. This is the normal temperature of the avian gut where they easily colonize. *Campylobacter* are also encountered in the human gut, which is at 37 °C, but generally they do not grow below 30 °C. Temperature has a great impact on the growth and survival of *Campylobacter* during its detection from food. The response of *C. jejuni* to low temperatures has been studied and findings reflect a slow gradual decline in growth rate near minimum growth temperature and a sudden decline below 30 °C (Hazeleger and others 1998). Bacteria such as *E. coli* and *Salmonella* produce characteristic cold shock proteins that give them the ability to withstand temperatures below optimum growth and thus multiply. However, analysis of the *C. jejuni* genome sequence (Parkhill and others 2000) indicates that it does not produce typical cold shock proteins. This may explain *C. jejuni* inability to replicate at temperatures associated with refrigerated storage.

Presence of cold-inducible proteins is a key to adaptation of many bacteria to lower temperatures. On the contrary, although *C. jejuni* is not able to multiply at low temperature, it is metabolically active below minimum temperatures of growth (30°C) and performs respiration at temperatures as low as 4 °C (Park 2002). Hazeleger and others (1998) further reported that during refrigeration, although viability is lost rapidly, *Campylobacter* is fully motile and the cells are capable of moving towards favorable environments through chemotaxis. This may explain why, even after rapid loss of viability at lower temperatures, *Campylobacter* can still be isolated from frozen meats and poultry (Fernandez and Pison, 1996) although freezing reduces the viable numbers

significantly (Humphrey and Cruickshank 1985). *Campylobacter* can survive at 4 °C but cannot grow below 30 °C. Although several factors are responsible for the freeze-induced injury of bacterial cells (i.e. ice nucleation and dehydration) Stead and Park (2000) have shown that oxidative stress is also a contributory factor to the freeze-thaw induced death of *Campylobacter*.

High temperature

Campylobacter are sensitive to heat and are thus inactivated by heat treatments such as pasteurization and conventional cooking processes. Studies on other bacterial cells such as *E. coli* have revealed that exposure of bacteria to higher than optimum temperatures elicits a heat shock response producing heat shock proteins (HSPs). Major HSPs have been cited as molecular chaperones and proteases that are important for cell survival since they prevent aggregation and refolding of proteins (Arsene and others 2000). Several HSPs have been identified for *C. jejuni* and some have been identified as GroELS, DnaJ, DnaK and Lon protease (Konkel and others 1998; Thies and others 1999a; Thies and others 1999b). Although HSPs have been identified in *C. jejuni*, major heat shock regulatory factors found in *E. coli* and *Bacillus subtilis* are absent in *C. jejuni* and the mechanism involved in the heat shock response has not yet been revealed (Park 2002). In culturing, *Campylobacter* are incubated at 37 °C and 42 °C, both of which are within the optimum temperature for their growth.

Influence of oxidative stress

When bacteria are exposed to oxygen, formation of reactive oxygen intermediates such as superoxide radicals takes place and if not neutralized, can damage nucleic acids, proteins and cell membranes. In addition, when bacteria enter the host, they are exposed to numerous host-killing mechanisms such as reactive oxygen species including superoxide, hydrogen peroxide and halogenated oxygen molecules (De Melo and others 1989; Kiehbauch and others 1985). For this reason, most bacterial cells, including *C. jejuni* that are able to grow in the host have mechanisms to induce responses against these radicals by inducing anti-oxidant enzymes. Several enzymes such as superoxidase dismutase (SOD) (Purdy and Park 1994; Pesci and others 1994; Purdy and others 1999) and catalase (Stead and Park 2000) act as a defense system in *Campylobacter*. The SOD plays a role in the defense against oxidative stress and promotes aerotolerance particularly during survival when growth has stopped. It catalyzes the conversion of oxygen radicals to H_2O_2 and O_2 and is the first line of defense against toxicity of reactive oxygen intermediates (Purdy and Park 1994). Several researchers (Purdy and others 1999; Stead and Park 2000) have demonstrated that SOD deficient mutants were less capable of survival in milk, poultry meat and were intolerant to freezing. These mutants were less capable of colonizing animal models (Purdy and others 1999) and less capable of invasion of mammalian cell lines in vitro (Pesci and others 1994) suggesting that SOD has a role in *Campylobacter* pathogenesis. Catalase in *C. jejuni* is encoded by a single gene *katA* and plays a role in the prevention of oxidative stress through the conversion of H_2O_2 into H_2 and O_2 . Day and others (2000) demonstrated that catalase plays a role in hydrogen peroxide resistance and intracellular survival in vitro. Although

Campylobacter has mechanisms for surviving exposure to toxic oxygen metabolites, during culturing it is important to culture *Campylobacter* in media containing substances that can neutralize the toxic effects of oxygen and its metabolites. Several components such as lysed or defibrinated blood, haemin, charcoal, sodium pyruvate, sodium metabisulfite and ferrous sulfate are added to isolation agars and enrichment media to counteract the effect of these toxic oxygen metabolites.

Influence of osmotic stress

Campylobacter are less tolerant to osmotic stress than other foodborne pathogens. Doyle and Roman (1982) studied the response of *C. jejuni* to various concentrations of sodium chloride concentrations (0.5%, 1%, 1.5%, 2.0%, 2.5% and 4.5%) at 4°C, 25°C and 42 °C, and reported that it was better to grow *Campylobacter jejuni* in media containing 0.5% NaCl as concentration greater than 1% retarded growth or increased death rate. *Listeria monocytogenes* will grow at concentration of 10% sodium chloride and *Salmonella* at concentrations of 4.5%. Abram and Potter (1984) reported that *C. jejuni* survival at refrigeration temperature decreased as the sodium chloride concentration was increased from 0-2% and there was more rapid decline at room temperature.

Reezal and others (1998) studied the effect of osmolality in nutrient media on the growth and culturability of *Campylobacter* spp. and they found that in nutrient media with lower osmolalities (~ 130 mosmoles), *Campylobacter* exhibited a morphological change from rod/spiral to coccoid form as a stress response. The authors also noted that at 4 °C and medium osmolalities of 254 and 171 mosmoles, cells could still be recovered

after 4 days whereas at 130 mosmoles, there was a decline in culturability. This study confirmed that *Campylobacter* are sensitive to low osmolality nutrient conditions.

Influence of pH

Compared to other foodborne pathogens, *Campylobacter* is sensitive to low pH. *Campylobacter* loses viability rapidly at pH below 4.9 (Blaser and others 1980a).

Influence of stationary phase and starvation

When bacterial cultures are growing in laboratory media, they will grow in an exponential manner until they enter the stationary phase. Rees and others (1995) reported that for many bacteria, entry into the stationary phase is accompanied by structural and physiological changes that result in increased resistance to heat and cold shock, osmotic, oxidative and pH stress. In many foodborne pathogens, this process allows the organisms to adapt to procedures used in the food industry such as low temperature, low or high pH and osmolarity. The regulator for such physiological changes in Gram negative bacteria has been identified as the σ -factor RpoS that is critical for the survival of cells in stationary phase (Rees and others 1995). Homologues have been identified in most *Enterobacteriaceae*, *Pseudomonas*, *Vibrio* and *Legionella pneumophila* species. Although most foodborne pathogens possess the σ -factor RpoS responsible for the regulation of cell responses during the stationary phase, analysis of the *C. jejuni* NCTC 1168 genome sequence (Parkhill and others 2000) indicates that this factor is absent. This may explain the greater sensitivity of stationary cells of *C. jejuni* to mild heat and oxidative stress than the cells from the exponential phase (Kelly and others 2001).

The role of non-culturable cells

Moore (2001) described *Campylobacter jejuni* as a fastidious pathogen that grows only in vivo and transmission to a new host involves adaptation to a new hostile environment. Exposure of *Campylobacter* to unfavorable environments such as oxygen, changes in temperature and starvation, makes it revert to a viable but non-culturable form. This reversion to a non-culturable form has been reported in several studies (Rollins and Colwell 1986; Cappelier and others 1997; Tholozan and others 1999) and may have direct implications in the isolation of *C. jejuni* from food. The loss of culturability is often associated with change in cell morphology from a spiral to a coccoid form. Although some researchers suggest that the coccoid form is a dormant viable but non-culturable stage of *C. jejuni*, others have reported that the coccoid form is merely a degenerative form (Moran and Upton, 1987; Beumer and others 1992; Boucher and others 1994) and the reversion to a non-cultural state is a survival mechanism that the bacterium adopts when exposed to a nutrient lacking environment (Roszak and Colwell 1987).

Characterization of the viable-but-non-culturable (VNC) form

Several morphological forms of *C. jejuni* have been reported. Rod shaped forms, including spiral, s-shaped and characteristically curved cells predominate in young cultures, while non-culturable coccoid forms occur mainly in old cultures (Butzler and Skirrow 1979). In general, two morphological forms of *C. jejuni* have been described; culturable spiral forms and non-culturable coccoid forms. Reilly and Gilliland (2003) presented electron photomicrographs depicting the variable cellular morphology of this

organism. Transition from spiral to coccoid forms depends on numerous factors such as the strain, temperature, pH, osmolarity and medium (Boucher and others 1994; Hazelger and others 1995). Transformation of cells to the coccoid morphology is due to unfavorable conditions of an organism (Karmali and others 1981) and Moran and Upton (1986) further demonstrated that this transformation was due to exposure of *C. jejuni* to toxic oxygen derivatives. This was confirmed by observation of increased coccoid formation on agar surfaces incubated aerobically. Coccoid forms are not motile (Butzler and Skirrow 1979) and they are reported to be viable but non-culturable (Rollins and Colwell 1986). They have a lower content of cytoplasmic components and nucleic acids than the rod forms (Moran and Upton 1986) and during the conversion to coccoid forms, nucleotides leak from the cells.

Lazaro and others (1999) suggested that the conversion of spiral cells to coccoid cells and the conversion of viable cells to viable but non-culturable cells (VNC) should be considered separate but related events. Further more, Weichart and others (1997) reported that the VNC state involved two phases; the loss of culturability with maintenance of cellular integrity with intact RNA and DNA resulting in a potentially viable culture or degradation of RNA and DNA resulting into loss of viability. The degradation of RNA and DNA and lack of detectable amounts of intact ribosomes or chromosomes suggested lack of viability (Weichart and others 1997). This may explain the differences in findings among various workers on the existence and stability of the VNC cells within the *Campylobacter* species.

The reversion of *Campylobacter jejuni* cells to the VNC state has been described as dormancy “rest period of reversible interruption of the phenotypic development of an

organism” (Sussman and Halvorson 1966) which is exhibited by other pathogens such as *Salmonella* spp. in soil (Turpin and others 1993). The dormancy has been further characterized as “exogenous dormancy” as opposed to the constitutive dormancy that is exhibited by *Clostridium* and *Bacillus* spp. that may be triggered by the environment and is an innate property of the cell, under strict gene regulation. Rollins and others (1987) showed that the VNC form of *Campylobacter* was viable in water samples by detection using non-conventional culturing techniques but could not be detected by conventional culture methods. They concluded that the inability to culture these cells was due to several factors including lack of survival of the microorganism in the VNC state, persistence and adherence to surfaces as biofilms, and lastly the cells may be present below the detectable threshold necessary to establish growth on laboratory media.

Detection of viable but non-culturable cells (VNC) of *Campylobacter jejuni*

Reports by Rollins and Colwell (1986) and Cappelier and others (1997) have pointed out the difficulty of culturing the VNC cells of *C. jejuni* on conventional laboratory media that normally supports their growth. Alternative methods have been proposed and several methods for detecting the VNC forms have been reported. Rodriguez and others (1992) used a redox dye 5-cyano-2, 3-ditoyl tetrazolium chloride (CTC) for enumeration of respiring cells of the bacteria in a sample. Oxidized CTC is colorless and non-fluorescent; however on reduction via the electron transport activity, it is converted to fluorescent insoluble CTC-formazan, which is detectable in cells. Counterstaining of samples with DNA-specific fluorochrome allows enumeration of active and total bacteria within the sample. Another methodology involves the detection

of viable cells by counting cells that elongate in response to nutrient concentration in the presence of acridine orange, which intercalates with DNA, making the elongated cells visible (Kogure and others 1979). Other methods used to enumerate respiring cells involve the use of a combination of CTC with a specific fluorescent antibody (FA). Hegarty (1999) used the CTC-FA combination that gives information on the viability and metabolic activity of cells.

Recovery of viable but non-culturable cells

In some recovery studies, the term “resuscitation” has been used to denote “the transition of cells from non-culturable to culturable states with respect to a given medium”; it has also been defined as “a reversal of metabolic and physiological activities that characterize the non-culturability state” (Baffone 2006). Several methodologies have been used to recover the VNC form both in vitro and in vivo. In both methodologies, cells have been starved or stressed in an appropriate environment and periodically sampled for viable count till reversion to VNC is observed.

Recovery of VNC cells from in vitro and in vivo models

Rollins and Colwell (1986) suspended *C. jejuni* cells in stream-water microcosm and reported that VNC *C. jejuni* could be recovered by animal passage. This was further confirmed by Jones and others (1991) who demonstrated that after storage in water for six weeks, two of the four strains of *C. jejuni* in the VNC stage could be recovered in suckling mice. Stern and others (1994) fed one-day old chicks with an aqueous suspension with no viable cells and the chicks became colonized with *C. jejuni*. Saha and

others (1991) recovered freeze-thawed injured non-culturable cells of *C. jejuni* through a rat gut. Cappelier and others (1999) suspended three human isolates of *C. jejuni* in surface water and induced the VNC state after starvation for 30 days. Culturable cells were recovered from embryonated eggs and they adhered to HeLa cells after resuscitation. The authors used two animal models (murine and chicken) to demonstrate the recovery of *C. jejuni* VNC and found the mouse model more efficient in recovery than the chick model. Although these studies have demonstrated success in recovery of CNV cells of *C. jejuni* in animals, chicken and embryonated eggs, some researchers have reported no success in recovery of these cells. Medema and others (1992) were not able to recover cells that were non-culturable, but elongated in response to nutrients through feeding one-day old chicks or passage through the allantoic fluid of embryonated eggs. Further more, Beumer and others (1992) could not recover *Campylobacter* cells after inoculating $\sim 10^7$ cells into simulated gastric, ileal or colonic environments. No viable cells could be recovered by the fecal route after feeding coccoid cells to rats, rabbits and human volunteers nor was an immune response elicited; although ATP measurements and direct viable counts indicated cell integrity was intact. Other data that demonstrate the inability to recover the VNC forms include van de Giessen and others (1996) who were not able to recover VNC forms prepared in water microcosms from chicks and mice. Tholozan and others (1999) were able to recover only 3 out of 36 strains of *C. jejuni* that had been induced into the VNC form after 15 days in water at 4 °C.

There are contradicting reports about the recovery of VNC forms of *C. jejuni*. MacKay (1992) explained the significance of the VNC forms of *C. jejuni* by reporting that they play a major role in the maintenance of an infection cycle from the environment

to animals and subsequently to man. Beumer and others (1992) further noted that the importance of the contribution of the coccoid forms to the maintenance of an infectious cycle between man, animals and the environment has been underestimated and thus under reported. Little is known about the survival and transmission of *Campylobacter* spp. in the environment or how animals and birds that are a natural reservoir become infected. Several environmental sources have been implicated in *Campylobacter* outbreaks and yet *Campylobacter* spp. have not been isolated from these sources. Reliable detection methods must be available that allow detection of this form of pathogen.

Prevalence and transmission of *Campylobacter jejuni*

Campylobacteriosis has tremendous economic and social implications worldwide because *Campylobacter* exist naturally in birds and animals used in food production. It is the leading cause of acute human gastroenteritis worldwide (Friedman and others 2000). In the United States, in 2003, *Campylobacter* was confirmed as the cause of 12.6 cases per 100,000 people by the Center for Disease Control (CDC) and Prevention FoodNet active surveillance program; and was the most frequent agent identified in six of the nine FoodNet sentinel states (Anonymous 2004). It is estimated to cause approximately 2.1-2.4 million cases each year in the USA alone (Altekruse and others 1998; Sahin and others 2003). In 1996, 46% of laboratory-confirmed cases of bacterial gastroenteritis reported in the Center for Disease Control and Prevention/ U.S. Department of Agriculture/Food and Drug Administration Collaborating Sites Foodborne Disease Active Surveillance Network were caused by *Campylobacter* species (Altekruse and others 1999).

In prevalence, *Campylobacter* is followed by *Salmonella*, *Shigella* and *Escherichia coli O157: H7* infection at 28%, 17% and 5% respectively. Reports show that 80% of all *Campylobacter* related illness is transmitted through food and is responsible for 5% of food related deaths (Mead and others 1999). Although there are several identified species within the genus, *Campylobacter jejuni* is the most frequently implicated in clinical diagnosis (Stern and others 1994). *Campylobacter jejuni* is reported to have an isolation rate that exceeds *Salmonella* and accounts for greater than 90% of all *Campylobacter* infections (Park 2002). The main sources of infection are undercooked chicken, raw milk, untreated water and wild animals (Harris and others 1986; Jordan 2005). Other sources include sewage contamination and pet contact particularly pets with diarrhea. *Campylobacter* enteritis is considered to be a foodborne infection rather than food poisoning, with infection often being derived from a range of foods and water based environmental sources (ACMSF 1993). Members of the genus *Campylobacter* are ubiquitous because they colonize the gastro intestinal tracts of most animals and birds as commensals, but are associated with disease in humans. Poultry, including wild birds and animals are a natural habitat for *Campylobacter* therefore cells of the organism may enter the environment including the food and water chain through feces of animals, birds or infected humans (Blaser and others 1980b, 1984a).

Poultry as a source of transmission

Birds are regarded as a natural reservoir of *Campylobacter*. Different kinds of poultry, especially broiler chickens are some of the most important sources of *Campylobacter* infection (Pearson and others 1993). *Campylobacter* grow optimally at

42 °C and this may explain the prevalence in birds since this is the normal temperature in the intestines of birds. Freidman and others (2000) reported that exposure to contaminated chickens contributed to 50% of *Campylobacter* infections. *Campylobacter* is widely distributed in poultry production and is reflected in the isolation rate reported; for example studies in the United States have shown that 80% of commercial chicken carcasses are positive for *Campylobacter* (Kramer and others 2000; Oyarzabal and others 2004). Willis and Murray (1997) reported 69% of chickens bought from local supermarkets were contaminated with *C. jejuni*. In Minnesota, a survey carried out by the Department of Health reported that 88% of all retail poultry was positive for *Campylobacter* species (Hingley 1999) while Cory and Ataby (2001) reported that 80% of all raw chicken in the UK were contaminated with *Campylobacter*. A survey conducted to investigate the prevalence of pathogens revealed that 88.2% of broiler chickens and 59.8% of ground chicken samples were positive for *C. jejuni* and *C. coli* (Ransom and others 1998) while another survey reported that 90% of the flocks in the United States were colonized (Stern and others, 2001). Jacobs-Reitsman (2000) studied the distribution of *Campylobacter* in newly slaughtered broiler chickens and hens and found 89% of chicken neck skin samples and 75% of the subcutaneous samples to be positive for *Campylobacter*. When poultry is inoculated with *Campylobacter* experimentally, colonization occurs exclusively in the intestinal mucosal layer in the crypts of the epithelia of the ceca, large intestines and the cloaca (Park 2002).

Although findings (Doyle 1984; Pearson and others 1996) suggest that vertical transmission of *Campylobacter* from parent to chicks is highly unlikely, horizontal transmission is reported to occur and sources of transmission include poultry sheds,

water, litter, feed, fauna and footwear of farm workers (Clark and Bueschkens 1988; Shanker and others 1990). Experimental studies indicate that *C. jejuni* spreads rapidly within chicken flocks and can spread from an infected bird in a susceptible flock within 72 hours (Shanker and others 1990) and in larger flocks of about 2000 birds, the spreading is logarithmic (Montrose 1985). Within the colonized flocks, the organism spreads so fast that the colonization rate is close to 100% (Pearson and others 1993; Cory and Atabay 2001). Ramabu and others (2004) investigated the sources of transmission of *C. jejuni* in broiler chickens and reported isolation rates from pallets, crates, truck beds, truck wheels, catcher's and driver's boots, forklifts and tractors. Rapid cross contamination in poultry carcasses during processing was also reported by Pearson (1996) and Corry and Atabay (2001). This could explain the high rate of isolation of *C. jejuni* from poultry and this identifies the major source of cross-contamination since *Campylobacter* is widely distributed in the environment. Although several interventions have been proposed and implemented to reduce the risk of exposure to *Campylobacter* such as reducing the flock colonization or biosecurity measures at farms and improving processing and slaughter house hygiene, these measures have not been sufficiently effective (Van Gerwe 2005). The strategy to prevent colonization of the flock has been successful with *Salmonella* but unsuccessful with *C. jejuni*. This could be explained by the differences in their physiology, ecology and epidemiology.

