EFFECTS OF COLD SHOCKING ON THE SURVIVAL AND INJURY OF *ESCHERICHIA COLI* O157:H7 UNDER FREEZING CONDITIONS

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Jill Bollman

In Partial Fulfillment of the

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of MASTER OF SCIENCE

DEDICATION

This thesis is dedicated to the memory of my grandparents. All of whom played a special role in making me the person I am today. They all will live on in my heart forever.

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ABSTRACT

The survival and injury of *Escherichia coli* O157:H7 strains in both frozen foods and trypticase soy broth (TSB) was investigated following cold shocking at 10°C for 1.5 h. Using both trypticase soy agar (TSA) and violet red bile agar (VRBA) as recovery media, it was demonstrated that survival levels between cold shocked (CS) and non-cold shocked (NS) *E. coli* in ground beef or ground pork were not significantly different ($p \leq 0.05$). In contrast, cold shocking *E. coli* in either milk, whole egg or sausage resulted in a significant ($p \leq 0.05$) enhancement in survival. For milk, survival levels of CS *E. coli*, after 28 days of frozen storage, were 1.89 and 1.66 $\log_{10}$ CFU/ml higher on TSA and VRBA respectively, when compared to NS cells. In egg, these values were 0.64 and 1.31, while in sausage, values of 0.74 and 1.19 were obtained. In TSB (pH 7) survival of CS *E. coli* for some strains was about 3 $\log_{10}$ CFU/ml higher when compared to NS cells. Acidification of TSB (pH 5), however, appeared to negate the protective effects of the cold shock treatment. In milk, increasing the differential between the growth and the cold shock temperatures resulted in higher numbers of survivors. In this regard, the growth-cold shock temperature protocol giving optimum survival was 37-10°C. In contrast, growth of *E. coli* at 20°C followed by cold shocking at 10°C did not result in any significant increase in survival. In addition, increased survival due to cold shocking was correlated with the appearance of a novel protein with a pI of 4.8 using isoelectric focusing analysis. Preliminary studies also indicated no clear survival pattern between cold and non-cold shocked cells following heat treatment at 48 and 51°C.

INTRODUCTION

Most microorganisms react to a wide variety of stresses by inducing the synthesis of proteins (Watson, 1990). Some of these stress responses such as the production of heat shock proteins have been well characterized, while others such as the cold shock response require further research. The cold shock response in *E. coli*, initially described by Jones *et al.* (1987) generally occurs when cells are exposed to an abrupt downshift in temperature of at least 13°C (Jones & Inouye, 1994). Temperature downshifts are known to induce a specific pattern of gene expression which is characterized by: the induction of a set of proteins termed cold shock proteins or Csps; the repression of synthesis of heat shock proteins; and the continued synthesis of many transcriptional and translational proteins that are normally repressed during periods of non-growth (Jones & Inouye, 1994).

Much of the research concerned with the cold shock response in bacteria has focused on gene regulation and the associated molecular biology of the cold shock proteins. These proteins, which are produced in increased amounts at low temperatures, and their roles are only now beginning to be understood (Bayles *et al.*, 1996). Although relatively little work has been done to examine the advantage that this response may confer upon the bacteria, two studies indicate that cold shocking may increase an organism's ability to better survive freezing conditions. In this regard, Goldstein *et al.*(1990) reported that when *E. coli* grown at 37°C, were frozen and thawed after pre-incubation at 10°C (cold shock temperature) for 6 hours, a seventy fold increase in survival resulted compared to a similar protocol without cold shocking. In the other study, Willimsky *et al.* (1992) examined the major cold shock protein (CspB) in *Bacillus subtilis*. By creating a mutant which no longer produced CspB, and exposing it to -80°C temperatures, their

results indicated that the wild cells that were able to produce CspB had an increased thermotolerance over their mutant counterparts. From these results it appears that bacteria cold shocked prior to freezing have an increased level of survival. With respect to foods, this would result in a higher microbial load that may be better adapted for cold temperature growth and survival upon thawing and refrigerated storage (Berry & Foegeding, 1997). Bacteria that are adapted to low temperatures exhibit shorter lag times when exposed to cold temperatures. This reduced lag time may impact food safety and quality depending upon the length and condition of storage (Berry & Foegeding, 1997).

There have been numerous studies to indicate that the induction of one stress response may provide protection to the bacteria against other stresses (Watson, 1990; Jenkins *et al.* 1990). Information regarding the cross-protective effects of cold temperature stress and other stresses, and their possible significance to foodborne microorganisms is lacking. Bacteria often enter our food from harsh environments that impose a variety of stresses; thus bacteria in food may be predisposed to survive and possibly grow, despite the presence of preservative barriers such as low temperature and low pH. Research indicates that bacterial response to environmental stresses such as those imposed as barriers in foods can induce stress tolerance, and in some cases increase virulence (Berry & Foegeding, 1997). The significance of the cold shock response to the safety of our food supply is not yet known.

Rapid cooling and freezing are important techniques used to maintain quality and safety particularly in the dairy and meat industries. Although cold shocking has not been previously thought to enhance the survival of pathogens such as *E. coli* in foods, cooling regimes do exist in the food industry which could potentially induce a cold shock phenomenon. Therefore, the

purpose of the present study was to evaluate the impact of cold shocking on a variety of *E. coli* O157:H7 and non-pathogenic *E. coli* strains in various foods and culture broths by examining their survival and injury during frozen storage. *E .coli* O157:H7 was selected for this study because of the number of recent outbreaks associated with this bacterium and the severe consequences associated with ingestion of this strain of *E. coli.* Preliminary studies were also conducted to determine if the induction of the cold shock response provides this bacterium with an increased ability to survive not only cold stress, but also other stresses such as heat and acid environments that bacteria may be exposed to during processing and in consumer homes.

LITERATURE REVIEW

1. *Escherichia coli* O157:H7

i. Characterization of Foodborne *E. coli*

For approximately 100 years, *Escherichia coli* has been considered to be a potential pathogen of humans and domestic animals (Kornacki & Marth, 1982). In 1971, *E. coli* was established as a foodborne pathogen (Jay, 1996). *E. coli* strains involved in foodborne illness are placed into four main groups based on their disease characteristics, effects on certain cell cultures and serologic groupings: Enteroinvasive (EIEC), enteropathogenic (EPEC), enterotoxigenic (ETEC) and, enterohemorrhagic (EHEC) (Jay, 1996). EIEC strains generally do not produce enterotoxins, but cause a dysentery-like syndrome which resembles that of *Shigella* (Jay, 1996). This group usually resides in the colon, and produces bloody or non-bloody voluminous diarrhea. EPEC do not produce enterotoxins, but they can cause diarrhea, especially in infants and young children (Kornacki and Marth, 1982.) EPEC exhibit a specific type of attaching and effacing adherence to intestinal mucosal epithelial cells (Karmali, 1989). In this regard they destroy microvilli and adherence to epithelial cell membranes by the formation of cups or pedestals located at the base of the attached bacterium. It is these lesions that are thought to be responsible for the diarrhea (Karmali, 1989). ETEC are the major cause of bacterial diarrhea in third world countries and of travelers' diarrhea (Karmali, 1989). They are characterized by the production of enterotoxins. These bacteria attach to the small intestine enterocytes using specific fimbriae and secrete enterotoxins which leads to fluid secretion and watery diarrhea (Karmali, 1989). The final group, EHEC or verotoxigenic *E. coli* (VTEC) are the most recently described class of diarrheagenic *E. coli*. The main virulence determinant of

this pathogen is the production of cellular cytotoxins (Neill, 1997). The nomenclature of these toxins varies. They have been called Shiga-like toxins (SLTs) since they are immunologically and functionally similar to these toxins. They have also been called verotoxins (Vts) and verocytotoxins because of their cytotoxic activity to a line of African green monkey kidney cells known as Vero cells. At present more than one hundred *E. coli* serotypes have been shown to produce verotoxins (Neill, 1997). All these strains have been called Shiga-like toxin producing *E. coli* (SLTEC). The EHEC are a subset of the SLTEC that cause bloody diarrhea. The most common and easily identified SLTEC serotype is *E. coli* O157:H7 (Neill, 1997).