During slaughter and processing, the gut of the birds has been cited as the principal source of *Campylobacter*, which is transferred to the skin (Rivoal and others 1999). These researchers provided evidence of cross-contamination at slaughterhouses by using flagellin genes to type the strains found in the different batches of birds in

slaughterhouses. Louge and others (2003) also noted that most carcass contamination occurred during transport. During production, the entire process contributes to the contamination event. During the slaughter process, *Campylobacter* are spread from the intestinal contents to the carcass, therefore the greatest risk arises from handling of the raw chicken, cross contamination within the kitchen and consumption of undercooked poultry products (Bryan and Doyle 1995). In the home kitchen when frozen chicken is thawed, cross contamination can easily occur from either the carcass or any associated purge and this could be an important source of contamination within the kitchens (Birk and others 2004).

Drinking water as a source of transmission

Campylobacter has ability to survive in water but their survival is dependent on the source of the water and the season (Jones, 2001). Municipal water systems have been implicated as a source of waterborne outbreaks of *Campylobacter* (CDC 1978). These findings could explain several major waterborne outbreaks of *C. jejuni* infections such as 11 of 57 reported outbreaks of *Campylobacter* infections in the United States between 1978 and 1986 (Tauxe 1992). These cases of infection were related to drinking unboiled surface water, contamination of ground water with surface water, inadequate disinfection or contamination of drinking water by avian wildlife feces. *Campylobacter* has been isolated from water reservoirs and drinking water in particular has been reported to be a source of infection and a significant risk factor (Pearson 1993). Thomas and others (1999) and Jones (2001) reported water to be one of the main sources of transmission of campylobacteriosis. Drinking water was a contributor to the sporadic

infections and outbreaks of campylobacteriosis. Snelling and others (2005) further in their experimental studies, found that although chlorine levels used in drinking water should render it potable, waterborne protozoa were capable of acting as protection for *C. jejuni* and concluded that this could partly explain the findings by Stern and others (2002) that chlorination of broiler drinking water had no effect on *C. jejuni* colonization of broilers. Cools and others (2003) investigated the survival of *C. jejuni* in drinking water at 4°C. All 19 strains tested were recovered after enrichment in Bolton broth after 64 days of storage. Although the recovery was dependent on strain origin, recovery was also dependent on the type of media used. In this study the researchers noted that Bolton broth, which is a selective medium, supported resuscitation and recovery of *C. jejuni* while the non-selective agar medium did not, over extended period of time. Most of the 19 strains though, decreased to below detection levels more quickly when viability was tested using Karmali selective agar medium than with Columbia blood agar (CBA), which is a non-selective agar medium. These findings in general suggest that broiler drinking water is a major risk factor in the transmission of *C. jejuni* to the poultry and could explain the persistence of *C. jejuni* despite all the efforts in its eradication from the poultry flock. The potential for transmission exists in abattoirs and food processing facilities where potable water is used extensively. Carter and others (1987) isolated *Campylobacter* spp. from a variety of aquatic habitats in central Washington and concluded that no untreated water should be considered free of *Campylobacter* including mountain streams.

Animals as a source of transmission

Campylobacter has been isolated from intestines of healthy calves and adult cattle (Stanley and others 1998) as well as from calves exhibiting signs of enteritis (Terzalo and others 1987). Prevalence of *Campylobacter* is higher in poultry, thus poultry products are specifically considered to be the main sources of *Campylobacter* infections in humans. This organism also has been isolated from feces of other farm animals including beef and dairy cattle, and sheep. Unpasteurized bovine milk and milk products are frequent vehicles for campylobacteriosis outbreaks and it is likely that the cattle carcasses are contaminated at slaughter by direct or indirect fecal contamination although at a lower rate than in poultry (Besser and others 2005). Wesley and others (2000), Stanley and others (1998) and Fitzgerald and others (2001) have implicated cattle as a major player not only in transmission of campylobacteriosis by milk, but in the environmental and water contamination through the disposal of abattoir effluents and slurries to the land. There are many species of *Campylobacter* in the cattle feces, but *C. jejuni* is frequently shed in higher numbers than others (Inglis and others 2005).

Genotyping has implicated cattle as an important source of human-pathogenic *Campylobacter* (Nielsen and others 2000). Seasonal variation of bovine fecal shedding of *Campylobacter* has been observed with peaks occurring in spring and fall seasons (Stanley and others 1998). Although studies have been undertaken to compare the prevalence of *Campylobacter* in cattle, it has been difficult due to varied isolation methods, specimen types, farm and husbandry systems, seasons, ages of animals and specimen types (Besser and others 2005) and thus the relative direct or indirect contribution of cattle to sporadic infections are not known (Frost 2001). However,

molecular typing evidence suggests the significance of cattle, sheep and other animals as sources of human infections have been previously underestimated (Stanley and Jones 2003).

In addition to cattle, *Campylobacter* colonize the gastro-intestinal tracts of a wide range of other animals, especially animals raised for human consumption such as sheep and swine. *Campylobacter* can be isolated from pig feces at a wide range of frequencies up to 100% (Meng and Doyle 1998; Nesbakken and others 2002) but in comparison, pig carcasses are not frequently contaminated with *Campylobacter*. The range of carcasses positive for the organism varies from 2.9% to 10.3% (Pezzoti and others 2003). The prevalence on lamb is higher than beef. *Campylobacter* is more frequently isolated from lamb lairs than are other pathogens (Small and others 2002). Approximately 73% of lamb offal is contaminated with *Campylobacter* (Kramer and others 2000). This data in general suggests that like poultry, beef, dairy cattle, sheep and swine are all potential sources of human *Campylobacter* infections.

Domestic pets such as dogs and cats carry *Campylobacter* in their gastrointestinal tracts although they are asymptomatic (Blaser and others 1980b). As a result, pets are a natural reservoir for *Campylobacter* and since they are associated with humans, the organism is likely to be transmitted directly to humans through direct contact by pets such as cats and dogs. The species most associated with dogs and cats is *C. upsaliensis* and *C. helveticus* (Baker and others 1999). *Campylobacter* species have also been recovered from exotic pets such as turtles (Harvey and Greenwood 1985) and hamsters (Fox and others 1983).

Fresh produce as a source of transmission

Research has been done on the prevalence of *Campylobacter* spp. on fresh produce since eating patterns have changed in recent years with changing emphasis on eating 'fresh' produce for a healthy diet. Park and Sanders (1992) isolated *Campylobacter* from spinach, lettuce, radishes, green onions, parsley and potatoes sampled from farmers' outdoor market at a rate of 3.3%, 3.1%, 2.7%, 2.5%, 2.4% and 1.6% respectively, but found all samples from the supermarket to be negative. Thunberg and others (2002) analyzed fresh produce at the retail level for human pathogens and reported absence of *Campylobacter* species. Sago and others (2003) investigated the microbiological quality of retail bagged ready-to-eat salad vegetables and of all the samples tested, 99.3% were found to be of satisfactory quality and *Campylobacter* was reported to be absent. Although these findings reflect fresh produce as minimum source of risk for *Campylobacter* species contamination, several outbreaks associated with different types of fresh produce have been reported worldwide (Blaser and others 1992; Roels and others 1998). In some of the outbreaks, the food handlers and cross contamination in the kitchen were cited as the source of the organism. It is assumed that with cross contamination from poultry products as a risk factor, consumption of fresh produce could still be a risk factor in the transmission of *Campylobacter* spp.

Isolation of *Campylobacter jejuni* from food products

Like other food-borne pathogens, isolation of *Campylobacter* from food is often difficult because the pathogen may be present in low numbers and often in an environment with a high level of competitor organisms. Furthermore, the cells may have

undergone sublethal-injury during the harsh procedures used in food industry; these sublethally-injured cells often exhibit greater sensitivity to hydrogen peroxide, photochemically induced radicals and selective agents used in traditional culture media (Ray and Johnson 1984). Coupled with complex growth requirements, this has led to a difficulty in the isolation of *Campylobacter* from various food matrixes. Different studies have been undertaken to improve the recovery of *Campylobacter* based on modifications of existing isolation and enrichment media, improved understanding of needed growth requirements such as temperature and time of incubation, addition of components that reduce the toxic effects of oxygen and its derivatives and use of combinations or complete elimination of antibiotics (Bolton and others 1984; Humphrey 1986 a, b and 1990; Corry and others 1995). However, there is still no standardized procedure for the isolation of *Campylobacter* from various food matrixes. This has led to inconsistent data on the prevalence of *C. jejuni* in various foods, which could explain inconsistencies in isolation of *Campylobacter* in the food chain. The use of conventional tests for the differentiation and species identification of *Campylobacter* has proven difficult because they are fastidious, assaccharolytic and possess few distinguishing biochemical characteristics (Engvaal and others 2002). This is all further complicated if they enter the viable but non-culturable phase. The failure of culture techniques to isolate the organisms can result in failure of their detection from sources implicated in outbreaks. *Campylobacter jejuni* also lacks the alternative sigma factor RpoS (shown to produce greater stress resistance in stationary phase cells in a variety of foodborne pathogens such as *Salmonella* and *Escherichia coli*) and are thus fastidious, requiring complex media and environment for growth. This promotes extra sensitivity to environmental stresses such

pH, heat, cold and oxidation and osmolarity. Genes under RpoS control are involved in providing stationary phase cells with resistance to a number of processing procedures used in the food industry. *Campylobacter jejuni* often are unpredictable during cultivation with continuous sub-culturing leading to the formation of the coccoid or elongated filamentous forms (Reilly and Gilliland 2003). The decline in viability and difficulty in culturing can occur after exposure of *Campylobacter* cells to unfavorable environments such as oxygen, starvation and changes in temperature; any of which may cause them to enter the VNC state.

Rapid and improved methods have been proposed and used for the detection of *Campylobacter jejuni* based on 16S rRNA gene including polymerase chain reaction (PCR). Several specific oligonucleotides have been used as primers and probes for the PCR. Although PCR is used in a wide range of applications in the detection of pathogens, Giesendorf and others (1992) observed that the complex composition of food matrices can hinder the PCR and lower its sensitivity. Islam and others (1993) used PCR to detect the VNC form of *Shigella dysenteriae* from water microcosm and although the system detected cells, using PCR they could not differentiate between the viable but non-culturable and the non-viable cells. Additionally, *Taq* DNA polymerase is affected by specific inhibitors in foodstuff, which may inhibit the direct detection of low numbers of cells in food (Giesendorf and others 1992). It is therefore important that a reliable, simple and less tedious method for isolation and detection of *Campylobacter* from food is developed. Although the FDA-BAM procedure (Hunt and others 2001) has been standardized and stipulates protocols for isolation of several food products, it is tedious and cumbersome; and at times is inconsistent in recovery of *Campylobacter* (Tran 1998;

Baserisalehi 2004). The FDA-BAM procedure requires the utilization of blood supplement which is not only expensive, but can be a major source of contamination (Karmali 1986). The use of blood in developing countries would be critical where availability of sterile blood may be limited (Karmali 1986).

Currently used media components for isolation of *Campylobacter*

Different conventional culturing methods are being used for isolation of *Campylobacter* in foods all of which involve the use of selective enrichment media followed by culturing on selective agars. Isolated colonies are confirmed using various biochemical tests. Many agar media and enrichment media have been formulated and several have been modified to improve selectivity and sensitivity in isolating *Campylobacter* from food over the years but in general, they are all dependent on antibiotics for suppression of background microflora. Some of the antibiotics currently in use include cefoperazone, trimethoprim, vancomycin, rifampicin, cephalothin, polymyxin and amphotericin B in various combinations. Most of these methods have been compared for their effectiveness to recover species of *Campylobacter* in the presence of background flora naturally found in some food components. It also has been recognized that food samples not only carry background microflora, but have a complex matrix which may make *Campylobacter* isolation difficult (Lubeck and others 2003). To this end, the composition of several enrichment media and isolation agars has been more often than not improved upon by replacement of one component with another or complete removal of one component or in some cases changes in the concentration of the selective agents.

Media Components

The discovery of the Skirrow medium opened the door to successful isolation of *Campylobacter* and provided the link between *Campylobacter* infections to food contamination particularly chicken (Skirrow 1977). Skirrow agar contains peptone as a source of nutrients, lysed horse blood and antibiotics to prevent the growth of competitor microorganisms. The media used for isolating *Campylobacter* from food and water were derived from those first developed for isolation of *Campylobacter* from feces (Jacobs-Reitsman 2001). Most liquid enrichment media were developed with the intention to aid recovery of low numbers or sublethally injured cells. In general all *Campylobacter* media contain peptone and antibiotics, some contain blood, many include quenching agents to overcome the effects of toxic oxygen species such H₂O₂ and superoxide (Bolton and others 1984a). Apart from agents that are used to select *Campylobacter*, other ingredients have been added to media to help neutralize the toxic effects of oxygen. Ingredients added to media that protect *Campylobacter* from the toxic effects of oxygen derivatives include lysed or defibrinated blood, charcoal, a combination of ferrous sulfate, sodium metabisulfite and sodium pyruvate (FBP) and haemin (Corry and others 1995).

Development of *Campylobacter* Plating Media

Bolton and Robertson (1982) gave a detailed justification for their formulation of Preston and Charcoal Cefoperazone Deoxycholate (CCD) media. Development of the Preston media was based on a survey carried out by these workers on the minimum inhibitory concentration (MICs) of four antibiotics namely: polymyxin, rifampicin,

vancomycin and trimethoprim against 104 strains of *Campylobacter* spp. and competitive organisms. The workers further determined that campylobacter grew best on Solidified Nutrient Broth No. 2 after comparing it with other media such as Columbia blood agar base and Veal or Brain heart infusion. The Nutrient Broth No.2 is a non-complex media that contains less thymidine, a trimethoprim antagonist, than other media. Alternatives to blood for neutralizing the toxic effects of oxygen were tested and the best combination was found to be 0.4% charcoal, 0.25% ferrous sulfate and 0.25% sodium pyruvate (Bolton and Coates 1983a; Bolton and others 1984a). Results from a study of the effects of 11 dyes, 17 chemical compounds and 14 therapeutic agents on several strains of *C. jejuni* biotypes 1 and 2 and a group of Gram negative and Gram positive competitive microorganisms indicated deoxycholate and cefazolin to be the most effective selective agents (Bolton and others 1984b). Apart from Karmali's agar, Charcoal Cefoperazone Deoxycholate Agar (CCDA) is the most widely used plating medium that does not use blood which is advantageous since blood is expensive, has a short shelf life and can easily be contaminated (Corry and others 1995). In the later years, cefazolin (10 mg/L) was replaced by cefoperazone (32 mg/L) to allow fewer contaminants; this combined with incubation of plates at 37 °C is called the modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA). Amphotericin B was used to suppress overgrowth of yeasts and molds able to grow at 37 °C but not at 42 °C (Hutchinson and Bolton 1984). The rationale could explain why the FDA-BAM procedure (Hunt and others 2001) proposes the use of mCCDA agar in its protocol for isolation of *C. jejuni* from various foods and why most food microbiology laboratories in which use of blood is not common, prefer mCCDA agar to other media.

Nutrient source

Since *Campylobacter* are assacharolytic, peptone is included in the media as a major nutrient source. Preston broth (Bolton and Robertson 1982) and Exter broth (Martin and others 1996) contain meat extract or Lab Lemco (Oxoid) at a concentration of 10g per liter and peptone at 10 g per liter. Bolton's broth and *Campylobacter* enrichment broth (CEB) have nutrient formulations that consist of peptone, yeast extract and α -ketoglutaric acid, a tri-carboxylic cycle intermediate (Bolton and others 1983a).

Blood Supplement

Campylobacter media often contain lysed or defibrinated blood from various animal sources such as horses and sheep, at a level of 5 to 7% (v/v) to quench the toxic oxygen compounds such as H_2O_2 (Bolton and others 1984a) that form in the media when it is exposed to light. In addition, blood plays a role in neutralizing trimethoprim antagonists (Corry and others 1995). Although blood-free media for isolation of *Campylobacter* have been described (Tran 1998), the efficiency has been evaluated and found to be not only comparable to Bolton broth but with inconsistent performance with different foods. Another blood-free enrichment broth; modified charcoal cefoperazone deoxycholate broth was reported to be less selective than the Preston broth (Jacobs-Reitsma and de Boer 2001). Bloodless media use combinations of ferrous sulfate ($FeSO_4 \cdot 7H_2O$), sodium metabisulfite and sodium pyruvate (FBP) to counteract the toxic effects of oxygen (Hoffman and others 1979). Some media have been suggested that contain FBP supplements and blood (Stern and others 1992).

Antibiotics

Antibiotics are normally added to laboratory media used for the isolation of *Campylobacter* in order to suppress growth of unwanted microorganisms and thus provide the maximum recovery of *Campylobacter*. *Campylobacter* are resistant to several antibiotics including vancomycin (inhibits Gram positive cocci); polymyxin B is generally active only against Gram negative bacteria although *Proteus* are sometimes resistant (Corry and others 1995); colistin which has the same spectrum of activity as polymyxin; trimethoprim (inhibits *Proteus* spp. and Gram positive cocci) and cephalosporins such as cephalothin and cefoperazone which have a wide spectrum of activity against Gram positive bacteria (inhibit *Enterobacter* spp., *Serratia* spp., *Pseudomonas aeruginosa*, some *Proteus* spp., *Yersinia Enterocolitica*). In Preston media (Bolton and Robertson 1982) rifampicin was substituted for vancomycin because rifampicin showed a wide spectrum activity against Gram positive and Gram negative bacteria while vancomycin had limited activity against Gram negative bacteria. Humphrey (1990) reported that rifampicin was inhibitory to *C. jejuni* through synergism with H₂O₂. Cycloheximide (actidione) was widely used in *Campylobacter* media to inhibit yeasts and molds but in later years, it was found to be too toxic for mammalian cells to be included in microbiological media. Amphotericin B was substituted for cycloheximide and it was found to be satisfactory as an antifungal agent (Martin and others 2002). Although cycloheximide and Amphotericin B have the same effect (inhibition of yeast and mold growth), they have different modes of action. While cycloheximide inhibits the translation of mRNA by ribosomes, thus preventing protein

synthesis, Amphotericin B causes impairment of fungal membranes but has no effect on mammalian cells (Martin and others 2002). A number of other antibiotics such as cephalothin, colistin and polymyxin B inhibit the recovery of *Campylobacter* species or strains of *C. jejuni*, *C. coli*, *C. fetus* subsp *fetus*, *C. jejuni* subsp. *doylei* and *C. upsaliensis* (Goossens and others 1986). Some protocols have proposed a delayed addition of antibiotics to enhance the recovery of thermotolerant *Campylobacter* (Jones and others 1999).

Pre-enrichment step

Pre-enrichment procedures are utilized in the laboratory to increase the recovery of *Campylobacter* from food and environmental samples that are normally contaminated with low numbers or sub-lethally injured cells. Protocols specifying a pre-enrichment step have been recommended in the analysis of food and water samples (Bolton and others 1984b). The enrichment procedure normally begins with a resuscitation step consisting of incubation for 4 hours at 37 °C (Humphrey 1989; Bolton 2000; Hunt and others 2001) after which the enrichment is transferred to a 42 °C incubator. Goossens and Butzler (1992) proposed that pre-enrichment is limited to 4 hours to prevent overgrowth of background flora. After pre-enrichment, an aliquot from the enrichment culture can be sub-cultured to a selective agar for incubation at 42 °C for 48 hours in some protocols, 37 °C is used in the FDA-BAM protocol.

Pre-enrichment broths

Several pre-enrichment broths have been described including Preston broth, Exter broth, Bolton broth, CEB (*Campylobacter* enrichment broth). Pre-enrichment and enrichment broths are developed based on their ability to permit growth of *Campylobacter* while suppressing the growth of background flora. Many of them have been improved over the years by incorporation of additional components or removal of components. The FDA-BAM protocol (Hunt and others 2001) specifies the use of Bolton broth for both pre-enrichment and enrichment procedures.

Preston broth was proposed by Bolton and Robertson (1982), after reporting that the Skirrow agar was not effective in recovering *Campylobacter* from animal and environmental samples. Preston medium can be used for either the broth for pre-enrichment or as an agar medium for isolation of colonies. It does not contain yeast extract, a trimethoprim antagonist but includes 5% (v/v) lysed horse blood. The antibiotics included are: polymyxin B (5 IU/mL), rifampicin (10 µg/mL), trimethoprim (10 µg/mL) and cycloheximide (100 µg/mL). Rifampicin was found to be effective in inhibiting Gram positive bacteria. Bolton and others (1984a) later reported a modification of the Preston formulation by the addition of sodium pyruvate, sodium metabisulfite and ferrous sulfite (FBP), which assist in quenching the toxic oxygen metabolites. Inclusion of FBP allows for aerobic storage of the broth for a period of 7 days at 4 °C but the containers must be tightly closed and the headspace should be less than 1 cm.

Bolton broth is recommended by the U.S. Food and Drug Administration Bacteriological Analytical Method (Hunt and others 2001). Bolton broth contains

peptone and yeast extract, α -ketoglutaric acid, sodium pyruvate, sodium metabisulfite and haemin. The role of haemin is to counter trimethoprim antagonism as a result of inclusion of yeast extract. Sodium pyruvate and sodium metabisulfite allow aerobic incubation, sodium carbonate is included to generate carbondioxide during growth. The complete medium has in addition, 5% (v/v) lysed horse blood and antibiotics namely: cefoperazone (20 $\mu\text{g}/\text{mL}$), vancomycin (10 $\mu\text{g}/\text{mL}$), trimethoprim (10 $\mu\text{g}/\text{mL}$) and cycloheximide (50 $\mu\text{g}/\text{mL}$).

The original formulation for Exter broth was a nutrient broth that included 5% lysed horse blood but was later amended to include oxygen quenchers (FBP mixture). The amendment also excluded yeast extract, which is a trimethoprim antagonist. Exter broth has incorporated antibiotics: cefoperazone (15 $\mu\text{g}/\text{mL}$), Polymixin B (5 IU/mL), trimethoprim (10 $\mu\text{g}/\text{mL}$), rifampicin (10 $\mu\text{g}/\text{mL}$), and amphotericin (2 $\mu\text{g}/\text{mL}$), after a study by Humphrey and Cruikshank (1985) reported that stressed cells of *C. jejuni* were damaged by rifampicin but cefoperazone optimized the recovery of *C. coli*. Exter broth can be solidified by addition of 15 g/L of agar but the complete media contains FBP supplement, blood and antibiotics. The protocol for enrichment using the Exter broth requires aerobic incubation in containers fitted with a screw cap with a head space that is less than 1 cm, for 4 hours at 37 °C to allow resuscitation of injured cells (Humphrey 1986) followed by 24-48 hours at 42 °C (Martin and others, 2002).

Campylobacter enrichment broth (CEB) has the same formulation as Bolton broth but is different in the substitution of natamycin for cycloheximide in the antibiotic supplements.

The base formula for Park and Sanders broth is Brucella broth (Difco) which contains peptone, glucose, yeast extract, sodium pyruvate and sodium metabisulfite. The protocol using Park and Sanders broth involves both the addition of lysed horse blood (5 %v/v) and two antibiotics namely vancomycin and trimethoprim, then incubation for 4 hours at 32 °C. After that, the remaining antibiotics (vancomycin and trimethoprim) are added and the enrichment is transferred to 37 °C for 4 hours and finally to 42 °C for 42-44 hours. The use of Park and Sanders broth was recommended by International Organization for Standardization (ISO 1995) for samples that contained stressed cells caused by procedures such as freezing.

Selective Agar Media for isolation of *Campylobacter*

After the pre-enrichment step, isolation of *Campylobacter* involves the streaking of the enrichment culture onto selective agar medium plates. There are a variety of selective agar media that are being used but Bolton and others (1984a) reported that *Campylobacter* growth is affected when plates are stored in or exposed to light or air. Corry and others (1995) proposed that the plates be used immediately after pouring and if stored, to be stored (anaerobically at room temperature or aerobically at refrigeration temperature) for not more than 5 days. Plates are dried at 42 °C in an incubator to remove excess moisture from the surface but should not be dried in the laminar flow with lids off because the slightest exposure to air, limits the recovery of *Campylobacter* (Hunt and others 2001). Buck and Kelly (1981) also reported that *Campylobacter* are sensitive to the moisture levels in plating media which can cause changes in colony morphology. However such a change in colony morphology also could be due to inhibitory oxygen

derivatives formed during the storage and drying of plates. Various types of agar media have been used; the ones that include blood (normally 5-7%) are Skirrow, Campy-Cefex, Butzler, Preston and Exter media. Blood free selective agar media include charcoal as an oxygen quencher. Examples include Karmali or mCCDA (modified Charcoal Cefoperazone Deoxycholate agar) (Corry and others 1985). Some that contains neither blood nor charcoal is Abeyta-Hunt-Bark Agar (Hunt and others 2001).

Performance comparison of different media

Some studies have been done to evaluate the effectiveness of different isolation media for their abilities to recover *Campylobacter* from artificially and naturally contaminated samples. Baylis and others (2000) compared the performance of three *Campylobacter* enrichment broths namely: Bolton broth, *Campylobacter* enrichment broth (CEB) and Preston broth. Laboratory inoculated and naturally contaminated food samples were used to evaluate the three enrichment broths. The mean log₁₀ populations recovered were reported as follows: Preston broth, 7.11; Bolton broth, 7.09 and CEB, 6.57. While Preston broth supported growth of the highest number of *Campylobacter*, it did not deter the growth of some competitor organisms. In contrast, CEB inhibited all competitor microorganisms but failed to recover the strains of *Campylobacter* of interest. The difference in recovery between Bolton broth and CEB was surprising to the investigators, since both have the same base and antibiotic composition; however Bolton broth presented the best overall performance. Peterz (1991) conducted a trial among six laboratories that tested chicken liver which had been artificially inoculated with strains of *C. jejuni* and compared the recovery performance of mCCDA and Preston agar. Both

agar media exhibited similar isolation rates but mCCDA allowed less growth of contaminating flora. Gun-Munro and others (1987) evaluated the performance of six different isolation media based on ability to recover *C. jejuni* while suppressing the background flora. They evaluated the performance of Skirrow medium (Skirrow 1977), Butzler medium (Goosens and others 1983), Blaser-Wang medium (Blaser and others 1979), Preston medium (Bolton and Robertson 1982), Charcoal based selective media (Karmali agar) described by Karmali (Karmali and others 1986) and modified charcoal cefoperazone deoxycholate agar (mCCDA) described by Bolton and others (1984b). Findings showed that modified CCDA and Karmali agar supported the best growth of *C. jejuni* while inhibiting background flora. Results observed for mCCDA and Karmali agar were similar although Karmali agar produced the most easily identifiable colony morphology of strains tested. The researchers further pointed out that the selective feature in both media was cefoperazone, which is a broad-spectrum cephalosporin with powerful activity against species of *Pseudomonas* and members of the *Enterobacteriaceae* family. They further noted that there was more suppression of fecal flora after 24 hours than 48 hours, which could have direct implications in the recovery of *Campylobacter* from food. It is envisaged that recovery of *Campylobacter* spp. from food matrix in the presence of naturally existing background microflora of food may be better achieved after 24 hours than after 48 hours. The most frequent background flora reported includes the family *Enterobacteriaceae*, *Pseudomonas* spp., streptococci and yeasts (Bolton and others 1983b). Oyarzabal and others (2005) compared the effectiveness of six agar media to recover *Campylobacter* spp. from chicken rinses. Although 63.3% of all the combined plates were positive for *Campylobacter* spp., no

statistically significant difference was reported among the five agar media tested namely: Campy-cefex, mCCDA-Cefex, Campy, mCCDA and Karmali.

Standard methods for isolation of *Campylobacter jejuni*

International Organization for Standardization

The International Organization for Standardization (1995) method for isolation of *Campylobacter* (ISO) in food and feedstuff includes three methods, two methods that include a pre-enrichment procedure and one that is used only for products with large numbers of *Campylobacter* suspected and involves direct plating (Jacobs-Reitsma 2000). The pre-enrichment protocols are based on either Preston broth (Bolton and Robertson 1982) or Park and Sanders broth (Park and Sanders 1991). The Preston broth formulation does not include FBP or a resuscitation step. The Park and Sanders protocol involves addition of part of the antibiotics in the first step and incubation at 32 °C for 2 hours; then addition of the remaining antibiotics and re-establishment of the modified atmosphere and incubation at 37 °C for 2 hours and finally the third step involves incubation at 42 °C for 40 to 42 hours. After enrichment in both protocols, selective detection is done by plating on two agar media, one which must be Karmali. The second agar medium could be modified Butzler, Skirrow, CCDA or Preston. Incubation of all agar media plates is done under microaerophilic conditions at 42 °C for 24-72 hours.

Food and Drug Administration/Bacteriological Analytical Method (FDA/BAM)

The Bacteriological Analytical Manual (BAM) protocol (Hunt and others 2001) for isolation of *Campylobacter* species from food and water was designed by the US Food and Drug Administration (FDA) and includes protocols for isolation from specific samples such as water, shellfish, and milk and cheese products. For all samples, incubation is done under microaerophilic atmosphere. The pre-enrichment and enrichment broth specified in all protocols is Bolton broth and a pre-enrichment is specified to increase the recovery of stressed cells. Two isolation agar media namely Abeyta-Hunt-Bark agar and mCCDA are specified for use by the protocol.

A four-hour pre-enrichment is carried out if the sample has been produced within the past 10 days. Pre-enrichment is done at 37 °C for 4 hours under microaerophilic conditions. A five-hour pre-enrichment is done if the product has been under refrigerated storage for ≥ 10 days. All water and shellfish are pre-enriched by this method.

Incubation is performed at 30 °C for 3 hours, then 37 °C for 2 hours. This method yields the greatest recovery for severely stressed cells (Hunt and others 2001).

Identification and confirmation is done by examination of wet mount for typical cellular morphology. Catalase and oxidase tests are done on the colonies on the streak plates. Further tests include biochemical tests for hippurate and antibiotic resistance. Carbol fuchsin is used as a counter stain in the Gram stain test.

The Food and Agricultural Products Center (FAPC) method for the detection of *Campylobacter* in foods

Recovery of *Campylobacter* from food remains dependent on selective media and use of antibiotics to suppress or eliminate competing microflora. *Campylobacter jejuni* has a low infective dose, therefore it is necessary to detect low numbers which necessitates an enrichment step. The enrichment step also will improve recovery of stressed cells that have undergone sub-lethal injury. The FDA-BAM procedure described by Hunt and others (2001) has been used successfully in the isolation of *Campylobacter* spp. but it is time consuming, labor intensive, cumbersome and relies on the use of blood, growth supplements and specialized equipment to achieve a microaerophilic atmosphere (5% oxygen, 10% carbon dioxide, 85% nitrogen) necessary for growth of *Campylobacter* (Tran, 1998; Baserisalehi, 2004). Some studies have reported difficulty in the culturing of *Campylobacter* from food because the cells can undergo a morphological transformation from spiral shaped rods to a coccoid form believed to be associated with the non-culturable state.

Reilly and Gilliland (2003) reported an improved culturing technique for *Campylobacter* based on the type of culturing vessel, incubation time, growth atmosphere and frequency of sub-culturing. They compared the effect of culturing *Campylobacter* in different shapes and sizes of growth vessels (Erlenmeyer flask, test tubes and vented tissue culture flasks) and reported that there was less growth in the test tubes and Erlenmeyer flasks than in the tissue culture flasks. Using vented tissue culture flasks agitated at 60-90 rpm in a microaerophilic atmosphere attained the best growth. They further observed a higher percentage of coccoid forms when Erlenmeyer flasks and test

tubes were used in comparison to the vented tissue culture flasks. The vented tissue culture flasks had a higher surface-to-volume ratio (250 mm²/mL) in comparison to the Erlenmeyer flask and test tubes (150-mm length, 14-mm diameter). The higher surface-to-volume ratio favored maximization of atmospheric exchange between the modified atmosphere and the cultures in the broth. The best procedure for providing the needed atmosphere was to place the flasks in a sealed chamber in which the atmosphere was created by Gas Pak system designed for *Campylobacter*. The effect of incubation time for enrichment was investigated by incubating *Campylobacter* for 24, 48 and 72 hours. Findings showed that there was a 2.0 to 3.2-log₁₀ reduction of *Campylobacter* after 48 hours, and counts decreased further after 72 hours of incubation. This correlated proportionately with the number of coccoid cells formed. The longer the incubation time, the higher the percentage of coccoid cells formed. This could explain reduction in plate counts after longer incubation periods; the concept of non-culturable forms that could not be detected on agar media plates.

The authors further showed that *Campylobacter* could be recovered successfully after incubation at 37 °C for 24 hours as opposed to 48 hours at 42 °C in the FDA-BAM protocol. This study revealed that incubation at 37 °C for 24 hours without a pre-enrichment step not only recovered similar counts as incubation at 37 °C for 24 hours but fewer coccoid forms were observed in comparison to incubation at 42 °C for 48 hours. The method was shorter, less cumbersome and easier to use.

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CHAPTER III

VALIDATION OF AN IMPROVED ISOLATION AND DETECTION METHOD FOR *CAMPYLOBACTER JEJUNI* IN VARIOUS FOODS

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ABSTRACT

To validate an improved method for isolation of *Campylobacter jejuni* from food, *Campylobacter jejuni* ATCC 29428 and 33560 were inoculated separately into 25-g portions of beef muscle, ground beef, chicken skin and 50-g portions of milk to yield approximately 10-100 cells per gram of food sample. The samples were stored at ~ 4 °C for 10 days. On days 0 (no storage), 3, 7 and 10, the samples were drawn for enrichment in Bolton broth supplemented with antibiotics, with and without blood supplement, for 24 and 48 hours using the Food and Agricultural Products Center (FAPC) and the Food and Drug Administration (FDA) procedures as described by Reilly and Gilliland (2003) and Hunt and others (2001) respectively. Enumeration of the organisms in the enrichment cultures was done on pre-poured *Campylobacter* Karmali selective agar media plates after 24 and 48 hours of enrichment. There was no significant difference between counts recovered using the FDA and the FAPC methods for detection of *Campylobacter jejuni* for either strain in all the food products tested ($P>0.05$). No significant difference was observed in performance of enrichment broth supplemented with and without blood ($P>0.05$). After 48 hours of enrichment, the counts recovered were similar for all products except milk. No cells could be detected after 10 days of storage in milk samples inoculated with either strain of *C. jejuni* after 24 or 48-hour enrichment period using either method for recovery. In contrast, cells were detectable on all days of storage in raw chicken skin, beef and ground beef samples after 24 and 48 hours of enrichment. Recovery of cells was possible after 24 hours of enrichment on all days of storage indicating that there is no need for 48-hour enrichment described in the FDA method. The results from the FAPC method for detection of *C. jejuni* from food were not different

from the FDA method. Incubation at 37 °C was adequate in the recovery of *C. jejuni* as opposed to the 42 °C used by the FDA.

INTRODUCTION

Campylobacter is the most common cause of human gastroenteritis worldwide and in the United States alone; it is estimated to cause approximately 2.4 million (1%) cases a year (Altekruse and others 1998; Tauxe 1992). Several problems have been cited regarding its detection and isolation from food and environmental samples.

Campylobacter is microaerophilic; it requires specialized conditions to grow. In addition, campylobacters do not ferment nor oxidize carbohydrates; instead they obtain energy from other compounds such as amino acids, tricarboxylic acid intermediates and respiratory quinones (Vandamme 2000). They require complex media for growth and are unable to degrade complex substances (Ketley 1997). They are known to revert to a viable but non-culturable form as a result of environment stress and starvation, which explains why culture techniques have failed to isolate *Campylobacter* from environmental sources implicated in major outbreaks of water or foodborne illnesses (Rollins and Colwell, 1986). The traditional methods for isolation of *Campylobacter* currently in use are time consuming, laborious and inconsistent because *Campylobacter* not only has a slow growth rate, but may also normally occur in food samples in low numbers (Baserisalehi and others 2004, Tran 1998). The current standard method described in the U.S. Food and Drug Administration's Bacteriological Analytical Manual (FDA/BAM) protocol (Hunt and others 2001) requires the use of blood supplement in the enrichment broth and in addition, a prolonged enrichment is prescribed. The procedure has been described as laborious, rendering the isolation and detection of *Campylobacter* from food problematic. In this study, a method that is faster and simpler was used; no

blood supplement was necessary and no pre-enrichment step was prescribed. The enrichment procedure was reduced to 24 hours as opposed to 48 hours in the FDA/BAM protocol described by Hunt and others (2001).

The aim of this study was to validate an improved culturing technique for detection of *Campylobacter* in beef, ground beef, milk and chicken skin and compare it with the FDA/BAM procedure described by (Hunt and others 2001) for detection of *Campylobacter* from food.

MATERIALS AND METHODS

Source, Maintenance and Preparation of Cultures

Two strains of *Campylobacter jejuni* ATCC 33560 and 29428 were purchased from the American Type Culture Collection; ATCC, (Manassas, VA, 20108). The strains were rehydrated in 10 mL Bolton broth (Oxoid Ltd; Basingstoke, Hampshire, England) in 25 cm² tissue culture flasks (Falcon Brand; Becton Dickinson Labware, Franklin Lakes, NJ) equipped with a 0.2- μ m-pore size vented cap. The cultures were incubated in a GasPak chamber (BBL) charged with a mixture of O₂ (5%), CO₂ (10%) and N₂ (85%) generated from Campy-Pak microaerophilic gas generating system (BD BBL™ Campy-Pak™ System; Becton Dickinson Microbiology Systems, Cockeysville, MD). The Gas-Pak chamber (BBL) was placed on an orbital bench top shaker (Innova™ 2000, New Brunswick Scientific Model) set at 85 rpm and incubated for 18 to 24 hours in a thermostatically controlled incubator at 37 °C. The cultures were concentrated by centrifuging at 10,000 x g at 3-5 °C for 10 minutes. Ten mL of sterile phosphate buffer (85 ppm) was used to wash the cell pellets. The pellets were resuspended in 1 mL non-fat dry milk (10%). One drop (~0.05 mL) of the resuspended cells was aseptically placed onto 5-6 sterile glass beads (2 mm diameter) contained in a sterile 8 x 150 mm glass tube, vacuum dried at ambient temperature and sealed under vacuum (10mm Hg). The dried cultures (stock cultures) were stored at -20 °C until needed. Prior to the performance of each experiment, the stock cultures were rehydrated in 10 ml of freshly prepared Bolton broth (made following the manufacturer's instructions) in sterile non-pyrogenic polystyrene (25-cm²) tissue culture flasks with 0.2 μ m vented cap (Corning Inc. NewYork). The cultures were streaked onto charcoal based Karmali agar (Oxoid Ltd.

Basingstoke, UK.) plates and incubated microaerophilically (5% O₂, 10% CO₂ and 85% N₂) in Gas-Pak (BBL) jar system for 48 hours at 37 °C. One isolated colony was transferred from the Karmali agar to 10 ml of Bolton broth in a 25-cm² tissue culture flask and incubated for 18-24 hours with constant shaking (85 rpm) at 37 °C. Prior to beginning all experiments, the two strains were checked for purity using Gram staining procedure, microscopic examination (Nikon, Phase contrast 1.25, Alphaphot 2- YS2 Japan) and biochemical assays (API Campy® strips, Biomerieux Inc., Hazelwood, MO, USA).

For food inoculation experiments, preparation of concentrated cell suspension was done using the procedures of Reilly and Gilliland (2003). Cultures (10 mL) were transferred to sterile centrifuge bottles and centrifuged at 10,000 x g at 3-5 °C for 10 minutes. Before centrifugation, the empty tubes were weighed in order to estimate the pellet weight. After removal of the supernatant, 10 mL of phosphate buffer (85 ppm) was added to the pellet and vortexed to resuspend the pellet followed by centrifugation. This procedure was performed twice. After the final centrifugation, the supernatant was removed and the pellet was resuspended in buffer twice its weight. Serial dilutions were carried out using peptone water (0.1%). Samples were inoculated with 200 µl of an appropriate dilution of the cell suspension to yield the desired inoculum levels of *Campylobacter* per gram of food sample.