ii. Distinguishing Characteristics of *E. coli* O157:H7

E. coli O157:H7 isolates are typical of most *E. coli;* however, they do exhibit some distinguishing characteristics. This serotype of *E. coli* possesses a 60-MDa plasmid and expresses an uncommon 5,000 - 8,000 molecular weight outer membrane protein (Doyle *et al.*, 1997). It also produces some biochemical reactions that are not typical of most other *E. coli.* More than 90% of *E. coli* isolates of human origin have the ability to ferment sorbitol within 24 h, and to produce the enzyme β-glucuronidase, whereas the majority of *E. coli* O157:H7 isolates do not (Doyle, 1991). This strain of *E. coli* also can produce enterohemolysin which is a characteristic of verotoxin producing *E. coli* but not of other *E. coli* strains (Doyle, 1991). Additionally, it does not grow at 44-44.5^{0}C, an incubation temperature used to recover fecal coliforms (Doyle, 1991). These characteristics have led to the formation of various selective screening methods for this bacterium such as the use of MacConkey-sorbitol agar or latex agglutination tests (Doyle, 1991). Traditional methods used to detect *E. coli* in foods may

therefore overlook this organism during routine analysis (Doyle, 1991).

iii. Mechanism of Action

VTEC produce one or both of at least 2 antigenically distinct verotoxins, VT1(SLT-I) and VT2 (SLT-II) (Karmali, 1989). Verotoxins of *E. coli* O157:H7 are immunologically indistinguishable from Shiga toxin and also share the same biological activities (Padyhe & Doyle, 1992). However, only the cytotoxicity of VT1 can be neutralized by the Shiga toxin antiserum (Gyles, 1994). Both verotoxins are composed of an A-B subunit structure with molecular weights of approximately 33 kDa and 7.5 kDa respectively(Gyles, 1994). The A subunit exhibits the biological activity, whereas the B subunit possesses the binding site for the toxin molecule (Gyles, 1994).

VT1 and VT2 have identical biological activities despite being immunologically different (Padyhe & Doyle, 1992). On a weight basis, VT2 is less active by a factor of 1000 on Vero cells than VT1. It has also been shown to have a 100-fold higher LD_{50} for rabbits than VT1. The receptor for both VT1 and VT2 is a globotriosyl ceramide (Gb3) on the eukaryotic cell surface. After the B subunit of the toxin binds to the Gb3 receptor on the cell, it is internalized by receptor mediated endocytosis, followed by fusion with lysosomes, translocation to the golgi apparatus and transfer to the cytosol (Gyles, 1994). In the cytosol, the A subunit undergoes partial proteolysis and is reduced into 2 fragments. The smaller fragment binds to the 60S ribosomes to inhibit protein synthesis and causes cell death (Doyle, 1991). The toxins specifically cleave the N-glycosidic bond in 28S ribosomal RNA. This causes the release of a single adenine residue and prevents the elongation-factor-1-dependent binding of

aminoacyl-tRNA to the ribosome (Gyles, 1994). This results in cell death by preventing further peptide chain elongation thereby suppressing protein synthesis (Doyle *et al.*, 1997). The susceptibility of various cell lines to verotoxins is dependent on the presence of the glycolipid receptor on the cell surface (Gyles, 1994). It is believed that VTEC colonize the intestine and produce verotoxins that are absorbed into the circulation, causing damage to tissues rich in Gb3 receptor sites (Gyles, 1994). Gb3 is plentiful in the cortex of the human kidney and is present in primary human endothelial cell cultures (Padyhe & Doyle, 1992). The presence of these receptors in the kidneys is consistent with an etiologic role of VTEC in hemolytic uremic syndrome (HUS), in which endothelial cells of the renal vasculature system are the principle sites of damage (Padhye & Doyle, 1992).

In the past it had been suggested that this organism is non-invasive and does not enter the circulatory system because afflicted patients have little or no fever. (Padyhe & Doyle, 1992). It was thought that the bacterium colonized the intestinal tract where it released its toxins. It has recently been reported that *E. coli* O157:H7 is able to invade certain tissue culture cells such as HCT-8 ileocecal cells but not others such as Hep-2 cells (Doyle *et al.*, 1997). Further research is required to determine the role of *E. coli* O157:H7 in infection. In addition, there have been conflicting reports as to how this organism attaches to the gut, although most researchers agree that attachment to epithelial cells is mediated by constituents in the outer membrane of the bacterium (Padyhe & Doyle, 1992).

Weeratna and Doyle (1991) found that VT1 can be produced in food. They demonstrated that VT1 was produced both in ground beef and in milk with maximum production at 37^{0}C. More toxin production was seen with agitation indicating that aeration is an

important factor for toxin synthesis. Since VT1 is a cell-associated toxin, large numbers of cells would have to lyse in order to detect sufficient toxin quantities in food. Therefore, the presence of toxins in food could be indicative of improper food handling (Weeratna & Doyle, 1991).

The verotoxins have been shown to cause host specific diseases. For example, both enteric and systemic diseases are observed in humans, while cattle only display enteric disease and pigs only exhibit a systemic disease (Gyles, 1994).

iv. Pathogenicity

Escherichia coli O157:H7 is an important, common human pathogen which causes a wide variety of diseases and symptoms. In addition it has been implicated in numerous worldwide outbreaks of foodborne illness. This organism is among the most frequently recovered enteric human pathogens (Tarr, 1994) and has been recognized as the most important etiologic agent of hemorrhagic colitis (Bopp *et. al*, 1987). The symptoms of infection may include severe abdominal cramps, nonbloody diarrhea, bloody diarrhea (hemorrhagic colitis), hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). Patients with hemorrhagic colitis generally develop severe abdominal pain with watery nonbloody diarrhea that lasts approximately 24 h, followed by the production of bloody diarrhea which can last from 2-9 days (Neill, 1989). Incubation varies between 1 and 14 days with the duration of the illness averaging about 8 days (Stavric & Speirs, 1989). Other symptoms may include nausea and vomiting with little or no fever. Although all age groups are at risk, the young and the elderly are at increased risk for serious complications such as HUS and TTP (Stavric & Speirs, 1989). Hemolytic uremic syndrome is one of the primary causes of renal failure in young

children worldwide, having a 5% mortality rate in North America (Stavric & Speirs, 1989). This disease is a combination of acute renal failure, thrombocytopenia and microangiopathic haemolytic anemia (Gyles, 1994). VTEC infections have been implicated in 88-90% of patients with HUS. *E. coli* O157:H7 was responsible for approximately one half of these cases (Jaradat & Marquardt, 1997). Gyles (1994) reported that 10-20% of patients that are diagnosed with hemorrhagic colitis go on to develop HUS.

v. Epidemiology

E. coli O157:H7 was first reported in 1970 in Ireland as a cause of piglet enteritis (Hockin & Lior, 1986). The first identification in Canada was in 1978 at the Laboratory Centre for Disease Control (LCDC, Ottawa) (Hockin & Lior, 1986). Between 1978 and 1982 this serotype was isolated 5 times in different parts of Canada. All these isolates were non-invasive and did not produce enterotoxins. In 1982, the Center for Disease Control in Atlanta linked this serotype to two outbreaks of hemorrhagic colitis in the United States (Hockin & Lior, 1986). In this outbreak, a link was drawn between the infection and the consumption of ground beef at a chain of fast food restaurants in the Michigan and Oregon area (Chapman, 1995). In the same year another outbreak of hemorrhagic colitis involving *E. coli* O157:H7 was implicated in a nursing home in Ottawa where 17 of the 55 affected residents died (Hockin & Lior, 1986). In 1983, LCDC first reported that *E. coli* O157:H7 produced a toxin for Vero kidney cells (Hockin & Lior, 1986). In 1983, Karmali and coworkers were the first to establish an association of HUS with prior VTEC infection (Chapman, 1995). Since these initial reports, outbreaks and sporadic cases of HUS and HC linked to VTEC infections have been reported worldwide.

Reported cases of HUS in the UK and in North America are steadily increasing (Chapman, 1995). In 1989, Todd estimated that the number of VTEC infections in North America could be as high as 13,000 and that the cost per case was higher for VTEC O157 than for any other food-borne diarrhoeal disease (Chapman, 1995). In 1995, Boyce estimated that there were over 20,000 infections and up to 250 deaths per year due to *E. coli* O157:H7 in the United States alone. It is difficult to determine the actual incidence of this pathogen because few laboratories routinely culture stool specimens for these bacteria (Boyce *et al.*, 1995). Although over 100 serotypes of *E. coli* that produce verotoxins have been isolated from humans, not all serotypes produce illness. *E. coli* O157:H7 from both a clinical and a public health standpoint is the most important serotype in North America (Boyce *et al.*, 1995). More than 90% of VTEC strains isolated in North America and the UK belong to serotypes O157:H7 or O157:H- (Chapman, 1995). VTEC infections appear to be more prevalent in Canada, USA, and the UK. It is not known if these trends simply reflect the fact that these countries may have better epidemiological and microbiological systems to more effectively identify outbreaks and sporadic cases of *E. coli* O157:H7 (Chapman, 1995). VTEC infections are seasonal, showing higher incidence in the warmer months of the year. This may be due to the increased use of barbeques and the potential for undercooked ground beef (Chapman, 1995).

E. coli hemorrhagic colitis is mainly a food borne illness, but person to person transmission can also be very important (Stavric & Speirs, 1989). The majority of outbreaks have been due to the consumption of undercooked ground beef . Although *E. coli* O157:H7 is present in the intestines of only 1% of healthy cattle (Boyce *et al.*, 1995) the musculature may become contaminated during the slaughtering and dehiding process thereby dramatically

increasing its spread. Ground beef is particularly at risk because the grinding of the meat moves the pathogens from the outside to the inside of the meat. In addition, ground beef may be comprised of meat from numerous carcasses, therefore a small number of infected animals can contaminate a large supply of meat (Boyce *et al.*, 1995). This pathogen has also been transmitted in unchlorinated drinking water, raw milk, apple cider, apple juice, raw vegetables, persons swimming in fecally contaminated lakes and secondary person to person transmission. In terms of milk, dairy cattle have been identified as one major reservoir for these bacteria (Doyle, 1991). A significant association between the incidence of VTEC infections and cattle density has been found (Wilson *et al.*, 1997). A three year Canadian study of *E. coli* O157:H7 infections of children under the age of 15 demonstrated that contact with persons with diarrhea and exposure to rural settings were important risk factors for infection (Wilson *et al.*, 1997).

A survey of retail meats from Wisconsin and Alberta found that *E. coli* O157:H7 was present in 2-4% of ground beef, 1.5% of pork and poultry plus 2% of lamb (Doyle & Schoeni, 1987). However, it was not determined if these organisms were present on the animal at the time of slaughter or whether cross contamination occurred during processing or at the point of retail (Chapman, 1995). A survey in Ontario targeting retail products for VTEC reported incidence rates of 36.4% in beef and 10.6% in pork; but no isolates were O157:H7. In addition, no VTEC was isolated in chicken samples (Padhye & Doyle, 1992). In Manitoba, a survey of raw ground beef from retail grocery stores and fast food restaurants reported VTEC in 12% of samples but only 2% were O157:H7 (Padhye & Doyle, 1992).

Much emphasis has been placed on foods of bovine origin as the primary source of VTEC infections (Wilson *et al.*, 1997). In one Canadian study researchers found that the

consumption of improperly cooked ground beef was attributed to only 17% of VTEC infections (Wilson *et al.*, 1997). This study indicated that there may be other important risk factors associated with these infections. One newly identified risk factor is direct contact with cattle (Wilson *et al.*, 1997). Antibodies to VT1 were found in 41% of farm residents, and only 7.7% of urban residents. Similarly, 12.5% of farm residents had antibodies to O157 lipopolysaccharide as compared to 4.7% of urban residents (Wilson *et al.*, 1997). This indicated that farm residents with increased exposure to VTEC may have an increased resistance compared to urban dwellers (Wilson *et al.*, 1997). A case in point occurred where all urban relatives visiting a farm became ill with VTEC infections while the farm relatives were unaffected (Wilson *et al.*, 1997). Consumption of contaminated water from shallow rural wells is also a known risk factor for VTEC infections (Wilson *et al.*, 1997).

2. The Cold Shock Response

i. Overview

All organisms, from bacteria and yeast to plants and animals react to a wide range of environmental stresses by inducing the synthesis of stress proteins (Watson, 1990). Despite numerous studies that have examined the stress response, the function of the associated proteins often remains unknown. Part of the confusion has been due to the complexity of the stress responses and the fact that most of the proteins that are produced under stress are also produced under normal physiological conditions (Watson, 1990). The large number of stresses that can induce an elevated synthesis of stress proteins has also added to this confusion. It is this muti-stress response that has led to contradictory interpretations of the function of these

responses. Most of the research dealing with stress responses has been aimed at gene regulation and the associated molecular biology of the stress proteins (Watson, 1990).

A stress response that has not been well characterized is the cold shock response, initially described by Jones and coworkers (1987) in *E. coli*. This cold shock response occurs when cells are exposed to an abrupt downshift in temperature. Abrupt temperature downshifts induce specific patterns of gene expression which are characterized by: the induction of a set of proteins termed cold shock proteins or Csps, the repression of synthesis of heat shock proteins and the continued synthesis of many transcriptional and translational proteins that are normally repressed during periods of non-growth (Jones & Inouye, 1994). Temperature shifts of 13^{0}C or more result in the complete induction of the cold shock response as long as the temperature does not fall below a temperature that is not permissive for protein synthesis. Generally, the greater the magnitude of the temperature shift, the more pronounced is the response (Jones & Inouye, 1994). The cold shock response is induced during the lag phase of growth following the shift to low temperatures. This response ends once reproduction is resumed (Jones & Inouye, 1994).

ii. Cold Shock Response of *E. coli*

When the temperature of growing *E. coli* is rapidly decreased, the synthesis of sixteen polypeptides is induced (Berry & Foegeding, 1997). Most of these polypeptides show a 2 to 10 fold increase in induction after the temperature shift. One polypeptide, designated CspA or CS7.4, undergoes a 200 fold increase in induction and represents the only cold shock protein that is not detected at normal growth temperatures (Goldstein *et al.*, 1990). At peak synthesis (1.5h), CspA becomes the most abundantly synthesized protein in the cell. The transcription of the

cspA gene, and the synthesis of CspA utilize most of the cell resources during the lag period before growth resumes at 10^0C (Jones *et al.*, 1992). Much of the research in the area of the cold shock response has been focused on this protein and its overall function.