Preparation and inoculation of food samples

Beef, ground beef and chicken skin samples

Fresh samples of beef muscle (sirloin steak), ground beef (ground beef sirloin steak) and chicken thighs were purchased from a local supermarket and stored under

refrigeration ($\sim 4^{\circ}\text{C}$) until needed (not longer than 12 hours). For the whole beef muscle sample, small cubes ($\sim 2\text{ cm}^3$) were prepared from the internal portion of the muscle using sterile utensils. Ground beef did not receive any additional preparation. Both samples (beef strips and ground beef) were weighed (25 g) and aseptically transferred to sterile net lined filter bags (Nasco Whirl-Pak[®]). Using sterile utensils, the skin was removed from the chicken thighs. The skin was cut and weighed in uniform portions ($\sim 25\text{ g}$). In order to reduce the background microflora, the skin was soaked in ethanol solution (70%vol/vol) for 15 minutes. They were then rinsed twice in sterile water to remove residual ethanol. After rinsing, each portion of skin was transferred into a sterile net lined filter bag. Sufficient numbers of bags were prepared for each food to permit one bag of each for analyses at the desired time intervals. All samples were kept on ice prior to inoculation. Each sample was inoculated with 200 μl of the appropriate dilution of the concentrated suspension of either of the two strains of *Campylobacter jejuni* (ATCC 33560 and 29428) and stored for up to 10 days at refrigerated temperature ($\sim 4^{\circ}\text{C}$). Samples were removed on days 0, 3, 7 and 10 for detection of *Campylobacter*.

Milk

Raw milk was obtained from the Oklahoma State University Dairy Cattle Center. Upon receipt, the pH of the milk was measured aseptically using a pH meter (Fisher Scientific - Accumet Research benchtop pH meter AR25). The pH was adjusted to 7.5 ± 0.2 by addition of sterile sodium hydroxide (1-2 N) in order to neutralize the lactoperoxidase system in milk since *Campylobacter* is sensitive to this system. Milk samples (50 g portions) were weighed into sterile net lined filter bags (Nasco Whirl-Pak[®] and

pasteurized in a water bath (Fisher Scientific, Isotemp Model 220) at 63 °C for 30 minutes. Following pasteurization, the samples were rapidly cooled to approximately 4 °C in an ice-water bath. The samples were inoculated with 200 µl of concentrated cell suspension of either strain of *C. jejuni* (ATCC 33560 and ATCC 29428). Samples were kept at 4 °C and removed on days 0, 3, 7 and 10 for testing.

Uninoculated controls composed of 25 g of beef, ground beef, raw chicken skin and 50 g of milk samples were included to confirm that none of the food samples contained *C. jejuni* as part of their background flora.

Preparation of food samples

Beef, ground beef and chicken skin

Enrichment of samples was carried out using Bolton broth (100 mL) prepared according to the manufacturer's instructions and supplemented with 2% sodium cefoperazone (Sigma Aldrich, St Louis MO), 2% trimethoprim lactate (Sigma Aldrich, St Louis MO), 2% vancomycin (Sigma Aldrich, St Louis MO) and 2% amphotericin B (Sigma Aldrich, St Louis MO). In some experiments, samples were enriched in broth with or without lysed defibrinated horse blood (5% v/v) to determine if the blood was necessary. All samples (25 g portions) were suspended in enrichment broth (100 mL) and shaken gently for 5 minutes on a bench top orbital shaker set at 25 rpm. After mixing, the liquid filtrate was recovered using the sterile net lined bags. This constituted the samples for enrichment using either the FDA method or the FAPC method. In the FDA method, the liquid filtrate was transferred to a new sterile filter bag. For the

improved method, the liquid filtrate was placed into sterile 150 cm² polystyrene sterile tissue culture flasks with a 0.2 µm vented cap (Corning Inc, NY).

Milk

On days 0, 3, 7 and 10 of storage, the 50-g portions of milk in sterile filter bags were transferred to sterile centrifuge bottles and centrifuged at 20,000 x g for 40 minutes. The supernatant was discarded and the pellet was resuspended in Bolton broth (10 mL) supplemented with 2% sodium cefoperazone (Sigma Aldrich, St Louis MO), 2% trimethoprim lactate (Sigma Aldrich, St Louis MO), 2% vancomycin (Sigma Aldrich, St Louis MO) and 0.2% amphotericin B (Sigma Aldrich, St Louis MO), with or without lysed defibrinated horse blood (5% v/v). After resuspending the pellet in Bolton broth (10 mL), it was added to the remaining Bolton broth (90 mL). The enrichment culture was then transferred to either sterile filter bags for the FDA method of enrichment or 125-cm² polystyrene sterile tissue culture flasks with a 0.2 µm vented cap (Corning Inc, NY) for the FAPC method of enrichment.

Enrichment procedures

The Food and Drug Administration (FDA) method for detection of *Campylobacter* prescribes the use of two enrichment steps for detection of *Campylobacter*; a pre-enrichment step at 37 °C for 5 hours and enrichment at 42 °C for additional 19 hours for the 24-hour enrichment and an additional 23 hours for a 48-hour enrichment. Net lined bags are used for both steps of enrichment.

The net-lined filter bags containing the enrichment cultures were loosely closed and placed in airtight containers with Campy-Pak used to generate a microaerophilic atmosphere of 5% O₂, 10% CO₂ and 85% N₂. All samples were pre-enriched by incubating for 5 hours (Hunt and others 2001) at 37 °C with agitation (85 rpm) as reported by Reilly and Gilliland (2003). After 5 hours of pre-enrichment, samples were transferred to an incubator set at 42 °C and enriched for an additional 19 hours. After 24 hours of enrichment, appropriate dilutions of the samples were plated onto Karmali agar. The samples were then reintroduced into airtight containers and re-gassed with fresh Campy-Pak to generate a microaerophilic atmosphere (5% O₂, 10% CO₂ and 85% N₂) and incubated for an additional 24 hours. After the 48-hour enrichment period, the samples were again plated on Karmali agar.

The Food and Agricultural Products Center (FAPC) method for enrichment involves the use of 125cm² tissue culture flasks with vented (0.2 µm) caps. The FAPC method has a single enrichment step and incubation is carried out at 37 °C. After gently mixing on a bench top orbital shaker, the liquid filtrate from the filter bag was transferred to 125 cm² tissue culture flasks. The tissue culture flasks were laid flat in the growth chamber to ensure maximum surface: volume ratio required for growth. Incubation of samples with agitation was done at 37 °C in airtight containers gassed with Campy-Pak system to generate a microaerophilic atmosphere (5% O₂, 10% CO₂ and 85% N₂) for 24 hours. After 24 hours of incubation, 1 mL of the culture was drawn and appropriate serial dilutions (made using 99 mL of 0.1% peptone water) were plated onto Karmali agar. The samples were re-introduced into the chamber which was regassed and incubated for 24 hours more (48-hour enrichment). After the 48-hour enrichment period,

1 mL of culture was drawn and appropriate serial dilutions were made and plated onto Karmali agar.

Lysing of horse blood cells

On receipt, fresh defibrinated horse blood cells (PML Microbiologicals, Wilsonville, OR) were resuspended gently and poured into sterile disposable centrifuge tubes (50 mL portions). The blood was frozen at -20 °C, thawed at room temperature and refrozen to complete cell lysis.

Enumeration procedure

Appropriate dilutions of the enrichment cultures were plated onto pre-poured Karmali agar plates using a spiral plater (Don Whitley Scientific, Shipley, England) attached to a vacuum pump (Microbiology International) set at 20 in Hg. Enrichment samples (50- μ l aliquots) were plated in a logarithmic mode setting. The mean readings from the duplicate plates from each treatment in each replication were used for statistical analyses. Plates were incubated in Gas-Pak (BBL) jar system charged with Campy-Pak system, for 48 hours at 37 °C. Counts were done using an automatic plate reader (Protocol; Synoptics Ltd., Cambridge, UK) and related software. A minimum of three readings was performed on each plate and the final count was taken from the means of the six readings of the duplicate plates. Characteristic colonies were counted and all cultures within each treatment combination isolated were identified according to the biochemical characteristics described in the Campy API kits (Biomérieux Inc., Hazelwood, MO, USA). The kits were used in accordance with directions provided by the manufacturer.

Confirmation of identity of detected organisms

Gram Stain

After enrichment, the culture was spread onto a pre-cleaned microscopic slide and the Gram stain procedure for *Campylobacter* was followed in which carbol fuchsin was used as a counter stain. The slides were air dried and observed under a microscope. Red spiral rods about 0.5-8.0 μm long were indicative of *Campylobacter jejuni*.

Oxidase test procedure

Drops of oxidase reagent (2-3) were placed on a Whatman No. 1 filter paper and a few seconds were allowed for absorption. Using a sterile loop, a colony was placed on the filter paper over a line 3-6 mm long and changes were observed. A dark purple color change within few seconds constituted a positive reaction for oxidase.

Identification of isolates using API Campy® identification system

Identification of isolates from the food products was done using API Campy® strips (Biomérieux Inc., Hazelwood, MO, USA). Prior to streaking, isolates were checked to determine purity by Gram staining and microscopic examination for the typical spiral rod morphology. The isolates were streaked onto Columbia Agar Base with 5% horse blood; (BBL) and incubated at 37 °C under microaerophilic conditions (Campy Pak) for 48 hours. After incubation, the culture was harvested using a sterile cotton swab and suspended in 3 mL of sterile API solution (0.85 % NaCl) to a turbidity equivalent of a number 6 McFarland Standard. The suspension (80-100 μL) was inoculated into appropriate portions of the aerobic test strip following the manufacturer's direction.

Small aliquots (150 µl) of the remaining suspension were added to AUX medium which consisted of ammonium sulfate (2.0 g), monosodium phosphate (6.24 g), potassium chloride (1.5 g), agar (1.5 g), vitamin solution (10.5 mL), trace elements (10.0 mL) dissolved in 1000 mL of mineralized water. The suspension in the AUX medium was used to inoculate the remaining test strips. The test strips were incubated according to manufacturer's instructions. The first portion (aerobic) of the strip was incubated aerobically at 37 °C for 24 hours and the second portion was incubated under microaerophilic conditions for 48 hours at 37 °C. After incubation, the biochemical reactions were read and recorded accordingly.

Detection of *Campylobacter jejuni* ATCC 33560 and 29428 in ground beef during storage

In order to determine the detection levels of *C. jejuni* ATCC 33560 and 29428 during refrigerated storage for 10 days, ground beef samples (25 g) were inoculated with 200 µL of the highest dilutions of *C. jejuni* strains ATCC 33560 and ATCC 29428 (10^{-6} , 10^{-7} and 10^{-8}) and kept under refrigerated storage (~ 4 °C) for 10 days. Samples were taken on days 0, 3, 7 and 10 and enriched in Bolton broth supplemented with antibiotics for 24 and 48 hours respectively. Using the FDA and FAPC methods, the samples were tested for *C. jejuni* on the appropriate days of storage. Enumeration was done on enrichment cultures after 24 and 48 hours of enrichment by plating on pre-poured Karmali agar plates.

Growth curves for *Campylobacter jejuni* ATCC 29428 and 33560

Frozen stock cultures of two strains of *Campylobacter jejuni* ATCC 29428 and 33560 were rehydrated in Bolton broth (10 mL) and incubated in 25 cm² vented tissue culture flasks in a GasPak jar system with a microaerophilic gas generating system. The cultures were incubated on a shaker set at 85 rpm for 18 to 24 hours at 37 °C. After incubation, the cultures were concentrated by centrifuging at 10,000 x g for 10 minutes at 3-5 °C. The pellets were resuspended and washed with 10 mL of sterile phosphate buffer (85 ppm). The suspension was centrifuged and the pellets were washed twice in sterile phosphate buffer. The pellets were resuspended in peptone water twice their weight and ten-fold dilution series were carried out using 250 mL of Bolton broth to yield 10-100 CFU for each strain. This volume of Bolton broth for each strain was distributed into twenty four-25 cm² tissue culture flasks with vented caps (0.2 µm). The cultures for each strain were incubated for 0 to 48-hour period. At every two-hour interval of incubation starting with 0 hour, one tissue culture flask with enrichment culture for each strain was removed and appropriate dilutions were carried out using sterile peptone water (0.1%). Enumeration was done by plating the culture of each strain onto pre-poured Karmali agar using a spiral plate. Plates were incubated in a GasPak jar system charged with CampyPak system to generate a microaerophilic atmosphere (N₂ 85%, CO₂ 10%, O₂ 5%) at 37 °C for 48 hours. Counting was done using an automated plate reader. Growth curves were generated by plotting counts (log₁₀ CFU/ mL) taken from 0 to 48 hours at the two hourly intervals against time for a 48-hour period.

Statistical Analysis

The experiment was a 2x2x2x4 completely randomized factorial design with three replications of each treatment. Blood supplementation, detection method, enrichment time, and storage period were the main treatment effects. The data was analysed by doing an analysis of variance using the GLM procedure in SAS/STAT® software, Version 9.1.3 (SAS Institute Inc. 2000-2004). The main and interaction effects were considered significant if $p \leq 0.05$. The least square means of counts recovered were also compared and the hypothesis of equality of the means rejected if $p \leq 0.05$.

Statistical Model

Model:

$$Y_{ijklm} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \gamma_k + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk} + \delta_l + \alpha\delta_{il} + \beta\delta_{jl} + \alpha\beta\delta_{ijl} + \gamma\delta_{kl} + \alpha\gamma\delta_{ikl} + \beta\gamma\delta_{jkl} + \alpha\beta\gamma\delta_{ijkl} + e_{ijklm}$$

Where Y_{ijklm} = the m^{th} response of $\alpha_i\beta_j\gamma_k\delta_l$

μ = Overall mean

α_i = storage main effect

β_j = method main effect

$\alpha\beta_{ij}$ = storage*method interaction effect

γ_k = time main effect

$\alpha\gamma_{ik}$ = storage*time interaction effect

$\beta\gamma_{jk}$ = method*time interaction effect

$\alpha\beta\gamma_{ijk}$ = storage*method*time interaction effect

δ_l = blood supplement main effect

$\alpha\delta_{il}$ = storage*blood supplement interaction effect

$\beta\delta_{jl}$ = method*blood supplement interaction effect

$\alpha\beta\delta_{ijl}$ = storage*method*blood supplement interaction effect

$\gamma\delta_{kl}$ = time*blood supplement interaction effect

$\alpha\gamma\delta_{ikl}$ = storage*time*blood supplement interaction effect

$\beta\gamma\delta_{jkl}$ = method*time*blood supplement interaction effect

$\alpha\beta\gamma\delta_{ijkl}$ = storage*method*time*blood supplement interaction effect

e_{ijklm} = random error, $e_{ijklm} \sim N(0, \sigma^2)$

CHAPTER IV

RESULTS AND DISCUSSION

Confirmation of isolates of *Campylobacter*

Identity of isolates from beef, ground beef, chicken skin and milk were confirmed to be Gram negative, slender spiral rods. Using the API Campy system all the isolates tested urease (URE) negative and hippurate (HIP) positive as shown in Tables 10A and B in the appendix. They tested positive for reduction of nitrates (NIT), esterase (EST), hippurate (HIP), gamma glutamyl transferase (GGT), reduction of triphenyl tetrazolium chloride (TTC) and the reaction was variable for pyrrolidonyl arylamidase (PyrA). The tests were negative for L-arginine arylamidase (ArgA) and L-aspartate arylamidase (AspA) but positive for alkaline phosphate (PAL). Tests were negative for production of hydrogen sulfide (H₂S) for all isolates, negative for assimilation of glucose (GLU), sodium acetate (ACE) and propionate (PROP) but positive for assimilation of sodium succinate (SUT). They were resistant to sodium cefazoline and sensitive to nalidixic acid and erythromycin. All the isolates were catalase positive. *Campylobacter jejuni* and *C. coli* are similar but can be distinguished through hippurate hydrolysis. While *C. coli* is hippurate negative, *C. jejuni* is hippurate hydrolysis positive.

Influence of supplementation of enrichment broth with horse blood on recovery of *Campylobacter jejuni*.

Beef, ground beef, chicken skin and pasteurized milk were inoculated with $\sim 4.0 \times 10^3$ to 5.0×10^3 cells of *C. jejuni* strains ATCC 29428 and 33560 and stored at 4 °C for 10 days. To investigate the effect of blood in the enrichment medium on the recovery of *C. jejuni* cells from the food products, the products were cultured in Bolton broth with and without horse blood after storage for 0 (without storage), 3, 7 and 10 days, following FDA (Hunt and others 2001) and the FAPC (Reilly and Gilliland 2003) procedures, for 24 and 48 hours. Table 1 shows the number of cells of each strain of *C. jejuni* recovered from each product on day 0, prior to storage at refrigeration temperature (4 °C-8 °C). Results show that in all the products, both methods recovered *C. jejuni* cells in high numbers with and without blood in enrichment broth. The number of cells recovered in broth with and without blood supplement using both methods, were comparable throughout the 10 day storage period and blood had no effect on the ability of the two enrichment methods to recover the *C. jejuni* cells (Tables 11-18). Supplementation of the enrichment broth did not have an effect on the recovery of either strain of *C. jejuni* after 24 and 48 hours of enrichment for any of the products. For beef inoculated with strain 33560, 8.9 \log_{10} CFU/mL were recovered after 24 hours of enrichment period in broth with horse blood and 8.8 \log_{10} CFU /mL were recovered without blood. After 48 hours of enrichment, 9.0 \log_{10} CFU /mL cells were recovered with horse blood and 9.1 \log_{10} CFU/mL without blood supplement using the FDA method. Similar results were obtained for strain 29428. The FAPC method followed a similar trend with cell counts not differing after culturing in broth with and without blood after 24 or 48 hours of

enrichment period for either strain in all the products. Both methods recovered high numbers of both strains of *C. jejuni* cells cultured in broth with and without horse blood after 24 and 48 hours of enrichment time. These findings in general suggest that supplementation of enrichment broth with blood played no role in the recovery of both strains of *C. jejuni* from all the products tested, after 24 or 48 hours of enrichment time. Most widely used media for detection of *Campylobacter* include blood, the purpose of which is to promote aerotolerance of *Campylobacter* and to protect it from the toxic effects of oxygen (Bolton and others 1984a). Most media contain trimethoprim antagonists. Skirrow and others (1982) reported that lysed horse blood was required to neutralize these antagonists in media. Bolton and Coates (1983), Bolton and others (1984a) and Bolton and others (1984b) found that media containing charcoal was as effective as media containing blood. Bolton and Coates (1983), and Bolton and others (1984 a and b) reported supplements other than blood such as iron salts, Filde peptic digest of blood, hematin, charcoal, sodium metabisulfite and sodium pyruvate that improved the aerotolerance in *Campylobacter*. These workers showed that these components quenched the photochemically generated toxic oxygen derivatives which are detrimental to *Campylobacter*. Karmali and others (1986) described a blood-free charcoal based selective media for isolation of *Campylobacter* which they reported to be effective in supporting the growth of *Campylobacter*. They supplemented their media with charcoal, hematin and sodium pyruvate but excluded blood. Findings in the present study suggest that blood supplementation in enrichment played no role on the recovery of *C. jejuni* cells from the food matrixes which confirms findings by Bolton and co-workers (Bolton and Coates 1983; Bolton and others 1984a and Bolton and others 1984b). Bolton

broth is supplemented with some of the components described above including charcoal and this may explain why there was no difference detected in mean cell \log_{10} cfu/mL counts recovered after 24 and 48 hours of enrichment in broth with and without blood supplement using the two methods of detection. Recovery of both strains of *Campylobacter* did not show that blood had an effect on the mean log counts recovered.

Effect of enrichment time and storage period on the recovery of *Campylobacter jejuni*
Chicken and ground beef

Three replications of this experiment were done for each product (data is shown in the appendix Tables 11-18). The data was analyzed using the GLM procedure and significant interactions tables were drawn and differences between means within the interactions are reported (Tables 2-6).