iii. Cold Shock Proteins of *E. coli*

a. Identity

Many of the cold shock proteins in *E. coli* have been identified. They include NusA, whose function involves both termination and anti-termination of transcription, initiation factors 2α and 2β which are involved in the initiation of translation, plus RecA which plays roles in the induction of the SOS response and in recombination. Also included are the dihydrolipoamide acetyltransferase subunit of pyruvate dehydrogenase and pyruvate dehydrogenase-lipoamide, and H-NS which is a nucleoid-associated DNA binding protein plus the GyrA protein which is a subunit of the topoisomerase DNA gyrase. Another protein recently identified as Hsc66 is a homolog of the heat shock protein Hsp70 that functions as a molecular chaperone in the heat shock response (Lelivelt & Kawula, 1995). In addition a molecular chaperone called trigger factor (TF) was found to be a cold shock protein. This protein is necessary for *E. coli* viability at low temperatures; however, its overexpression at high temperatures (50°C) was shown to reduce viability (Kandror & Goldberg, 1997). The most recent protein discovered has a molecular weight of 70 kDa, and is referred to as CsdA. It is a major ribosomal-associated protein with helix destabilizing activities (Jones *et al.*, 1996). The most well studied cold shock protein in *E. coli* is CspA which has been referred to as the major cold shock protein. Most of the cold shock proteins that have been identified to date function in either transcription or translation or their

genes are on operons that contain other transcriptional or translational genes (Berry & Foegeding, 1997)

b. CspA

CspA is a 70 amino acid residue protein with a molecular weight of approximately 7.4 kDa and a pI of 5.92 (Goldstein *et al.*, 1990). It is a very hydrophilic protein containing more than 20% charged residues (Lee *et al.*, 1994). Some discrepancy exists as to its secondary structure. The predominant view is that it is composed of five antiparallel β-sheets forming a five stranded β-barrel with a hydrophobic core and a hydrophilic exterior (Newkirk *et al.*, 1994).

After a shift to from 37^0 to10^0 C, the rate of CspA production can reach 13% of total protein synthesis within 1.5 hours. This is equal to an approximate level of 250, 000 molecules per cell (Jones *et al.*, 1996). After the protein has reached its maximum concentration, it subsequently drops to a basal level remaining quite stable in the cell after synthesis (Jones & Inouye, 1994). After twenty hours at 15^0C, only approximately 30% of the CspA was degraded. Another study demonstrated that after 1.5 hours at 37^0 C following cold shocking, there was no discernable decrease in the amount of CspA (Goldstein *et al*, 1990). Due to the stability of the protein, and its continual production after initial induction at 15^0C, it is believed that a constant level of the protein is probably maintained in the cell as long as it remains exposed to cold temperatures (Goldstein *et al.*, 1990). Although the protein is not actively degraded upon return to 37^0 C, the level of the protein will be rapidly lowered by cell division in the absence of further synthesis (Goldstein *et al.*, 1990).

iv. Function of Cold Shock Proteins

Currently the function of the cold shock response and CspA have not been fully elucidated and a number of theories have been proposed. CspA may act as an antifreeze protein that protects the cell from damage caused by the growth of ice crystals during freezing. Antifreeze proteins lower the freezing temperature and inhibit recrystallization and the growth of large ice crystals during frozen storage (Feeney & Yeh, 1998). Antifreeze proteins are low molecular weight proteins commonly found in high concentrations in the serum of polar dwelling marine fishes and in the hemolymph of insects that winter in sub-freezing climates (Goldstein *et al.*, 1990). They are divided into 4 classes based on their structural properties; however, CspA bears no similarity to any of these classes with exception of its small size and its considerable proportion of hydrophilic residues (Goldstein *et al.*, 1990). This high content of hydrophilic residues is thought to be important in hydrogen binding to the lattice of the forming ice crystals. Goldstein and coworkers (1990) found that when *E. coli*, grown at 37^0 C, were frozen and thawed after a pre-incubation at 10^0 C for six hours, there was as much as a 70 fold increase in survival, compared to cells frozen and thawed without the 10^0 C pre-incubation. This study demonstrated that this protein had some type of protective role in the cell. If this protein does not have antifreeze activities it is thought that due to the extremely large number of molecules present per cell (approx. 250,000), it would still have a role in protecting the cell from freeze damage by lowering the freezing point of the cell (Tanabe *et al.*, 1992).

An interesting characteristic of CspA is that it has a high sequence similarity with the eukaryotic DNA binding proteins (Lee *et al.*, 1994). Since CspA can bind to DNA or RNA it is thought that it may function as a transcriptional activator or enhancer that can recognize and

bind to the promoter region of other cold shock genes (Jones & Inouye, 1994). This theory was supported by the finding that binding sites for CspA have been identified in the promoters of several cold shock genes (Jones & Inouye, 1994). This protein may be initially induced in order to regulate the synthesis of other cold shock proteins. The overproduction of CspA can increase the rate of synthesis of other cold shock proteins (Jones & Inouye, 1994). For example, La Teana and coworkers (1991) demonstrated that CspA can bind to the *hns* cold shock gene and stimulate the transcription of the cold shock protein H-NS. Jones and coworkers (1992) also found that CspA can bind to the promoter region of the *gyrA* cold shock gene and act as a positive transcriptional activator during cold shock. Since it has been shown that CspA can bind to RNA, it has been hypothesized that this protein may be able to mask certain mRNA's of other proteins to inhibit their translation and prevent their synthesis (Jones & Inouye, 1994).

Qoronfleh and coworkers (1992) believe that CspA may serve a general function to prepare cells to reinitiate protein synthesis after a drop in temperature. Exposure to low temperatures results in the accumulation of 70S ribosomes and a block in the initiation of translation. CspA may also be essential for the transcriptional machinery at low temperatures in order to convert a closed complex to an open complex during transcription initiation, thereby functioning as a cold shock transcriptional activator (Jones & Inouye, 1994).

In addition, a 70 kDa cold shock protein, CsdA, was found to be produced almost exclusively at low temperatures and was a major ribosomal protein at these temperatures (Jones *et al.*, 1996). This protein possesses helix-destabilizing activities. It has been proposed that at low temperatures this protein is necessary for ribosomal function to increase translational efficiencies of mRNA's by unwinding stable secondary structures that are formed at low

temperatures (Jones *et al.*, 1996). CspA may bind to the mRNA following unwinding by CsdA to prevent mRNA's from annealing. Some *E. coli* mRNA's have been shown to have a secondary structure that becomes more stable at lower temperatures. These mRNA secondary structures can inhibit the initiation of translation and cause a pause in the translation elongation reactions. In general, these secondary structures are more deleterious at low temperatures (Jones *et al.*, 1996). Therefore, it may be necessary to have a cold-shock inducible helix-destabilizing protein in ribosomes in order to facilitate growth at low temperatures. Other factors that corroborate this theory are that ribosomes have been proposed to be the physiological sensor for the cold shock response and that two cold shock proteins are translation initiation factors (2α, 2β) (Jones *et al.*, 1996). It has also been shown that the overproduction of CsdA increased the stability of certain mRNAs, indicating that this protein may also have the function of a mRNA stabilizer at lower temperatures (Jones *et al.*, 1996).

Lelivelt and Kawula (1995) found that a member of the heat shock protein family Hsp70 called Hsc66, was induced in *E. coli* by cold shock and not by heat shock. Hsp70 proteins function as molecular chaperones to assist unfolded, misfolded, or aggregated proteins in retaining or attaining a specific conformation (Lelivelt & Kawula, 1995). They also are involved in protein translocation across cytoplasmic membranes and protein degradation. The trigger factor in *E. coli* is induced at low temperatures and is essential for cell viability at low temperatures (Kandror & Goldberg, 1997). Most cold shock proteins are important in enabling cells to grow at low temperatures, but TF is the only protein to date that has been shown to actually increase viability in the cold (Kandror & Goldberg, 1997). TF has been shown to associate with ribosomes and growing polypeptides, indicating it may allow protein synthesis

and folding to continue at low temperatures, where the solubility, aggregation, and folding properties of proteins are different than at 37°C (Kandror & Goldberg, 1997). TF may also be important in maintaining preexistant cell proteins in a functional form by assisting in the refolding of cold damaged proteins. Kandror and Goldberg (1997) suggested that the finding of both molecular chaperones (TF & Hsc66) may indicate that at low temperatures, protein folding or refolding may require distinct enzymatic machinery from that at high temperatures. Although TF overproduction increased the viability of *E. coli* cells at 4°C, it reduced viability at 50°C, while TF deficiency at the elevated temperature increased viability. In contrast, overproduction of some of the heat shock proteins can reduce viability at 4°C, explaining why the heat shock proteins may be repressed at cold temperatures (Kandror & Goldberg, 1997).

v. Regulation of the Cold Shock Response

a. Transcription

Recent studies of the cold shock response have attempted to determine how the response is regulated. There have been conflicting reports as to at which level of protein synthesis this regulation is occurring. Many studies indicate that the regulation is at the level of transcription. A downshift in temperature results in an increase in the mRNA of the *cspA* gene, suggesting that the cold shock induction of CspA occurs at transcription (Jones & Inouye, 1994). The *cspA* gene expression caused by cold shock is temporary, and subsequently drops to a basal level several hours after treatment. This decrease in *cspA* expression was found to be blocked by the addition of chloramphenicol which indicates that the synthesis of the *cspA* gene repressor is inhibited and/or the *cspA* activator is constitutively active (Jiang *et al.*, 1993). The mRNA of

the *cspA* gene has been found to be very unstable at optimal growth temperatures (Jones & Inouye, 1994). The pattern of *cspA* mRNA induction appears to agree with the pattern of the CspA production after cold shock. Tanabe and coworkers (1992) found that there is a cold shock factor(s) which binds to a region upstream from the promoter region of the *cspA* mRNA at 14^0C, but not at 37^0C.

The exact mechanism of cold shock regulation is not known; however, a link has been drawn between two unusual nucleotides and this response. The induction of the cold shock response is accompanied by a decrease in two nucleotides, guanosine 5' triphosphate-3-diphosphate and guanosine 5' diphosphate-3-diphosphate ((p)ppGpp), which is proportional to the magnitude of the temperature shift (Jones & Inouye, 1994). These nucleotides seem to play a role in the temperature adaptation of *E. coli*. A decrease in (p)ppGpp positively affects the induction of the cold shock response which suggests that variations in the (p)ppGpp level may affect the transcription of the *cspA* gene (Jiang *et al.*, 1993). In addition, (P)ppGpp has also been shown to affect the transcription of some other *E. coli* genes and variations in the (p)ppGpp levels have been implicated as modulators of the cold shock response. These nucleotides are small molecule effectors whose activity is mediated by their ability to bind to proteins and alter their conformation. In addition, they also may be used to inhibit transcription of some genes (Lewin, 1990). Both of these nucleotides are produced by ribosomes that are stalled by amino acid starvation, carbon starvation, nutrient downshift and deficiencies in charged tRNA's (Goldstein *et al.*, 1990). The cold shock signal may be sent to the transcriptional apparatus, causing a rapid decrease in the transcription of genes encoding the components of the translational apparatus (Goldstein *et al.*, 1990). Along with this decrease in translational

components there is an increase in the synthesis of enzymes necessary for biosynthetic pathways (Berry & Foegeding, 1997). In contrast to the decrease of (p)ppGpp levels with a temperature downshift, an upshift in temperature results in an increase in the (p)ppGpp level. In both cases, the change in (p)ppGpp level is proportional to the magnitude of the temperature shift (Berry & Foegeding, 1997). If this decrease in (p)ppGpp does not control the cold shock response it may serve as a signal for the response.

During periods of non-growth, proteins involved in both transcription and translation are not usually produced. However, during the lag period after a temperature downshift, synthesis of many of the components of the transcriptional and translational machinery still continue (Jones & Inouye, 1994). The (p)ppGpp level has been shown to affect the synthesis of many of these transcriptional and translational proteins. If the levels of these nucleotides are increased prior to a temperature downshift, it results in the decrease in the induction of many cold shock proteins and a decrease in the synthesis of many transcriptional and translational proteins (Goldstein *et al.*, 1990). Inhibitors of translation, such as group C antibiotics, that cause a decrease in the (p)ppGpp level, can result in the induction of the cold shock response at increased temperatures such as 37°C (Jiang *et al.*, 1993). A mutant strain of *E. coli* deficient in (p)ppGpp was found to express high levels of the major cold shock protein CspA constitutively during steady state growth at 24° C (Jones *et al.*, 1992). This caused the cells to respond to a downshift in temperature similar to a cold adapted cell meaning the *E. coli* continued to grow exponentially when shifted to 10° C without the typical lag period (Jones *et al.*, 1992). A partial block in the initiation of translation caused by a downshift in temperature may produce a signal indicating the cell has insufficient translational machinery, causing the lowering of the level of

(p)ppGpp and the induction of the cold shock response (Jones *et al.*, 1992).

Alternatively, it was found that although these nucleotides may play a role in the cold shock response, a double mutant for both genes that produce these nucleotides showed that (p)ppGpp is neither sufficient nor necessary to induce the cold shock response (Jones *et al.*, 1992). Although (p)ppGpp prevents the induction of the cold shock response, its absence does not lead to induction. The decrease in the (p)ppGpp level following a temperature downshift may act to modulate the activity of another regulator (Jones *et al.*, 1992). Not all cold shock proteins are negatively regulated by (p)ppGpp, indicating that more than one regulon may comprise the cold shock response (Jones *et al.*, 1992).

b. Translation

Other investigators believe that the cold shock response is regulated at the level of translation. Shifting *E. coli* to lower temperatures results in an temporary inhibition of the initiation of translation; this may induce the cold shock response causing the cell to increase its translational capacity (Jones & Inouye, 1996). For example, one piece of evidence for translational regulation was the discovery that the RbfA protein of *E. coli* was a cold shock protein. This protein is involved in ribosomal maturation and/or initiation of translation (Jones & Inouye, 1996). If this protein is not present when an *E. coli* mutant was shifted to a lower temperature, it had a slower growth rate. A mutant that overproduces RbfA results in an increase in total protein synthesis accompanied by faster growth adaptation to the lower temperature.

It is believed that cold shock proteins can be translated by ribosomes under conditions that are not translatable for most mRNA's. Since certain inhibitors of translation can induce

either the cold shock or heat shock response, it has been proposed that the ribosome may be the sensor for these responses (VanBogelen & Neidhardt, 1990). A shift to 5°C results in the accumulation of 70S ribosomes which are thought to indicate a block in the initiation of translation. A shift to 10° C, on the other hand, results in a growth lag of 4 hours followed by renewed growth (Goldstein *et al.*, 1990). Therefore, it has been suggested that since some of the proteins that are induced during cold shock are involved with protein synthesis, the response may be to prepare cells to reinitiate protein synthesis during exposure to cold temperatures (Goldstein *et al.*, 1990).

It was also demonstrated that *cspA* mRNA was not translated at elevated temperatures, which indicated that *cspA* mRNA is not stable at these higher temperatures (Goldenberg *et al.*, 1996). The translation of *cspA* mRNA is less efficient at 37°C than at 10°C resulting in lower amounts of chromosomal *cspA* mRNA at 37°C. For example, the *cspA* mRNA level increased over 30-fold within 1 hour after transferring cells to 15°C (Goldenberg *et al.*, 1996). Goldenberg and coworkers (1996) showed that *E. coli* cells constitutively transcribe the *cspA* mRNA at 37°C, but the mRNA produced at this temperature is unstable and is degraded before it is translated. However, at lower temperatures the *cspA* mRNA becomes completely stable (Goldenberg *et al.*, 1996). These results suggest that the regulation of the cold shock response is post-transcriptional. The level of translation is a determinant of mRNA stability (Goldenberg *et al.*, 1996). A set of proteins including NusA and PNPase are induced following the cold shock response; these may function as feedback regulators that may cause the *cspA* transcripts to become destabilized (Goldenberg *et al.*, 1996). The CspA protein may play in a role in the regulation of the *cspA* gene (Goldenberg *et al.*, 1996). It is evident from the literature that the

regulation of the cold shock response is complex and probably involves more than one regulatory mechanism.