There was a significant two-way interaction between enrichment time and days of storage in ground beef and chicken skin (Table 2). Similar interactions were not observed for the beef and milk. The data shows that in both products, for both strains of *C. jejuni*, the counts for the 24 hour enrichment decreased significantly from days 0 to 3, 7 and 10 ($P < 0.05$) but were not significantly different after 48 hours of enrichment ($P > 0.05$). In both products, the number of cells recovered reduced with increase in storage time with chicken skin exhibiting the highest reduction in recovery of $\sim 3 \log_{10}$ CFU/mL over the 10-day storage period for the 24 hour enrichment. After 48 hours of enrichment, the cells recovered remained constant with increase in storage time and the number of cells recovered were not different over the storage time ($P > 0.05$) for either chicken and ground beef inoculated with *C. jejuni* ATCC 33560 and 29428. Although the number of cells of

C. jejuni recovered after 24 hours of enrichment in broth reduced with storage time, cells were still recoverable after 10 days of storage. Thus even though the number of cells recovered after 24 hours were lower than after 48 hours, the process still served the purpose of enrichment to enable detection of the pathogen. Recovery studies have shown that survival of *Campylobacter* during refrigerated storage is dependent on the type of food matrix (Duffy and Dykes 2006). Chicken skin had the highest decline in numbers compared to the ground beef. Chicken skin had a reduction of 3.7 log₁₀ CFU/mL with strain 33560 and 3.5 with strain 29428 after 24 hours of enrichment. In contrast, for ground beef, the reduction was 0.63 and 2.7 log₁₀ CFU/mL for strains 33560 and 29428 respectively (Table 2). Factors intrinsic to the food matrix may have played a role in their survival and protection of the cells and in addition meat may offer better protection than the chicken skin at low temperature. Subcutaneous layers of fat have also been reported to offer insulating protection to other bacteria on freezing (Dykes and Moorehead 2001). These findings in general are in agreement with studies done by Hazeleger and others (1998) who studied the physiological activity of *C. jejuni* at below minimum growth temperature (32 °C-36 °C). They reported that *C. jejuni* was able to perform respiration at 4°C and the electron transport chain was active and was able to produce ATP. This explains why after 10 days both methods still detected *C. jejuni* cells from ground beef and chicken skin samples. Loss of viability over storage time could be explained by injury to some cells to the extent that they could not be recovered in selective enrichment media (Ray and Johnson 1984). Another possibility could be the effect of oxidative damage, since samples were not kept in a microaerophilic atmosphere

under the refrigeration conditions. Although the interactions were significant, both methods were effective in recovering *C. jejuni* cells after 10 days of sample storage.

Effect of detection method and enrichment time on the recovery of *Campylobacter jejuni*

Chicken skin and ground beef

Analysis of data showed that there was significant two-way interaction between the detection method and enrichment time in chicken skin and ground beef ($P < 0.05$) as shown in Table 3. Similar interactions were not found for beef and milk. Averaging over blood supplementation and storage period, comparison of the two methods of recovery showed that there was no difference between the FAPC and FDA methods of recovery after either 24 or 48 hours of enrichment for chicken skin samples (Table 3). Both methods recovered similar counts after enrichment for 24 and 48 hours ($P > 0.05$) from chicken samples inoculated with both strains of *C. jejuni*. In comparison, ground beef inoculated with strains 33560 had significantly different counts using both methods after 24 and 48 hours of enrichment ($P < 0.05$) but counts recovered were similar for ground beef inoculated with strain 29428 ($P > 0.05$) using both the FDA and FAPC methods. Although the two-way interaction between enrichment time and method was found to be significant in two of the products tested, namely chicken and ground beef, inoculated with strains ATCC 33560 and 29428, these results confirm that there is no difference between the two methods of recovery. Both methods recovered cells sufficiently high in numbers to serve the purpose of enrichment in detection of the pathogen. The purpose of enrichment is to maximize recovery of cells that have undergone sub-lethal injury and thus may not be detectable on selective agar or to recover cells that may exist in a food

matrix in very low numbers and may also not be detectable on selective agar (Bolton and others 1984 a and b). In this research, the test cultures were easily detectable after 24 hour enrichment thus this incubation time served the purpose of the enrichment procedure. These results confirm that the two methods for detection of *C. jejuni* are effective in recovering *C. jejuni* from food and are not different ($P>0.05$).

Effect of blood supplementation and storage time on the recovery of *Campylobacter*.

jejuni

Milk

A significant two-way interaction between blood supplementation with storage time was observed in milk inoculated with the two strains of *C. jejuni* (ATCC 33560 and 29428) as shown in Table 4. The milk samples with and without blood supplementation had the same number of cells recovered over the 10-day storage period except for day 0 and 7 in milk inoculated with ATCC 33560 and day 0 for milk inoculated with strain ATCC 29428 as illustrated by Figures 2A and B in the appendix. There was a decline in the number of cells recovered over the 10-day storage period with viability loss by day 10 of storage for both strains, either with or without blood supplementation. These findings confirm that blood supplementation had no effect on the recovery of *C. jejuni* from food. Although these results may suggest that *C. jejuni* does not survive well in milk during refrigerated storage, *Campylobacter* outbreaks have been reported where milk has been the source including an outbreak in a school that affected 2,500 children after consuming free school milk (Jones and others 1981). Results for chicken skin and ground beef

(Table 5) further underscore the lack of effect of blood supplementation. For both chicken and ground beef, blood had no effect.

Effect of detection method, storage period and enrichment time on the recovery of

Campylobacter jejuni

Beef and milk

Statistical analysis of data showed significant three-way interactions between method, storage time and enrichment time in beef and milk (this was not observed for other products). Averaged over blood supplementation, analysis of the interactions in beef inoculated with *C. jejuni* strains 33560 and 29428 showed that with increase in storage period, the number of cells of both strains recovered using both methods reduced after 24 hours of enrichment in broth ($P < 0.05$) but remained similar ($P > 0.05$) throughout the storage period after 48 hours of enrichment (Table 6A). Over the 10-days storage period, the number of cells of both strains recovered reduced with time of storage. Analysis of counts recovered in milk inoculated with *C. jejuni* (ATCC 33560 and 29428) showed reduction in numbers recovered after 24 and 48 hours of enrichment over the 10-day storage period. By day 7, in milk inoculated with strain 29428, there were no detectable cells after 24 hours of enrichment (Table 6B). In contrast, on day 7 some cells were recovered but on day 10 no cells could be detected after 48 hours of enrichment using either method. In general, these findings confirm that the FDA and FAPC methods for recovery of *C. jejuni* are not different. In the experiments performed, storage at 4 °C could have caused some injury and death and this may explain the loss of viability in milk on day 10. In comparison with other food products, fewer counts of both strains of

C. jejuni were recovered after the 7-day storage for milk. This agrees with observations by Duffy and Dykes (2006) that variation in survival was related to differences in the food matrices used. Milk used in this study was skim milk (10% non-fat milk solids reconstituted with water, without potentially protective components (fats and other molecules) which act as a shield for *Campylobacter* such as in beef, ground beef and chicken skin. These results confirm further that the two methods of detection are not different and are effective in recovering *C. jejuni* from various food matrices. These findings also confirm that the degree of survival of *C. jejuni* in food is dependent on the type of food matrix. In all the experiments performed, milk supported survival of *C. jejuni* least.

Growth curve of *Campylobacter jejuni* cultured in Bolton broth at 37 °C and 42 °C.

The FDA and FAPC methods for detection of *C. jejuni* were used in the recovery of *C. jejuni* inoculated in beef, ground beef, chicken skin and pasteurized milk. The FDA method prescribes two enrichment steps; a pre-enrichment step of the culture broth supplemented with horse blood incubated at 37 °C for 4 or 5 hours, followed by incubation at 42 °C. In contrast, the FAPC method involves enrichment in Bolton broth not supplemented with blood with incubation at only 37 °C. In the previous experiments, recovery of *C. jejuni* was done with and without blood supplementation and findings indicated that blood had no effect on the recovery of *C. jejuni* from all the samples, using both methods for recovery (Table 1). In order to confirm that incubation at 42 °C was not needed, *C. jejuni* ATCC 33560 and 29428 were grown over a 48-hour period in enrichment broth without blood supplement both at 37 °C and 42 °C. At two-hour

intervals, 1 mL of enrichment culture with either strain of *C. jejuni* was drawn and plated on pre-poured Karmali agar. The findings suggest that there was no difference between growth of *C. jejuni* in broth incubated at 37 °C and 42 °C as shown in Figures 1A and B. Both strains showed a similar growth pattern with a lag phase of ~8 hours and an exponential phase from 8-hours to a peak at 24 hours. Both strains reached maximum numbers after 24 hours and leveled off to a stationary phase. The over-lapping error bars on both curves for both strains indicate that the difference between the counts recovered at 37 °C and 42 °C is not different. These results are in agreement with results reported by Scates and others (2003). They investigated the effect of temperature on the isolation of *Campylobacter jejuni* genotypes and reported 11% false negatives with incubation at 42 °C and 7% at 37 °C. Although they proposed that both temperatures be used for maximum recovery of *Campylobacter*, they further noted that the two incubation temperatures used separately did not yield significant differences in terms of species detected. Other findings by Humphrey (1986a and b) were in agreement with findings by Ray and Johnson (1984) that damaged *C. jejuni* recovered better in media at 37 °C than 42 °C. Khanna and others (2006) determined the effect of temperature on the growth and chemotactic behavior of *C. jejuni* by determining the growth pattern of *C. jejuni* at 37 °C and 42 °C and reported that its growth rate was greater at 37 °C than at 42 °C. They further reported that at 37 °C chemotaxis was more pronounced than at 42 °C. These results suggest that *C. jejuni* has a better ability to express virulence determinants at 37 °C than at 42 °C. The human body temperature is 37 °C and more likely *C. jejuni*'s growth as a human pathogen would be favored at that temperature. The FDA method requires pre-enrichment to be done at 37 °C and enrichment at 42°C, while the FAPC

method uses only 37 °C incubation temperature for the entire enrichment process.

Results from this study confirm that it may not be necessary to incubate *Campylobacter* at 42 °C since it can be cultured to high numbers at 37 °C.

Previous research by Reilly and Gilliland (2003) showed that enrichment for 48 hours increased the number of coccoid forms compared to the numbers at 24 hours and suggested thus, that culturing of *C. jejuni* for 24 hours at 37 °C was more favorable than 48 hours at 42 °C.

Detection threshold of *Campylobacter jejuni*

The highest dilution (10^{-9}) of ATCC 29428 had a population of 0.9 log₁₀ CFU /mL before enrichment in Bolton broth but after 24 hours of enrichment, 4.6 Log₁₀ CFU/mL were recovered using the FDA method and 4.1 log₁₀ CFU/mL counts using the FAPC method (Table 7). The highest dilution (10^{-9}) of strain 33560 had no detectable cells (below the detection threshold) before enrichment in Bolton broth but after 24 hours of enrichment, 6.3 log₁₀ CFU/mL were recovered using the FDA method and 5.4 log₁₀ CFU /mL using the FAPC method. Initial counts of *C. jejuni* ATCC 33560 were 2, 1.1 and <1.0 log₁₀ CFU /mL for dilutions 10^{-7} , 10^{-8} and 10^{-9} respectively but after 24 hours of enrichment, 8.1, 7.2 and 6.3 log₁₀ CFU /mL counts were recovered using the FDA method and 7.6, 6.3 and 5.4 log₁₀ CFU /mL were recovered using the FAPC method respectively. *C. jejuni* ATCC 29428 showed the same trend. These results show that at very low levels, *C. jejuni* was not detectable by direct plating onto Karmali agar, which is a selective medium. However after 24-hour enrichment, cells were recovered on Karmali agar. These findings correlate with reports by Cools and others (2003) who found that

during storage, numbers of *C. jejuni* detected on Karmali agar decreased below detection levels. However, they remained detectable on Columbia blood agar which is a non-selective media. Ray and Johnson (1984) further noted that injured cells develop sensitivity to a mixture of antibiotics in liquid or solid media. Further, injured *E. coli* as well as other Gram negative bacteria have not shown sensitivity to non-selective media (Ray and Speck, 1973) and this could explain why *C. jejuni* cells could not be detected on Karmali agar which a selective media.

Tables 8 and 9 show the initial counts of strains ATCC 33560 and 29428 of *C. jejuni* in ground beef without storage on day 0 determined by direct plating of the sample, and counts recovered from enrichment culture (24 and 48-hour enrichment periods) made from the samples after 0, 3, 7 and 10 days of storage at 4 °C. The highest dilution (10^{-8}) of ATCC 29428 had initial counts of $1.3 \log_{10}$ CFU/mL but after 24 and 48-hour enrichment, 4.8 and 8.8 \log_{10} CFU/mL were recovered using the FDA method and 4.3 and 8.9 \log_{10} CFU/mL were recovered using the FAPC method respectively for day 0 (Table 9). Following 3 days of storage, both strains of *C. jejuni* were detected equally well by both methods from the samples even for the lowest level of inoculum ($1.3 \log_{10}$ CFU/mL). Recovery was not equal for both strains following 7 days of storage and some were not recovered after 10 days. Although both strains of *C. jejuni* were not recoverable on day 10 of storage, both methods were effective in recovering very low numbers of cells in the ground beef matrix. Failure to recover any at 10 days may have been due to death of the organism during storage or to metabolic injury. These results in general demonstrate the ability of both the FDA and FAPC methods to recover low numbers of

C. jejuni cells from the food matrix and thus have direct implications in the detection of *Campylobacter jejuni* from food.

Summary of findings

The purpose of a pre-enrichment step is to resuscitate cells that have been injured in order to increase the recovery of cells (Humphrey 1989; Bolton 2000). It also enhances the recovery of numbers that may be below direct plating detection methods. The lower incubation temperature (37 °C) used for pre-enrichment in the FDA method is supposed to aid repair and recovery of injured cells while the higher incubation temperature for enrichment (42 °C) allows thermotolerant *Campylobacter* to grow, while suppressing the growth of unwanted background microorganisms (Fernandez and Pison 1996). This study has shown that enrichment done entirely at 37 °C has no effect on the mean counts recovered and thus the two step enrichment in the FDA method can be omitted in order to save time and resources. Although incubation at 42 °C serves to inhibit the growth of non-thermotolerant *Campylobacter*, findings in this research show that with enrichment at 37 °C, *C. jejuni* was detectable from the food matrices tested. Although enrichment after 48 hours recovered higher numbers than 24 hours, the 24-hour enrichment counts are adequate for further tests for detection of *C. jejuni* and therefore 48-hour enrichment appears not be necessary although it is prescribed in the FDA protocol. Although the two methods of detection (FDA and FAPC) used in this study have different enrichment times, incubation times and enrichment regimens, this study has shown that there is no difference between the two methods ($P > 0.05$).

Blood supplementation has been used over the years for culturing *Campylobacter* and its role has been to promote the growth of *Campylobacter* during its isolation. Findings in this study indicate that it is not necessary to use blood in enrichment broth as required by the FDA protocol. Blood did not play any role in the recovery of *Campylobacter* in all the products tested, using both the FDA and FAPC methods.

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Table 1-Influence of supplementation of enrichment broth with horse blood on recovery of *Campylobacter jejuni* from foods.

Strain	Carrier Food ¹	FDA Method ²				FAPC Method ²			
		Horse blood		No Horse blood		Horse blood		No Horse blood	
		24 Hrs ³	48 Hrs ³	24 Hrs	48 Hrs	24 Hrs	48 Hrs	24 Hrs	48 Hrs
ATCC 33560	Beef	8.9 ⁴	9.0	8.8	9.1	9.7	8.7	9.6	8.6
	G. Beef	9.2	9.3	9.2	9.2	9.8	8.5	9.7	8.7
	Milk	7.9	9.0	8.4	9.0	9.8	9.1	7.6	9.0
	Chick. Skin	8.1	8.9	8.3	9.0	7.1	9.3	7.5	9.0
ATCC 29428	Beef	9.1	8.7	8.8	8.9	8.9	8.4	9.3	8.5
	G. Beef	8.7	8.7	8.8	8.9	8.8	8.3	9.4	8.6
	Milk	6.7	8.3	7.4	8.4	6.0	8.6	6.8	8.6
	Chick. Skin	8.6	8.6	8.5	8.5	7.0	8.9	7.1	8.6

¹Carrier foods inoculated with 4.0×10^3 to 5.0×10^3 CFU/g or mL prior to enrichment process

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003)

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth

⁴Each value is the mean \log_{10} CFU /g or mL from 3 replicate trials

Table 2-Interaction of time of enrichment with storage period in chicken and ground beef inoculated with *Campylobacter jejuni*.

Strain	Product	Enrichment Time (Hrs) ³	Days of storage period at 4 °C			
			0	3	7	10
33560	Chicken skin	24 ³	¹ 7.77 ^a	6.33 ^b	5.49 ^c	4.08 ^d
		48 ³	9.03 ^e	9.01 ^e	8.91 ^e	8.95 ^e
			SEM ² = p-value ² = df ² =	0.135 0.0001 3		
29428	Chicken skin	24	¹ 7.83 ^a	6.23 ^b	4.89 ^c	4.31 ^c
		48	8.65 ^d	8.63 ^d	8.61 ^d	8.60 ^d
			SEM = p-value = df =	0.239 0.0001 3		
33560	Ground beef	24	¹ 9.45 ^a	9.31 ^{ab}	8.79 ^c	8.82 ^c
		48	8.92 ^{cd}	9.02 ^{bd}	9.14 ^{bd}	9.20 ^{abd}
			SEM = p-value = df =	0.106 0.0001 3		
29428	Ground beef	24	¹ 8.94 ^a	8.84 ^a	7.32 ^b	6.28 ^c
		48	8.63 ^{ad}	8.42 ^d	8.45 ^d	8.63 ^{ad}
			SEM = p-value = df =	0.142 0.0001 3		

¹Values are mean log₁₀ CFU/g from three replications.

²P-values, Standard Error of the Means (SEM) and associated degrees of freedom (df) for storage period with time.

³Hours of incubation in enrichment broth.

^{abcde} Means with different superscripts within rows and columns are different (P < 0.05).

Table 3-Interaction of detection method with enrichment time in chicken skin and ground beef inoculated with *Campylobacter jejuni*.

Strain	Product	Enrichment Time (Hrs) ³	Method	
			FDA	FAPC
33560	Chicken skin	24 ³	¹ 6.14 ^a	5.69 ^a
		48 ³	8.83 ^b	9.07 ^b
			SEM ² = 0.096 p-value ² = 0.0014 df ² = 1	
29428	Chicken skin	24	¹ 6.16 ^a	5.47 ^a
		48	8.57 ^b	8.68 ^b
			SEM = 0.169 p-value = 0.0213 df = 1	
33560	Ground beef	24	¹ 8.92 ^a	9.26 ^b
		48	9.22 ^b	8.92 ^a
			SEM = 0.075 p-value = 0.0001 df = 1	
29428	Ground beef	24	¹ 7.79 ^a	7.90 ^a
		48	8.72 ^b	8.34 ^b
			SEM = 0.100 p-value = 0.0174 df = 1	

¹Values are mean log₁₀ CFU/g from three replications.

²P-values, Standard Error of the Means (SEM) and associated degrees of freedom (df) for detection method with time.

³Hours of incubation in enrichment broth.

^{abc} Means with different superscripts within rows and columns are different (P < 0.05).

Table 4-Interaction of blood supplementation with storage period in milk inoculated with *Campylobacter jejuni*.

Strain	Blood	Storage period			
		0	3	7	10
33560	With ³	¹ 8.46 ^a	7.30 ^b	3.32 ^c	1.00 ^e
	Without ³	8.46 ^a	7.33 ^b	3.86 ^d	1.00 ^e
		SEM =	0.103		
		p-value =	0.024		
		df =	3		
29428	With ³	¹ 7.34 ^a	6.64 ^c	2.28 ^d	1.00 ^e
	Without ³	7.81 ^b	6.58 ^c	2.24 ^d	1.00 ^e
		SEM ² =	0.095		
		p-value ² =	0.032		
		df ² =	3		

¹Values are mean log₁₀ CFU/mL from three replications.

²P-values, Standard Error of the Means (SEM) and associated degrees of freedom (df) for storage period with blood supplementation.

³Supplementation of blood in enrichment broth.

^{abcde} Means with different superscripts within columns and rows are different (P < 0.05).

Table 5-Effect of blood supplementation on the recovery of *Campylobacter jejuni* from ground beef and chicken skin.

Blood Supplementation.	Product	
	Chicken skin	Ground beef
With	¹ 7.39 ^a	¹ 8.04 ^a
Without	¹ 7.04 ^a	¹ 8.34 ^a
	SEM ² = 0.119 p-value ² = 0.042 df ² = 1	SEM = 0.071 p-value = 0.0038 df = 1

¹Values are mean Log₁₀ CFU/g of three replications.