vi. Cold Shock Response in Other Microorganisms

The cold shock response is not unique to *E. coli* or even to prokaryotes. Studies have examined the cold shock response in a variety of organisms including psychrotrophs, mesophiles and thermophiles. It was found that *cspA* homologs exist in a number of Gram-positive and Gram-negative organisms indicating that they may be found in most eubacteria (Graumann *et al.*, 1996). One bacterium that has been frequently examined is *Bacillus subtilis.* Graumann *et al.* (1996) determined the identity of 16 Csps and reported that the relative production of 38 proteins increased to a maximum level 30-60 min after being transferred from 37°C to15°C. The major cold shock protein of *B. subtilis* is CspB which has a 62% homology with the CspA protein in *E. coli* (Graumann & Marahiel, 1994). Both CspA and CspB are small hydrophilic proteins with CspB containing 67 amino acid residues and CspA having 70. The secondary structure of CspB is a β-barrel and is similar to the structure of CspA in *E. coli.* Graumann and Marahiel (1994) suggested that the binding of CspB could stabilize an open complex that may be a limiting step in the initiation of transcription. The sequence that CspB recognizes has been found in the promoter region of several cold shock genes. It has also been hypothesized that, similar to CspA in *E. coli,* CspB may stabilize mRNA or affect translation which is a limiting factor at lower temperatures (Graumann & Marahiel, 1994). Willimsky and coworkers (1992) attempted to determine the physiological role of CspB protein synthesized during the cold shock response. They constructed a mutant in which the *cspB* gene was eliminated. This mutant was

therefore unable to synthesize its major cold shock protein. The mutant was subsequently exposed to -80^{0}C temperatures and its survival was compared to cells that contained the *cspB* gene. The results indicated that CspB may play a role in the acquired thermotolerance of *B. subtilis*. Cells that were able to undergo the complete cold shock response had an increased survival over their mutant counterparts. Graumann and coworkers (1996) also inactivated the *cspB* gene to determine its function in the cold shock response. They found that the level of synthesis of 15 cold inducible proteins did not increase with a shift from 37 to15^{0}C. This indicated that CspB may be responsible for the regulation of several other cold shock proteins. After cold shock, a minimum of 75 proteins were seen, however, only 4 were synthesized *de novo* at the lower temperatures. This observation indicated that most of the cold inducible proteins also function under optimal conditions of growth, and suggests that although the cold shock response of *B. subtilis* is similar to the response in *E. coli*, it also shows some differences. The number of proteins synthesized is approximately three times that of *E. coli*. Also the response is twice as fast. This may be explained because *B. subtilis*, unlike *E. coli*, is a soil bacterium that will constantly be subjected to temperature changes (Graumann *et al.*, 1996).

Most studies examining the cold shock phenomena have been performed using mesophiles. Recently researchers have investigated the cold shock response in psychrotrophs and psychrophiles. Cold shock proteins were found in the psychrotrophic yeast, *Trichosporon pullulans* and in the psychrotrophic bacterium *Bacillus psychroplilus*. Ray *et al.* (1994) found *cspA* homologs in most of the Antarctic psychrotrophic bacteria examined. The psychrotrophic organisms often possessed another group of proteins called cold acclimation proteins (Caps) which are proteins that are produced at a higher level during prolonged growth at lower

temperatures as compared with growth at higher temperatures. Some of these cold acclimation proteins are cold shock proteins but others are not related (Whyte & Inniss, 1992). Cold acclimation and cold shock proteins can be differentiated by comparing the protein synthesis electrophoretic patterns between psychrophiles growing continuously at low temperatures and psychrophiles exposed to a sudden temperature decrease. Cold acclimation proteins have never been found in mesophiles (Berger *et al.*, 1996). Analysis of *Arthrobacter globiformis* SI55 demonstrated that the levels of very few proteins changed when grown at 30^{0}C or at 4^{0}C. However at least 18 more peptides expressed at the lower temperature were thought to be Caps. Caps have also been found in the psychrotrophic *B. psychrophilus* and *Pseudomonas fragi.* When cold shocked, *A. globiformis* SI55 increased the production of 28 peptides, of which 19 were transiently overexpressed (Csps) and 9 were still overexpressed in steady state growth at 4^{0}C (Caps). *A. globiformis* SI55 possessed a DNA/RNA binding region of a cold shock gene that had a high homology with *E. coli* CspA(78%) and CspB in *Bacillus* (56%). This protein (A9) correlates to a Cap but not a Csp as it is produced during prolonged growth at low temperatures. The level of the protein in *A. globiformis* SI55 is also lower than the level of CspA in *E. coli.* The A9 protein production can also be triggered by chloramphenicol at higher temperatures similar to CspA (Berger *et al.*, 1996). Therefore, it has been suggested that the production of CspA-like proteins is not regulated exclusively by temperature but rather by some biochemical event in thermophiles, mesophiles and psychrotrophs. This means that these proteins may play a more general role in the cell rather than just in cold acclimation processes (Berger *et al.*, 1996). Berger *et al.* (1996) suggested that more Csps are over expressed in psychrotrophs than in mesophiles. Julseth and Inniss (1990) reported the production of 26 Csps in the psychrotrophic

yeast, *Trichosporon pullulans.* Berger *et al.* (1996) suggested that mesophilic bacteria may have an incomplete cold shock response system which may only comprise a portion of the response of cold-adapted bacteria. This may be the reason why mesophiles can not obtain steady state growth at low temperatures.

Mayr and coworkers (1996) examined the cold shock response in the psychrotroph, *Bacillus cereus.* They identified six small proteins including a major cold shock protein called CspA. It was highly homologous to other cold shock proteins (Mayr *et al.*, 1996). For example, *B. cereus* CspA was 63% homologous with CspA of *E. coli*, 71% homologous with CspB of *B. subtilis* and 58% homologous with SC7.0 of *Streptomyces clavuligerus.* The CspA protein was highly expressed during both cold temperature growth as well as during cold shocking. When four strains of *B. cereus*, including psychrotrophic strains, were shifted from 30°C to 10°C all strains not only continued the synthesis of many proteins despite the abrupt temperature drop but also produced cold shock proteins (Berry & Foegeding, 1997).

Hebraud *et al.* (1994) found 20 proteins in *P. fragi* that were expressed at higher levels at 4°C than at 30°C. Their rate of synthesis increased systematically as the temperature dropped below the optimal growth temperature. Two proteins with molecular weights of 7.0 (C7.0) and 8.0 (C8.0) kDa were both over expressed at the lower temperature. These two proteins appear very close to *E. coli* CspA in 2-dimensional electrophoretic analysis. There is also a strong recognition of *E. coli* CspA by anti-C8.0 serum indicating that they have some sequence homologies. It has been hypothesized that they all belong to the same class of DNA binding proteins (Hebraud *et al.*, 1994). C8.0 may play a role in steady state cultures at low temperatures similar to that of CspA in *E. coli* under cold shock conditions.

The number of cold shock proteins produced in *B. psychrophilus* appeared to be correlated with the severity of the cold shock, with the greater cold shock producing the greatest number of cold shock proteins (Whyte & Inniss, 1992). Whyte and Inniss (1992) found that *Bacillus psychrophilus* produced nine cold shock proteins with a temperature shift from 20 to 0^0 C, seven were produced with a shift from 20 to 5^0 C, and only five were produced with a shift from 20 to 10^0 C. Caps are also produced at different levels corresponding to the temperature. In *B. psychrophilus*, a shift from 20 to 0^0 C resulted in eleven cold acclimation proteins (eight were identified as csps), 20 to 5^0C resulted in ten caps and a shift from 20 to 10^0 C resulted in only four caps (Bayles *et al.*, 1996). The correlation between the increase in numbers and relative synthesis of the csps and caps with lower cold shock or growth temperatures may suggest that the production of such proteins constitutes an adaptive response by the bacterium to permit or aid growth at lower temperatures.

Cloutier and coworkers (1992) examined the cold shock response in temperate and Arctic strains of rhizobia. A variety of cold shock regimes were employed and for all treatments the Arctic rhizobia had a survival rate slightly lower than that of temperate strains. These results suggest that the Arctic strains cold adaptation for growth may not provide them with a better survival rate under freezing conditions.

A large discrepancy exists in the number of cold shock proteins found in each genera and even among strains. For example, in a study of *Listeria*, Bayles and coworkers (1996) found 12 cold shock proteins, while a study published at the same time by other researchers found 32 cold shock proteins (Phan-Thanh & Gormon, 1995). It is unclear whether the discrepancy is due to the use of different strains or the use of different experimental conditions. The criteria for the

classification of a protein as a cold shock protein and the methods used to compare the intensities of spots and bands on protein gels vary among researchers and protein separating systems (Bayles *et al.*, 1996).

3. The Heat Shock Response of *E. coli*

i. Overview

A well characterized stress response is the heat shock response. It usually refers to an increase in temperature ranging from a few degrees to over 20^{0}C above the normal growth temperature. The increased synthesis of a small set of proteins by exposing the cells to a non-lethal heat shock has been found to be universal in all organisms examined (Watson, 1990). As with cold shock, the majority of research on the heat shock response has been completed using *E. coli.* The result of a heat shock is the acquired thermotolerance of the organism. A sub-lethal increase in temperature can cause a 2 to 20 fold increase in the synthesis of at least 17 polypeptides in *E. coli.* (Watson, 1990). These proteins vary in size (10,100 - 94,000 kDa), net charge, quantity and extent of inducibility by heat (Neidhardt, 1987). This increased rate of synthesis occurs much faster in heat shocking (with the peak being from 5-15 minutes) as opposed to cold shocking with the peak occurring after about 2 h. After the maximum rate of synthesis is reached, the levels of heat shock proteins decline to a steady state level with the initiation of normal protein synthesis (Watson, 1990).

ii. Regulation

The heat shock response is positively regulated at the level of transcription by the sigma 32 (σ^{32}) polypeptide (Ang et al., 1991). Normal proteins use σ^{70} to bind to their promoters while heat shock proteins use σ^{32}. Sigma-32 acts as a transcriptional factor of RNA polymerase. At 37^{0}C, the secondary structure of the mRNA of σ^{32} is stable and the starter codon is inaccessible. At 42^{0}C, melting of the protein begins and the secondary structure is no longer stable and unfolds allowing the ribosome to attach and begin translation. Once this new sigma factor is synthesized it allows the other heat shock proteins to be produced.

iii. Function

The primary function of the heat shock response is to confer thermal resistance to the organisms by the production of a subset of proteins. Most of the heat shock proteins that have been identified play a role in the major cell processes such as DNA replication, RNA synthesis, protein synthesis, protein processing and protein degradation (Neidhardt, 1987). Heat shock proteins play a variety of roles in the cell including prevention of denaturation by preventing folding and unwanted protein interactions, the repair of improperly folded proteins and as an aid in proteolysis of non-repairable proteins (Watson, 1990).

4. Relationship Between Different Stress Systems

i. Relationship Between Stress Systems (not including the CSR)

Many of the genes of *E. coli* are organized into regulons, in which unlinked and individually controlled genes can be coordinately controlled by a common regulatory gene.

Most of the regulons of *E. coli* are responsive to environment stress, nutrient limitation, or damage caused by toxic chemical or physical agents (VanBogelen *et al.*, 1987). There are numerous examples of how exposure to one stress makes an organism better suited to survive other adverse conditions. For example, there are multiple inducers of the heat shock response. These other inducers usually cause the production of about fifty percent of the major heat shock proteins. However heat is still the most potent inducer and is the only agent that causes this response under conditions which minimally affect microbial growth rate (Neidhardt, 1987). One agent that comes the closest to mimicking heat is ethanol since all 17 proteins are induced. Other agents that cause the induction of this response in the order of their effectiveness are: puromycin, viral infection, nalidixic acid, methylating and alkylating agents, cadmium chloride, hydrogen peroxide and amino acid restriction (Neidhardt, 1987). The targets of these inducers are not known but many of the agents that can induce this response also lead to the accumulation of damaged, denatured or incorrectly folded proteins (Watson, 1990). If *Salmonella typhimurium* becomes adapted to hydrogen peroxide, it not only becomes resistant to toxic doses of the chemical, but it is also resistant to destruction by heat (Watson, 1990). This demonstrates a link between heat and oxidative stress response systems. On the other hand, *E. coli* pre-treated with hydrogen peroxide will also become resistant to higher doses of the substance and to near-ultraviolet radiation, but not to heat as in the case of *Salmonella* (Watson, 1990). Meury and Kohiyama (1991) demonstrated that the heat shock protein DnaK is involved in the adaptation of *E. coli* to environments with high osmolarity. Other relationships between stress responses have also been noted. The production of starvation proteins caused an increase in thermal and oxidative resistance in *E. coli* (Jenkins *et al.*, 1990). This system also works in the reverse with

starvation proteins being induced by thermal or oxidative stress. During an osmotic shock, 5 previously identified starvation proteins and 2 heat shock proteins were identified (Jenkins *et al.*, 1990).

Spector and coworkers (1986) examined protein induction under various stress conditions in *S. typhimurium* including starvation, heat, and anaerobiosis. They found considerable overlap among the proteins induced under the various starvation conditions but the overlap was minimal between the starvation proteins and the heat shock or anaerobic proteins. These results suggested that different regulatory pathways control each of these stress responses (Spector *et al.*, 1986). Since some mutual proteins were induced it would also indicate interconnecting regulation (Spector *et al.*, 1986).

ii. Relationship Between the Cold Shock Response and Other Stress Response Systems

Exposure of microorganisms to lowered temperature is not the only stress that induces cold shock proteins. Welch and coworkers (1993) found that when *E. coli* was shifted to an area of increased hydrostatic pressure, 55 proteins were induced, 11 of which were heat shock proteins, while 4 were cold shock proteins. Although heat and cold shock responses can exert opposite reactions to a variety of conditions including temperature and antibiotics which target ribosomes, some notable similarities exist (Welch *et al.*, 1993). For example, both high pressure and low temperatures inhibit an early stage of translation. Therefore it has been suggested that the function of the cold shock response is to adapt to the facilitation of the expression of genes involved in the initiation of translation. Also high pressure and low temperatures allow the

continued production of stringently controlled transcriptional and translational proteins when the growth rate decreases (Welch *et al.*, 1993).

The cold shock response has also been linked to the acid shock system. For example Heyde and Portalier (1990) reported the induction of 16 polypeptides in *E. coli* by shifting the growth medium pH from 6.9 to 4.3. Four of these polypeptides were shown to correspond to heat shock proteins, while three others were stress proteins that were induced by osmolarity, low temperature or aerobiosis.

5. The Impact of Freezing on Microorganisms

During freezing, microorganisms may suffer from a variety of damages that may have immediate or subsequent consequences on the cell. The impact is dependent upon a number of factors including the rate and temperature of freezing, length and temperature of storage, and the composition of the suspending medium in which the cells are frozen (Doyle *et al.*, 1997)

Gram negative mesophilic bacteria appear to be more susceptible to death or injury due to freezing than Gram positive bacteria (Doyle *et al.*, 1997). Bacteria in the logarithmic phase of growth are also most susceptible (Frazier & Westhoff, 1988). When mesophilic bacteria are rapidly chilled from their normal growth temperature, immediate death to a proportion of the culture results. In addition to death, freezing may also cause metabolic injury, presumably by damage to the plasma membrane (Doyle *et al.*, 1997). The sudden drop in temperatures results in membrane phase transitions from liquid crystal to gel status without allowing lateral phase separation of phospholipid and protein domains. This may result in the loss of permeability of the plasma and outer membranes (Doyle *et al.*, 1997).