^aMeans with different superscripts within columns are different (P< 0.05).

²P-values, Standard Error of the Means (SEM) and associated degrees of freedom (df) for main effect blood.

Table 6A-Interaction of detection method with storage period and enrichment time in beef inoculated with *Campylobacter jejuni*.

Strain	Days of storage	Enrichment time			
		24 Hrs ³		48 Hrs ³	
		FDA	FAPC	FDA	FAPC
29428	0	¹ 8.97 ^{ab}	9.08 ^a	8.79 ^{ab}	8.45 ^{abc}
	3	8.68 ^{ab}	8.96 ^{ab}	8.73 ^{ab}	7.78 ^{cd}
	7	7.75 ^{cd}	6.99 ^e	8.32 ^{abc}	8.68 ^{ab}
	10	6.59 ^e	7.02 ^{de}	8.85 ^{ab}	8.29 ^{bc}
		SEM ² =	0.2727		
		p-value ² =	0.0016		
		df ² =	3		
33560	0	¹ 8.83 ^{cd}	9.65 ^a	9.06 ^c	8.66 ^{cde}
	3	9.02 ^c	9.60 ^{ab}	9.25 ^{abc}	8.64 ^{cde}
	7	8.99 ^c	8.86 ^{cd}	9.05 ^c	9.10 ^{bc}
	10	8.14 ^e	8.43 ^{de}	8.97 ^c	9.01 ^c
		SEM =	0.188		
		p-value =	0.022		
		df =	3		

¹Values are mean log₁₀ CFU/g from three replications.

²P-values, Standard Error of the Means (SEM) and associated degrees of freedom (df) for storage period with enrichment time and detection method.

³Hours of incubation in enrichment broth.

^{abcde} Means with different superscripts within rows and columns are different (P < 0.05).

Table 6B-Interaction of detection method with storage period and enrichment time in milk inoculated with *Campylobacter jejuni*.

Strain	Days of storage	Enrichment time			
		24 Hrs ³		48 Hrs ³	
		FDA	FAPC	FDA	FAPC
29428	0	¹ 7.07 ^b	6.35 ^c	8.33 ^a	8.60 ^a
	3	4.45 ^d	4.75 ^d	8.60 ^a	8.65 ^a
	7	1.00 ^f	1.00 ^f	3.30 ^e	3.73 ^e
	10	1.00 ^f	1.00 ^f	1.00 ^f	1.00 ^f
		SEM ² =	0.2727		
		p-value ² =	0.0016		
		df ² =	3		
33560	0	¹ 8.10 ^b	7.68 ^c	9.00 ^a	9.05 ^a
	3	5.38 ^d	5.58 ^d	9.07 ^a	9.22 ^a
	7	2.90 ^f	1.95 ^g	4.65 ^e	4.85 ^e
	10	1.00 ^h	1.00 ^h	1.00 ^h	1.00 ^h
		SEM =	0.134		
		p-value =	0.011		
		df =	3		

¹Values are mean log₁₀ CFU/mL from three replications.

²P-values, Standard Error of the Means (SEM) and associated degrees of freedom (df) for storage period with enrichment time and detection method.

³Hours of incubation in enrichment broth.

^{abcd^{efgh}} Means with different superscripts within rows and columns are different (P< 0.05).

Table 7-Detection threshold of *Campylobacter jejuni* ATCC 33560 and 29428 in ground beef using the FDA and FAPC methods.

ATCC Strain	Dilution	Initial counts	FDA Method ²		FAPC Method ²	
			24Hours ³	48 Hours ³	24 Hours	48 Hours
29428	10 ⁻⁷	2.6 ¹	6.5 ⁴	9.1	5.7	9.1
29428	10 ⁻⁸	1.7	5.1	8.6	5.1	9.2
29428	10 ⁻⁹	0.9	4.6	8.8	4.1	9.3
Un-inoculated Control			< 1.0	< 1.0	< 1.0	< 1.0
33560	10 ⁻⁷	2.0	8.1	8.8	7.6	9.5
33560	10 ⁻⁸	1.1	7.2	9.1	6.3	9.6
33560	10 ⁻⁹	< 1.0	6.3	9.5	5.4	9.6
Un-inoculated Control			< 1.0	< 1.0	< 1.0	< 1.0

¹Numbers of *Campylobacter jejuni* inoculated into ground beef (reported as log₁₀ CFU/g) prior to enrichment process

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003)

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth

⁴Each value is the mean log₁₀ CFU/g from 3 replicate trials

Table 8-Detection of *Campylobacter jejuni* ATCC 33560 inoculated in low numbers into ground beef during refrigerated storage.

Day at 4°C	Dilution	Initial counts	Without Blood supplement			
			FDA ² 24 hours ³	FAPC ² 24 hours	FDA 48 hours ³	FAPC 48 hours
0	10 ⁻⁶	2.9 ¹	7.3	6.8	9.3	9.3
	10 ⁻⁷	2.1	6.4	5.5	9.0	9.1
	10 ⁻⁸	1.7	4.0	< 1.0	9.1	< 1.0
3	10 ⁻⁶		8.2	7.0	9.2	9.3
	10 ⁻⁷		7.5	5.9	9.1	9.2
	10 ⁻⁸		7.7	5.6	9.2	9.0
7	10 ⁻⁶		7.8	6.4	9.0	8.9
	10 ⁻⁷		6.3	3.8	8.4	6.9
	10 ⁻⁸		3.6	< 1.0	6.8	< 1.0
10	10 ⁻⁶		< 1.0	< 1.0	< 1.0	< 1.0
	10 ⁻⁷		< 1.0	< 1.0	< 1.0	< 1.0
	10 ⁻⁸		< 1.0	< 1.0	< 1.0	< 1.0

¹Numbers of *Campylobacter jejuni* inoculated into ground beef (reported as log₁₀ CFU/g) prior to enrichment process.

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003).

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth.

⁴Each value is the mean log₁₀ CFU/g from 3 replicate trials.

Table 9-Detection of *Campylobacter jejuni* ATCC 29428 inoculated into ground beef in low numbers during refrigerated storage.

Day at 4°C	Dilution	Initial counts	Without Blood supplement			
			FDA ²	FAPC ²	FDA	FAPC
			24 hours ³	24 hours	48 hours ³	48 hours
0 ^a	10 ⁻⁶	3.2 ¹	5.7	6.2	9.1	8.7
	10 ⁻⁷	2.2	4.6	5.2	8.9	8.9
	10 ⁻⁸	1.3	4.8	4.3	8.8	8.9
3	10 ⁻⁶		6.5	6.3	8.8	9.0
	10 ⁻⁷		5.5	5.4	8.8	8.9
	10 ⁻⁸		4.6	4.2	8.8	8.3
7	10 ⁻⁶		3.8	< 1.0	8.8	6.3
	10 ⁻⁷		< 1.0	< 1.0	< 1.0	< 1.0
	10 ⁻⁸		< 1.0	< 1.0	< 1.0	< 1.0
10	10 ⁻⁶		< 1.0	< 1.0	< 1.0	< 1.0
	10 ⁻⁷		< 1.0	< 1.0	< 1.0	< 1.0
	10 ⁻⁸		< 1.0	< 1.0	< 1.0	< 1.0

¹Numbers of *Campylobacter jejuni* inoculated into ground beef (reported as log₁₀ CFU/g) prior to enrichment process

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003)

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth

⁴Each value is the mean log₁₀ CFU/g from 3 replicate trials

Figure 1A-Growth curve of *Campylobacter jejuni* ATCC 33560 cultured in Bolton broth at 37 °C (◆) and 42 °C (■). Each value is the mean log₁₀ CFU/ mL from 3 replications.

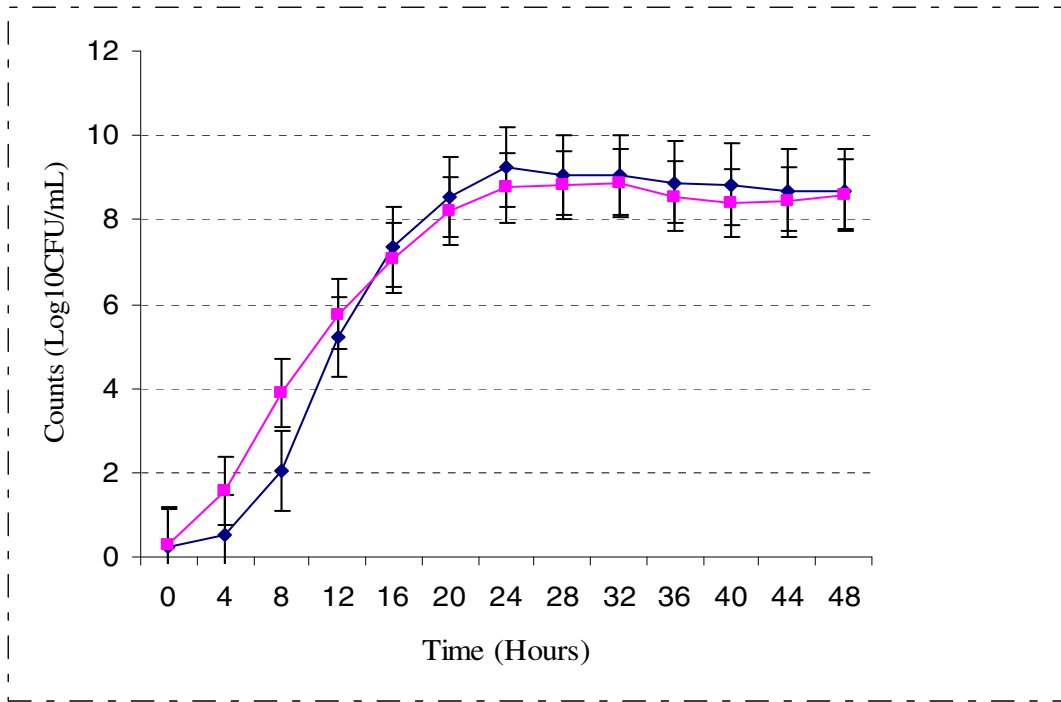
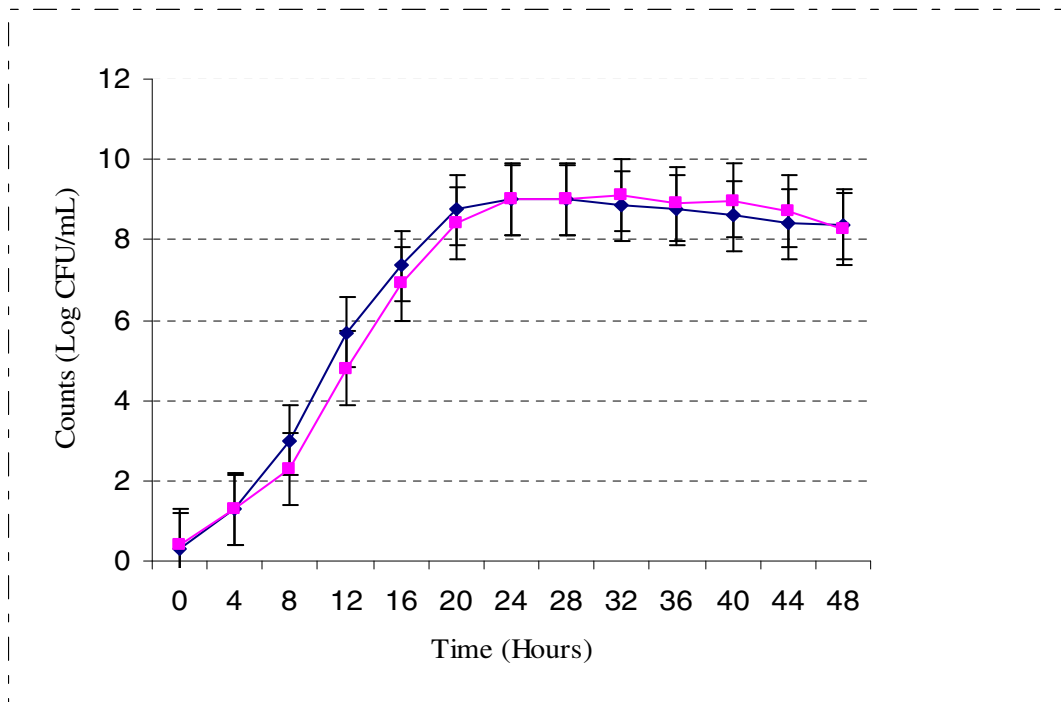


Figure 1B-Growth curves of *Campylobacter jejuni* ATCC 29428 cultured in Bolton broth at 37 °C (◆) and 42 °C (■). Each value is the mean log₁₀ CFU/mL from 3 replications.



Appendix

Table 10 A.-Fermentation pattern of *Campylobacter jejuni* ATCC 33560 and 29428 isolates from beef (B) and ground beef (G).

Blood	Method ¹	Sample	URE ⁴	NIT	EST	HIP	GGT	TTC	PyrA	ArgA	AspA	PAL	H ₂ S	GLU	SUT	ACE	PROP		
Without ²	FDA	B-33560 ³	-	+	+	+	+	+	V	-	-	+	-	-	+	-	-		
		B-29428	-	+	+	+	+	+	V	-	-	+	-	-	+	-	-		
		G-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		G-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
	FAPC	B-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		B-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		G-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		G-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
	With ²	FDA	B-33560	-	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
			B-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
			G-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
			G-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
FAPC		B-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		B-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		G-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		G-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-	

¹Method of detection used: FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003)

²Enrichment broth supplemented with or without blood

³Strain of *Campylobacter jejuni* isolated from either beef (B) or ground beef (G).

⁴Sugars and other compounds fermented by isolates of *C. jejuni* from beef and ground beef.

Table 10 B.-Fermentation pattern of *Campylobacter jejuni* ATCC 33560 and 29428 isolates from chicken skin (C) and pasteurized milk (M).

Blood	Method ¹	Sample	URE ⁴	NIT	EST	HIP	GGT	TTC	PyrA	ArgA	AspA	PAL	H ₂ S	GLU	SUT	ACE	PROP	
Without ²	FDA	C-33560 ³	-	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		C-29428	-	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		M-33560	-	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		M-29428	-	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
	FAPC	C-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
		C-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
		M-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
		M-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
With ²	FDA	C-33560	-	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		C-29428	-	+	+	+	+	+	V	-	-	+	-	-	+	-	-	
		M-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
		M-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
	FAPC	C-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
		C-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
		M-33560	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-
		M-29428	-	+	+	+	+	+	+	V	-	-	+	-	-	+	-	-

¹Method of detection used: FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003)

²Enrichment broth supplemented with or without blood

³Strain of *Campylobacter jejuni* isolated from either chicken skin (C) or pasteurized milk (M)

⁴Sugars and other compounds fermented by isolates of *C. jejuni* from chicken skin and pasteurized milk.

Figure 2A-Interaction of blood (Bld) supplementation with storage time in milk inoculated with *C. jejuni* ATCC 29428

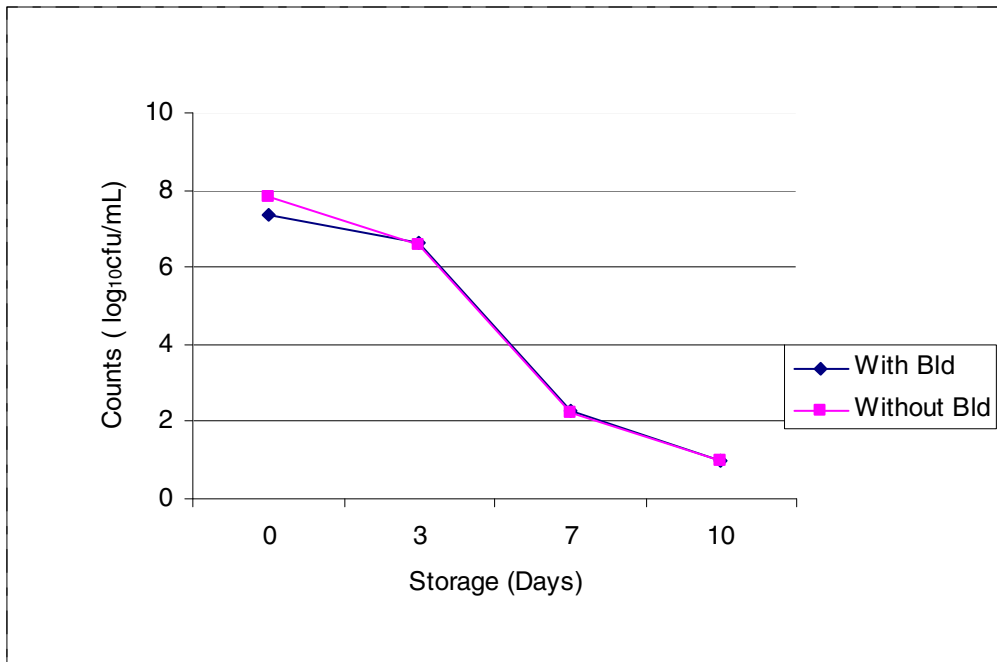


Figure 2B-Interaction of blood (Bld) supplementation with storage period in milk inoculated with *Campylobacter jejuni* ATCC 33560.

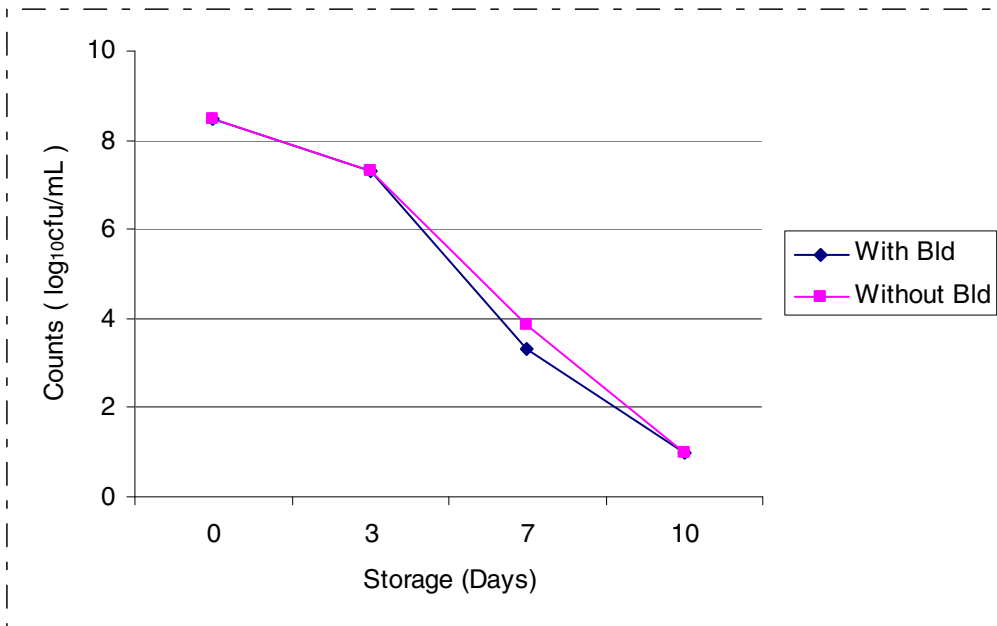


Figure 3-Interaction of method with storage period in chicken skin inoculated with *Campylobacter jejuni* ATCC 33560.

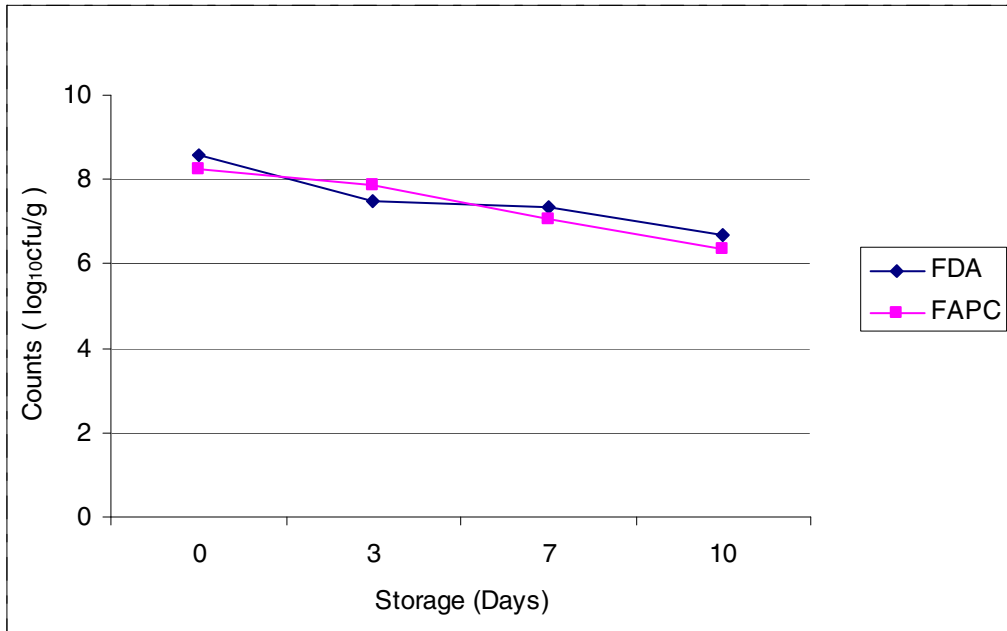


Figure 4A-Interaction of time with storage period in chicken skin inoculated with *Campylobacter jejuni* ATCC 29428.

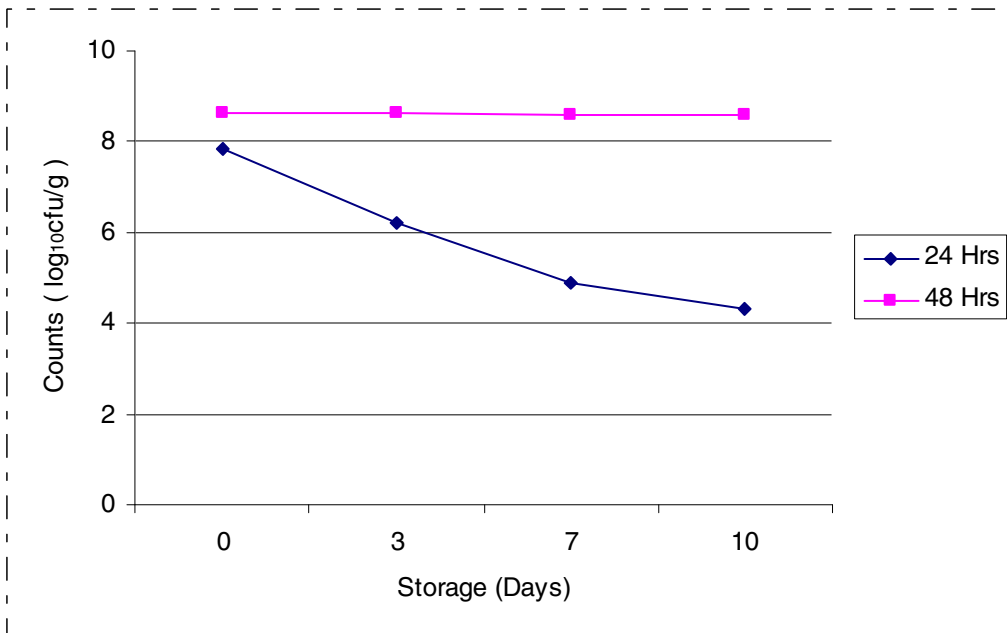


Figure 4B-Interaction of time with storage period in chicken skin inoculated with *Campylobacter jejuni* ATCC 33560.

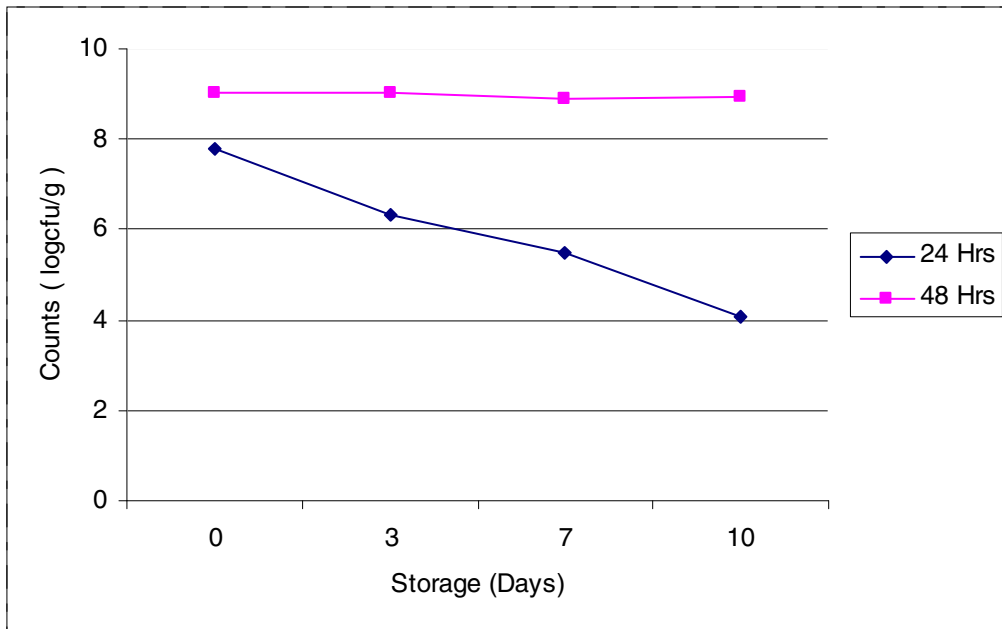


Figure 5A-Interaction of time and storage period in ground beef inoculated with *Campylobacter jejuni* ATCC 29428.

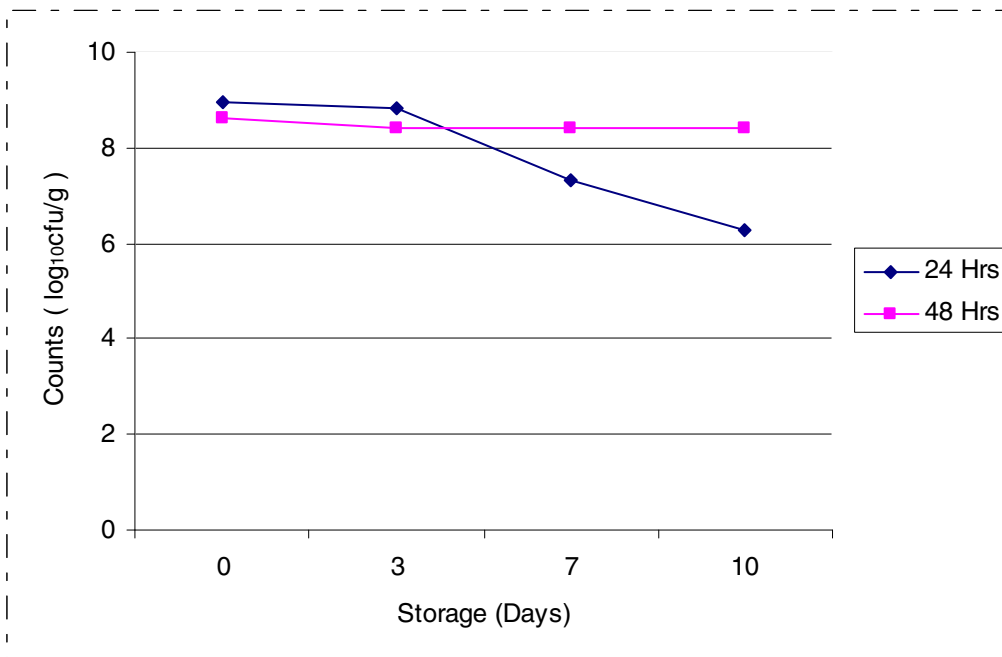


Figure 5B-Interaction of time with storage period in ground beef inoculated with *Campylobacter jejuni* ATCC 33560.

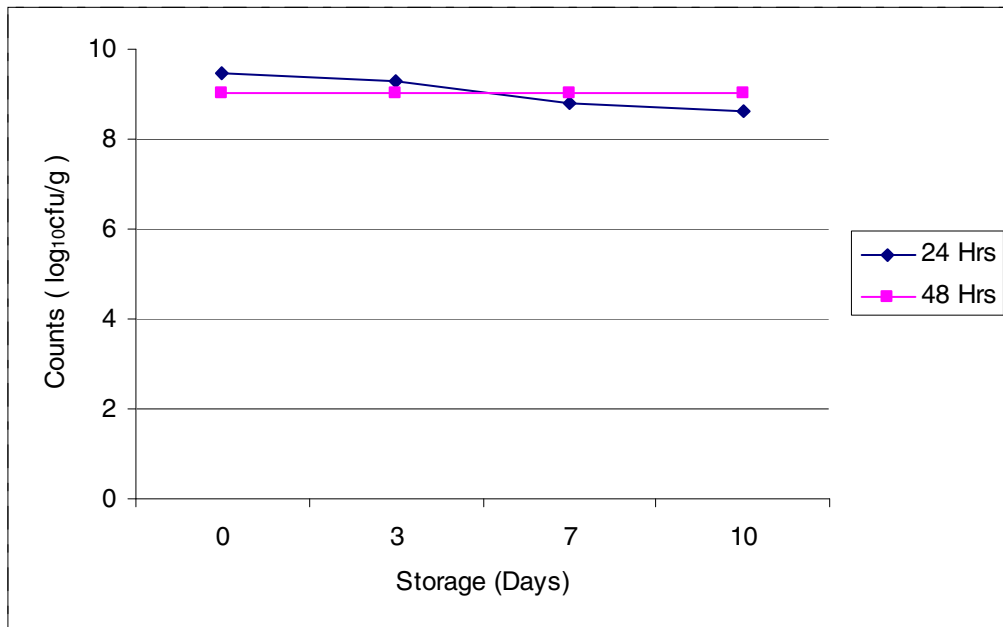


Figure 6A-Interaction of storage period with enrichment time and method of detection in beef inoculated with *Campylobacter jejuni* ATCC 29429 after 24 hours of enrichment.

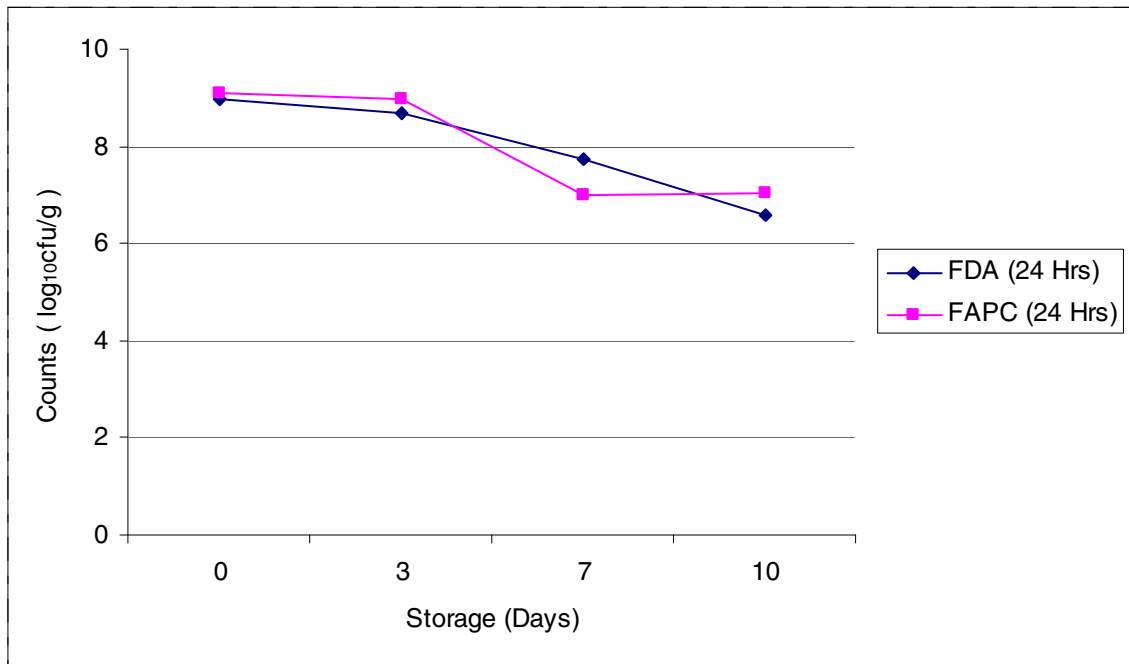


Figure 6B-Interaction of storage period with enrichment time and method of detection in beef inoculated with *Campylobacter jejuni* ATCC 29428 after 48 hours of enrichment.

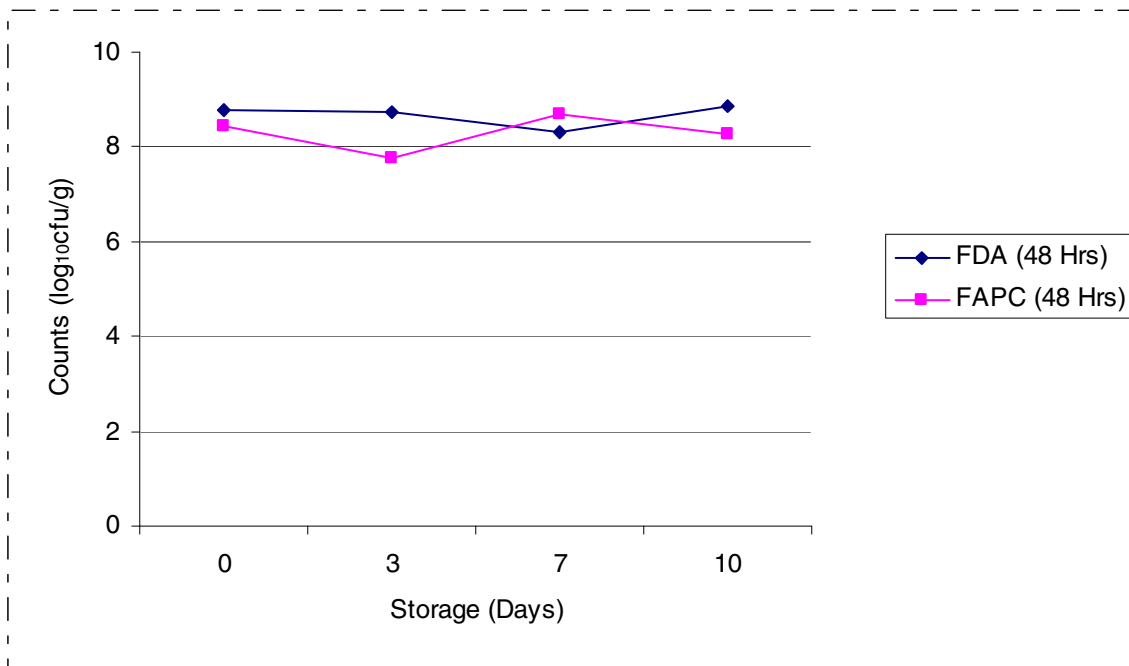


Figure 6C-Interaction of storage period with enrichment time and method of detection in beef inoculated with *Campylobacter jejuni* ATCC 33560 after 24 hours of enrichment.

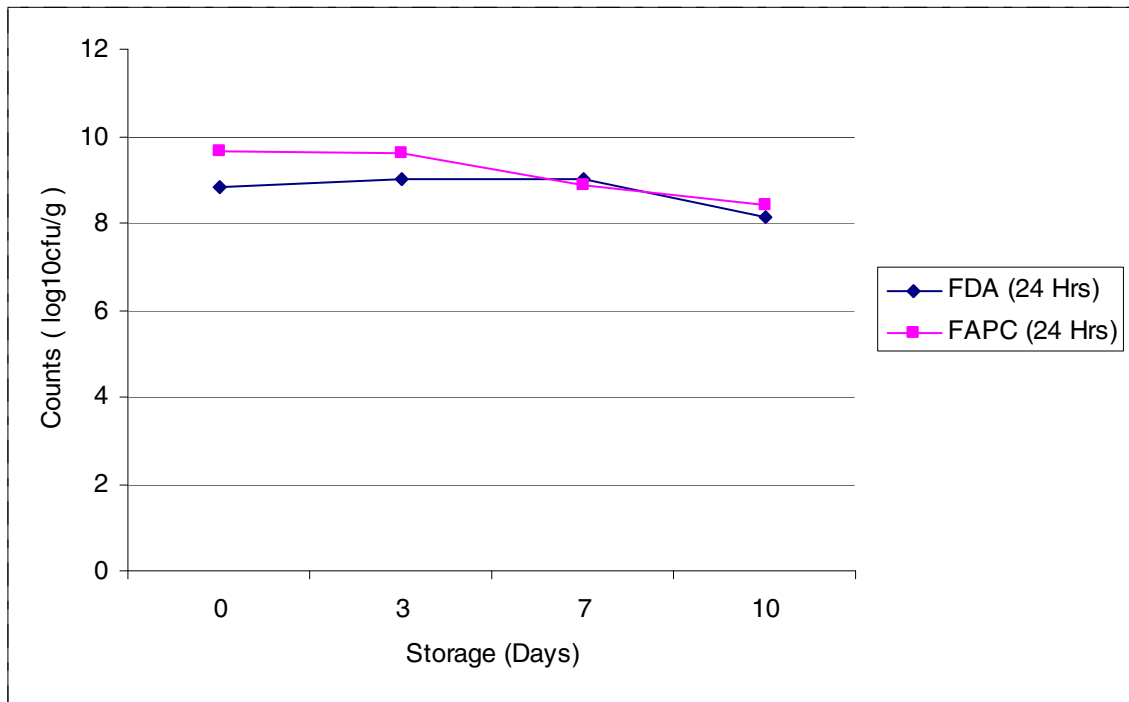


Figure 6D-Interaction of storage period with enrichment time and method of detection in beef inoculated with *Campylobacter jejuni* ATCC 33560 after 48 hours of enrichment.

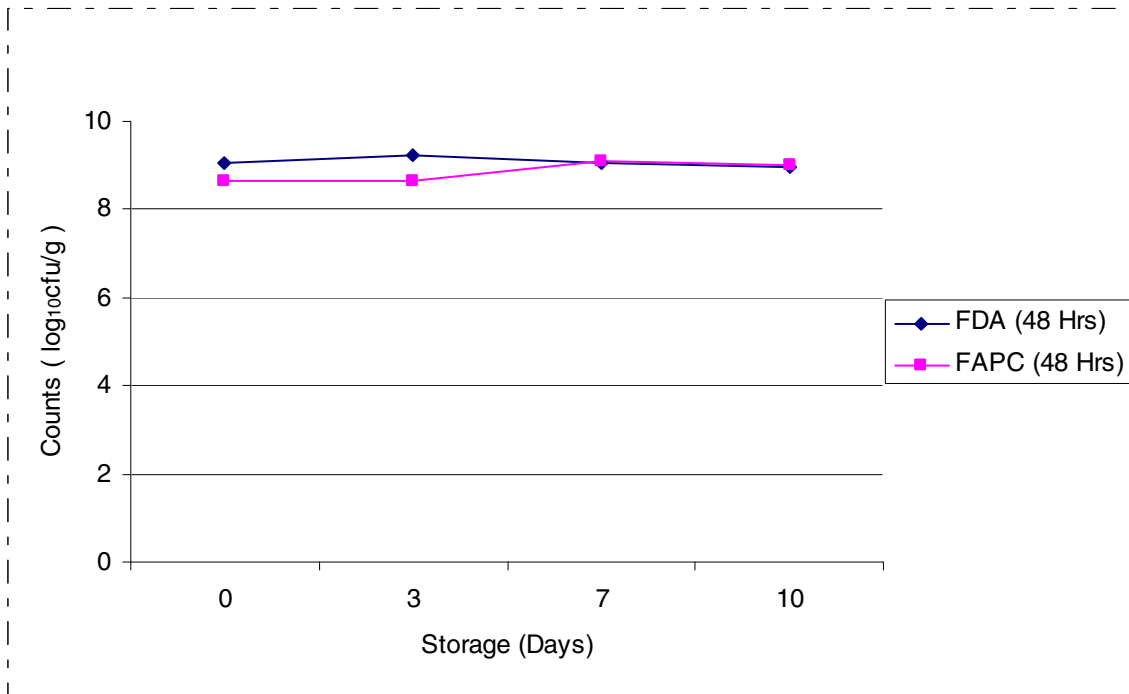


Figure 7A-Interaction of storage period with enrichment time and method of detection in milk inoculated with *Campylobacter jejuni* ATCC 29428 after 24 hours of enrichment

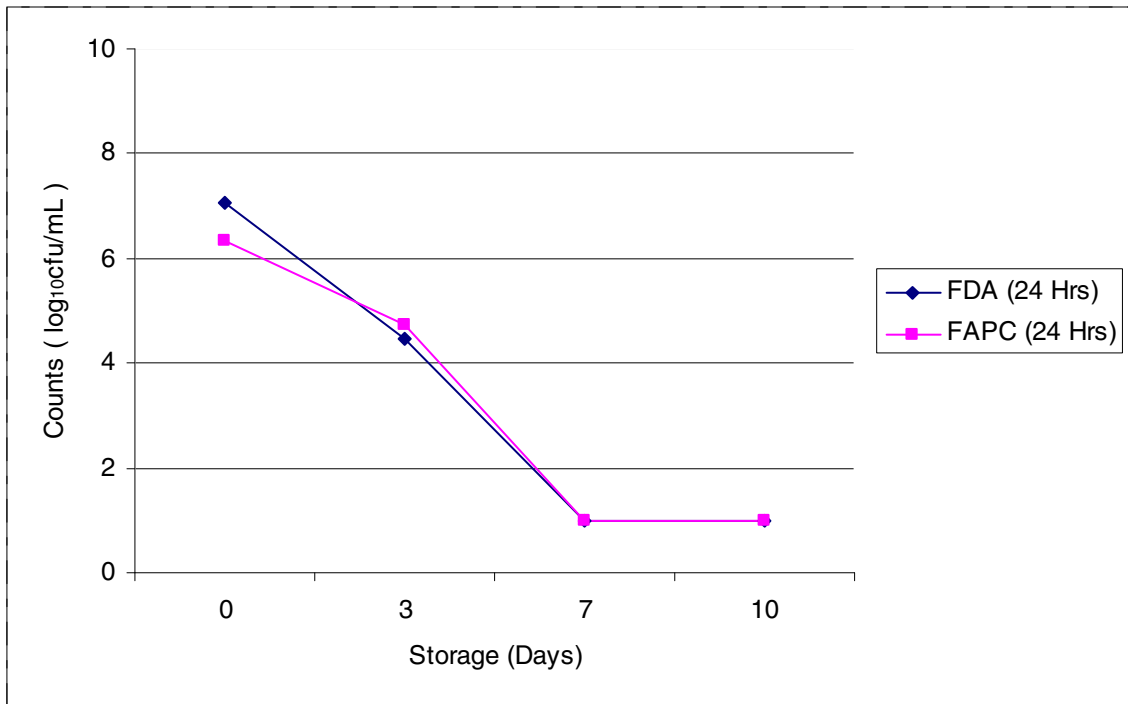


Figure 7B-Interaction of storage period with enrichment time and method of detection in milk inoculated with *Campylobacter jejuni* ATCC 29428 after 48 hours of enrichment

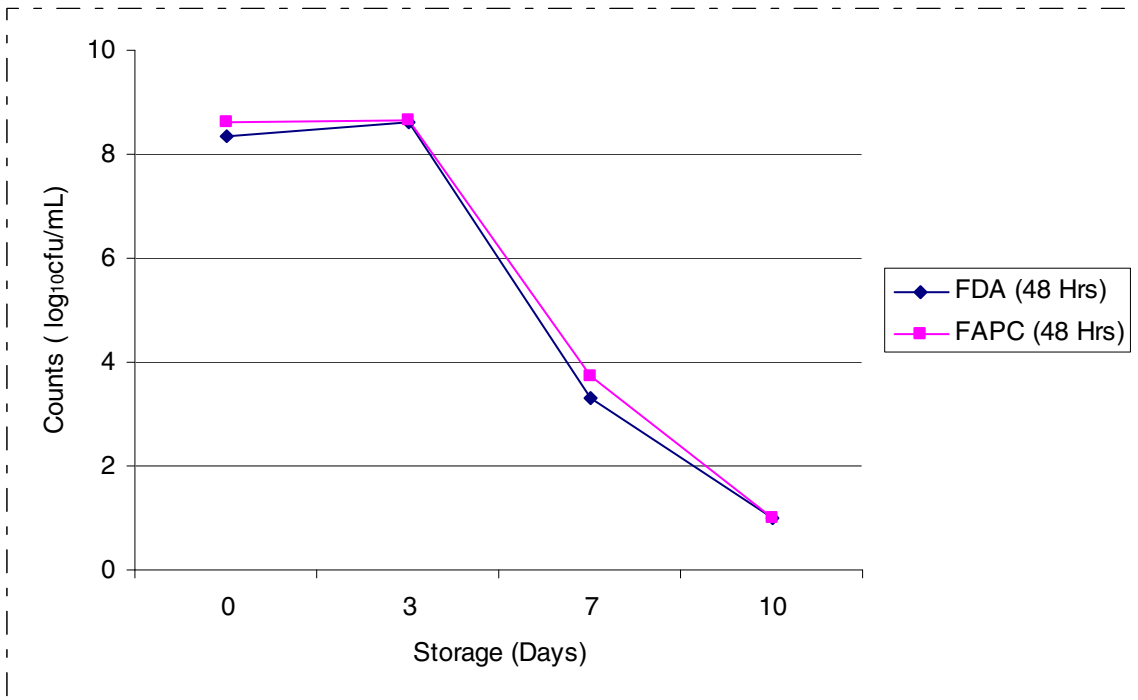


Figure 7C-Interaction of storage period with enrichment time and method of detection in milk inoculated with *Campylobacter jejuni* ATCC 33560 after 24 hours of enrichment

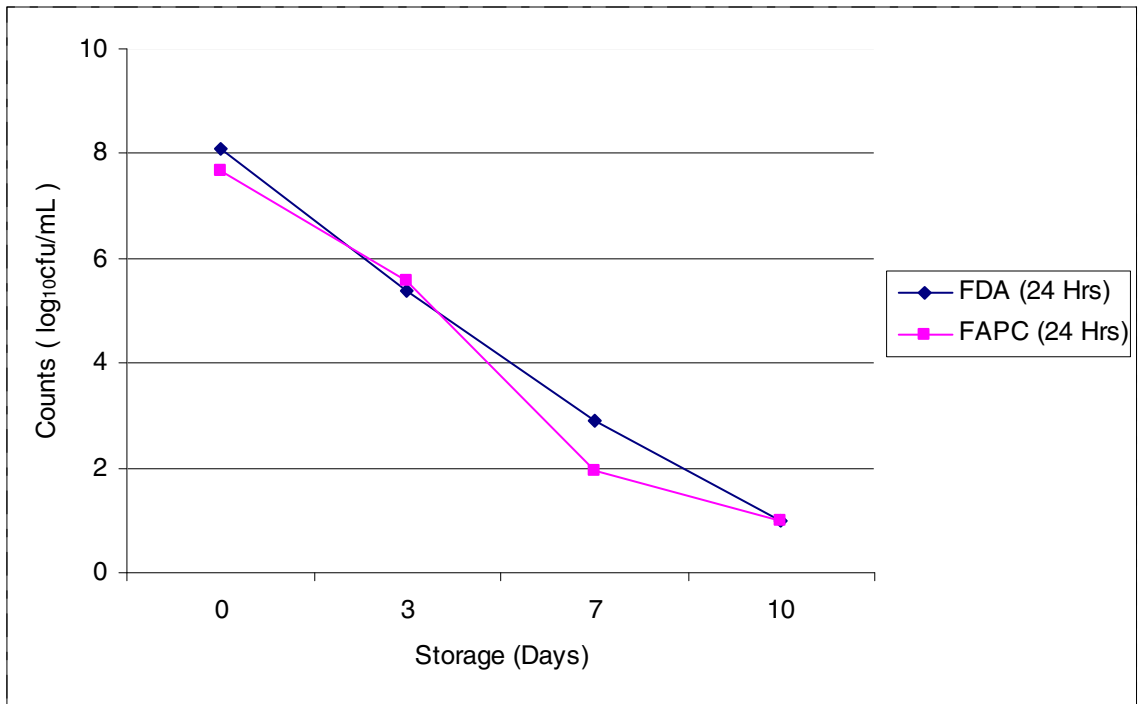


Figure 7D-Interaction of storage period with enrichment time and method of detection in milk inoculated with *Campylobacter jejuni* ATCC 33560 after 48 hours of enrichment

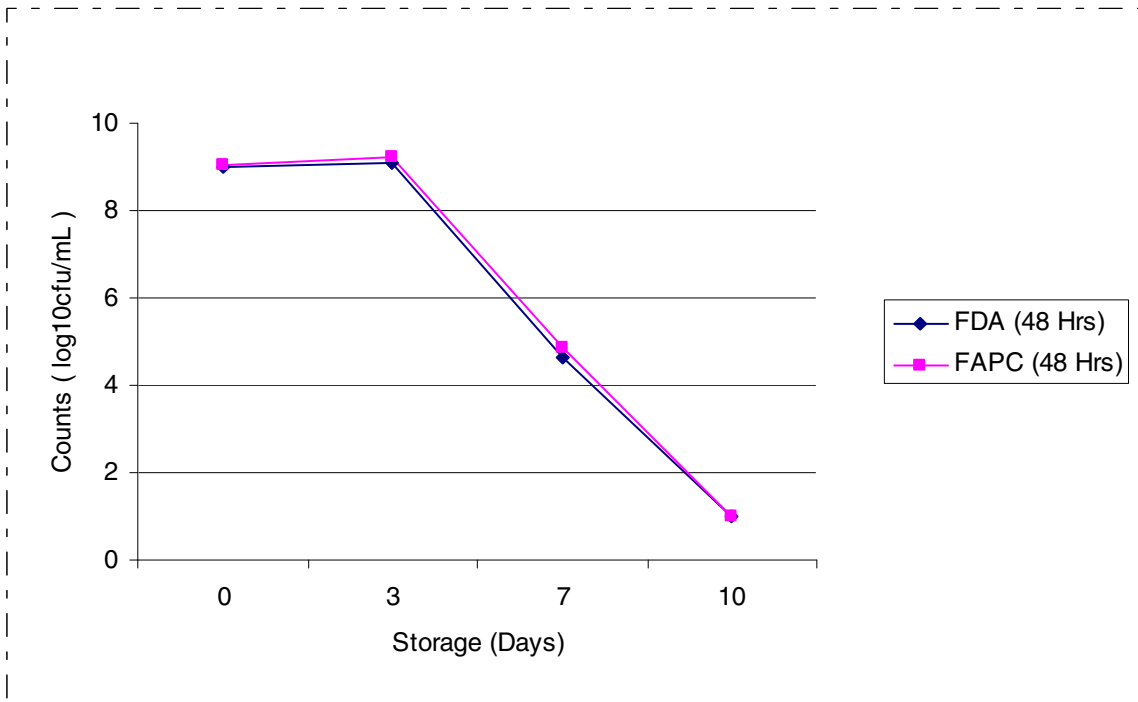


Table 11-Influence of blood supplementation of enrichment broth, enrichment time and detection method on the recovery of *Campylobacter. jejuni* ATCC 33560 from beef.

Method	Storage period (days)	Blood Supplement				
		Initial Counts ¹	Without	With	Without	With
			24 hours ³	24 hours	48 hours ³	48 hours
FDA ²	0	3.6	8.8 ⁴	8.9	9.1	9.0
	3		8.9	9.2	9.2	9.3
	7		9.1	8.9	9.1	9.0
	10		8.2	8.9	9.0	9.0
FAPC ²	0	3.6	9.6	9.7	8.6	8.7
	3		9.6	9.6	8.7	8.6
	7		8.9	8.8	8.9	9.2
	10		8.2	8.7	9.0	9.0

¹Beef was inoculated with 4.0×10^3 to 5.0×10^3 CFU/g prior to enrichment process.

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003).

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth.

⁴Each value is the mean \log_{10} CFU/g from 3 replicate trials.

Table 12. Influence of blood supplementation of enrichment broth, enrichment time and detection method on the recovery of *Campylobacter jejuni* ATCC 29428 from beef.

Method	Storage period (days)	Initial Counts	Blood Supplement			
			Without	With	Without	With
			24 hours ³	24 hours	48 hours ³	48 hours
FDA ²	0	3.7 ¹	8.8 ⁴	9.1	8.9	8.7
	3		8.9	8.4	8.7	8.8
	7		7.7	7.8	8.4	8.2
	10		7.2	6.0	8.8	8.7
Improved ²	0	3.7	9.3	8.9	8.5	8.4
	3		9.0	8.9	7.8	7.8
	7		7.5	6.5	8.6	8.7
	10		7.4	6.6	8.3	8.3

¹Beef was inoculated with 4.0×10^3 to 5.0×10^3 CFU/g prior to enrichment process.

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003).

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth.

⁴Each value is the mean \log_{10} CFU/g from 3 replicate trials.

Table 13- Influence of blood supplementation of enrichment broth, enrichment time and detection method on the recovery of *Campylobacter. jejuni* ATCC 33560 from ground beef.

Method	Storage period (days)	Blood Supplement				
		Initial Counts	Without	With	Without	With
			24 hours ³	24 hours	48 hours ³	48 hours
FDA Method ²	0	3.6 ¹	9.2 ⁴	9.2	9.2	9.3
	3		9.1	9.1	9.2	9.2
	7		8.8	8.5	9.2	9.3
	10		8.6	8.8	9.1	9.3
FAPC Method ²	0	3.6	9.7	9.8	8.7	8.5
	3		9.4	9.6	8.9	8.8
	7		8.8	9.1	9.0	9.1
	10		9.1	8.7	9.3	9.2

¹Ground Beef was inoculated with 4.0×10^3 to 5.0×10^3 CFU/g prior to enrichment process.

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003).

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth.

⁴Each value is the mean \log_{10} CFU/g from 3 replicate trials.

Table 14- Influence of blood supplementation of enrichment broth, enrichment time and detection method on the recovery of *Campylobacter. jejuni* ATCC 29428 from ground beef.

Method	Storage period (days)	Blood Supplement				
		Initial Counts	Without	With	Without	With
			24 hours ³	24 hours	48 hours ³	48 hours
FDA Method ²	0	3.7 ¹	8.8 ⁴	8.7	8.9	8.7
	3		8.6	8.9	9.0	8.7
	7		7.6	7.4	8.5	8.6
	10		6.4	6.3	8.8	8.6
FAPC Method ²	0	3.7	9.4	8.8	8.6	8.3
	3		9.2	8.5	8.0	8.1
	7		7.6	7.0	8.5	8.2
	10		6.9	5.9	8.8	8.3

¹Ground Beef was inoculated with 4.0×10^3 to 5.0×10^3 CFU/g prior to enrichment process.

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003).

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth.

⁴Each value is the mean \log_{10} CFU/g from 3 replicate trials.

Table 15-Influence of blood supplementation of enrichment broth, enrichment time and detection method on the recovery of *Campylobacter. jejuni* ATCC 33560 from pasteurized milk.

Method	Storage period (days)	Blood Supplement				
		Initial Counts	Without	With	Without	With
			24 hours	24 hours ³	48 hours	48 hours ³
FDA ²	0	3.3 ¹	8.4 ⁴	7.9	9.0	9.0
	3		6.4	5.2	8.9	8.9
	7		3.3	2.5	4.9	4.4
	10		< 1.0	< 1.0	< 1.0	< 1.0
FAPC ²	0	3.3	7.6	7.8	9.0	9.1
	3		5.6	5.6	9.1	9.3
	7		2.2	1.8	5.1	4.6
	10		< 1.0	< 1.0	< 1.0	< 1.0

¹Pasteurized milk was inoculated with 4.0×10^3 to 5.0×10^3 CFU/mL prior to enrichment process.

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003).

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth.

⁴Each value is the mean \log_{10} CFU/mL from 3 replicate trials.

Table 16-Influence of blood supplementation of enrichment broth, enrichment time and detection method on the recovery of *Campylobacter. jejuni* ATCC 29428 from pasteurized milk.

Method	Storage period (days)	Blood Supplement				
		Without	With	Without	With	
		Initial Counts	24 hours ³	24 hours	48 hours ³	48 hours
FDA ²	0	3.6 ¹	7.4 ⁴	6.7	8.4	8.3
	3		4.3	4.6	8.5	8.7
	7		< 1.0	< 1.0	3.3	3.3
	10		< 1.0	< 1.0	< 1.0	< 1.0
Improved ²	0	3.6	6.8	6.0	8.6	8.6
	3		4.7	4.8	8.8	8.5
	7		<1.0	<1.0	3.7	3.8
	10		< 1.0	< 1.0	< 1.0	< 1.0

¹Pasteurized milk was inoculated with 4.0×10^3 to 5.0×10^3 CFU/mL prior to enrichment process.

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003).

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth.

⁴Each value is the mean \log_{10} CFU/mL from 3 replicate trials.

Table 17-Influence of blood supplementation of enrichment broth, enrichment time and detection method on the recovery of *Campylobacter. jejuni* ATCC 33560 from raw chicken skin.

Method	Storage period (days)	Blood Supplement				
		Initial Counts	Without	With	Without	With
			24 hours	24 hours ³	48 hours	48 hours ³
FDA ²	0	3.3 ¹	8.3 ⁴	8.1	9.0	8.9
	3		6.0	6.2	8.8	8.9
	7		5.7	5.7	8.7	9.0
	10		4.3	4.5	8.6	9.0
Improved ²	0	3.3	7.5	7.1	9.0	9.3
	3		6.5	6.6	9.1	9.2
	7		4.9	5.4	9.0	9.0
	10		3.6	3.9	8.9	9.0

¹Raw chicken skin was inoculated with 4.0×10^3 to 5.0×10^3 CFU/g prior to enrichment process.

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003).

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth.

⁴Each value is the mean \log_{10} CFU/g from 3 replicate trials.

Table 18-Influence of blood supplementation of enrichment broth, enrichment time and detection method on the recovery of *Campylobacter. jejuni* ATCC 29428 from raw chicken skin.

Method	Storage period (days)	Initial Counts	Blood Supplement			
			Without	With	Without	With
			24 hours ³	24 hours	48 hours ³	48 hours
FDA ²	0	3.6 ¹	8.5 ⁴	8.6	8.5	8.6
	3		5.9	6.5	8.6	8.7
	7		4.6	5.8	8.3	8.7
	10		3.9	5.4	8.5	8.7
Improved ²	0	3.6	7.1	7.0	8.6	8.9
	3		5.8	6.7	8.6	8.7
	7		4.7	4.4	8.6	8.8
	10		3.9	4.1	8.6	8.6

¹Raw chicken skin was inoculated with 4.0×10^3 to 5.0×10^3 CFU/g prior to enrichment process.

²FDA (Hunt and others 2001); FAPC (Reilly and Gilliland 2003).

³Hours of incubation in enrichment broth prior to plate counts; a measure of growth in the broth.

⁴Each value is the mean \log_{10} CFU/g from 3 replicate trials.

VITA

Rose Odongo

Candidate for the Degree of

Doctor of Philosophy

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METHOD FOR *CAMPYLOBACTER JEJUNI* IN VARIOUS FOODS

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Scope and Method of Study:

The Food and Agricultural Products Center (FAPC) method, an improved method for the detection and isolation of *Campylobacter* in food, was compared to the Food and Drug Administration/Bacteriological Analytical Method (FDA/BAM) method in the recovery of *Campylobacter jejuni* inoculated in food samples. Beef, chicken skin, milk and ground beef samples were kept at 4 °C for 10 days and the effects of enrichment time, storage period, blood supplementation of the enrichment broth and detection method on the recovery of *Campylobacter* inoculated in the food samples were investigated over the storage period. The abilities of the two methods to recover two strains of *Campylobacter jejuni* ATCC 29428 and 33560, inoculated in the food samples over the storage period were compared.

Findings and Conclusions:

The results from FDA and FAPC methods for detection of *Campylobacter jejuni* from food samples were found not to be statistically different from each other. Blood supplementation of the enrichment broth had no effect on the recovery of *Campylobacter* from food in either method. Although the counts of *Campylobacter* cells recovered after 48 hours of enrichment were higher than those recovered after 24 hours, counts recovered after 24 hours served the purpose of enrichment and thus, there is no need for a 48-hour enrichment period. The growth curves of *Campylobacter* cultured at 37 °C and 42 °C demonstrated that it can grow well at either temperature and therefore it is not necessary to grow *Campylobacter* at 42 °C as required by the FDA protocol. One incubation temperature at 37 °C was adequate to recover *Campylobacter* cells from the food matrices tested.

ADVISER'S APPROVAL: _____ Dr. Stanley E. Gilliland