During freezing, bacteria will experience an osmotic shock in addition to the mechanical injury caused by ice crystal formation. As the temperature is lowered, and more water freezes, the remaining free water becomes more concentrated with solutes (Doyle *et al.*, 1997). This can alter the pH and ionic strength of the cytosol leading to the denaturation of proteins and impairment of the function of DNA, RNA plus cellular organelles. Ice crystals can form outside the cell (extracellular ice) causing the water to be drawn out of the cell resulting in a dehydration or concentration effect (Frazier & Westhoff, 1988). When cells are thawed, they are re-exposed to the osmotic effects.

The length of frozen storage also impacts the survival of bacteria. The number of viable organisms decrease with storage time. The initial death rate during freezing is rapid, but it is followed by a gradual reduction of microorganisms (Frazier & Westhoff, 1988). Bacterial cells may undergo reversible or irreversible injury during frozen storage. Irreversible injury repair can occur if nutrients, energy sources and specific ions are present and metabolism can commence. The temperature of storage also plays a role in survival. The lower the temperature of frozen storage, the slower the death rate of survivors (Doyle *et al.*, 1997)

The rate of freezing is an important factor influencing microbial viability. If freezing occurs slowly, crystallization occurs extracellularly. The bacterial cells lose water because they have a vapour pressure greater than that of the ice crystals; this water will freeze extracellularly (Doyle *et al.*, 1997). There is a further decrease of water output from the cells because the crystallization causes the concentration of the external solution to increase. A slow freezing causes increased injury because the bacteria are exposed to this osmotic effect for a longer time period (Doyle *et al.*, 1997). When freezing occurs rapidly, the survival rates

increase because the cells are exposed to the osmotic stress for a reduced length of time. There appears to be a critical range of temperatures which results in lethal effects (more organisms are inactivated at -4 to -10^{0}C than at -15 to -30^{0}C) (Frazier & Westhoff, 1988). However, if freezing rates are too high, crystal formation can occur intracellularly, resulting in an increase in injury and a decrease in survival rate (Doyle *et al.*, 1997). Intracellular crystals may form through the cell, resulting in altered permeability or "holes" in the membrane and cell wall. Intracellular ice is thought to be more harmful to cells than extracellular ice crystals (Frazier & Westhoff, 1988).

One of the most important factors in determining the effect of freezing on microbial cells is the composition of the suspending medium. Certain compounds present in the medium may either enhance or diminish the lethal effects of freezing (Doyle *et al.*, 1997). The composition of food influences the rate of death during freezing and storage. Sugar, salt, proteins, colloids, fat, and other substances may be protective, whereas high moisture and low pH may hasten killing (Frazier & Westhoff, 1988).

MATERIALS AND METHODS

1. Bacterial Strains and Growth Conditions

E. coli O157:H7 strains 7283 and 7282 (beef isolates), 7110, 7128, 7236 (human isolates), and 7174 (bovine isolate) were kindly donated by the Laboratory Centre for Disease Control, Ottawa, Canada. A non-enterohemorrhagic *E. coli* strain (MY20) was obtained from the Food Development Center, Portage la Prairie, MB. All strains were maintained on trypticase soy agar plates (TSA, BBL, Cockeysville, MD; pH 7.0) at 4°C. Cultures were activated by transferring loop inocula into 20 ml of trypticase soy broth (TSB, BBL; pH 7.0) at 37° C. Following two consecutive 24-h culture transfers, 10 μl were inoculated into TSB (20 ml) contained in 50-ml Erlenmeyer flasks and incubated at 37° C for 4 h using a rotary shaker (150 rpm). This protocol resulted in mid-exponential cultures (determined from previous growth studies).

2. Products

Regular ground beef, uncooked dinner sausage (casing removed after purchase) and medium ground pork (chub pack) were purchased at a local retail store; whole pasteurized egg was provided by Inovatech, Winnipeg, MB. The products (10g) were weighed into sterile sampling bags (180 g capacity, Fisher, ON) and spread to a uniform thickness of about 2 mm. Following freezing (-20°C) the samples were sterilized (25 kGy) using electron beam irradiation (Impela 1-10/1, AECL Pinawa, MB) and stored at -20°C until use. Additional samples (5 ml) consisting of: milk (2%, UHT; retail), sterile TSB (BBL, pH 7.0) and TSB, pH adjusted to 5.0 with 1M citric acid, were aseptically transferred to 15 ml sterile plastic tubes (Corning Inc., Corning, N.Y.) and held at 4°C until use.

3. Product Inoculation and Cold Shocking

Mid-log cultures (ca. 10^7 CFU/ml, 10μl) of *E. coli* strains grown at 37°C as previously outlined were separately inoculated into each product to achieve a level of ca. 10^6 CFU/ml or g with the exception of ground pork where a cocktail consisting of three (7282, 7110, 7283) strains was used as the inoculum (same total cell concentration). Following inoculation of the egg or meat products, the sample bags were throughly massaged by hand to distribute the organisms. The liquid/broth products were vigorously mixed for 30 sec. All food samples were initially tempered to 10°C prior to inoculation. After inoculation, the cells receiving cold shock treatment were placed at 10° C for 1.5 h. The samples were then placed in a freezer and maintained at -20 °C ± 5°C for up to 28 d. The controls were placed directly into the freezer (-20° C ± 5° C) following inoculation for up to 28 d.

4. Determination of Survivors Following Frozen Storage

E. coli survivors (CFU/g or ml) were assessed on predetermined days. The sampling times for several strains were varied in some products including milk and TSB. In these products 3 strains (7128, 7283, my20) were sampled on days 0,7,14, and 28 to obtain preliminary results. Since there were only small changes in survival between days 7 and 14, all subsequent strains were sampled only on days 0, 1,7, and 28. The samples were thawed (liquid samples 30 min.; solid samples 10 min.) at room temperature (~ 22°C) before analysis. Sterile peptone water (0.1%, 90 ml) was added to the meat and egg sample bags which were then massaged by hand. Following serial dilution (0.1% sterile peptone water) the samples were surface plated in duplicate (0.1 ml) or quadruplicate (0.25 ml) on TSA (BBL) and violet red bile

agar (VRBA, BBL) and incubated for 24 h at 37°C. The latter medium was used to assess injury incurred as a result of freezing. Colonies from one culture plate (square root number) per product were streaked onto Sorbitol MacConkey agar (Difco, Detroit, MN) to assess culture purity. Survivors (colony forming units; CFU/g or ml) and percent (%) injury were determined. The % injury was determined using the following equation: $\frac{\text{CFU TSA - CFU VRBA}}{\text{CFU TSA}} \times 100$

5. Temperature Shifts to Induce Cold Shocking

Mid-log cultures of *E. coli* O157:H7 (strain 7282) (ca. 10^7 CFU ml^{-1}) were grown in TSB at 37°C as previously outlined. Following inoculation into a series of sterile test tubes containing UHT milk (5 ml) maintained at 37°C, replicate tubes were either directly transferred to a freezer at -20°C ±5°C (control) or rapidly cooled on ice and maintained at 10°C for 1.5 h and then frozen. At 0, 1, and 7 days, tubes were thawed at room temperature and survivors were enumerated on both TSA and VRBA following incubation at 37°C for 24 h. Similar studies were performed using the following growth-cold shock temperature protocols: 37-10°C, 30-10°C, 30-15°C, and 20-10°C.

6. Thermal Resistance

A series of sterile plastic tubes (15 ml, Corning) containing TSB (5 ml, pH 7.0) were initially tempered in a water bath (Magni Whirl, Blue Island, IL) maintained at 48.5± 1°C. Following inoculation with cold shocked *E. coli* (strain 7282) (10µl), they were mixed and returned to the water bath. At predetermined time intervals the tubes were removed from the water bath and quickly mixed; aliquots (1 ml) were then pipetted into tubes containing pre-chilled peptone (0.1%, 9 ml) and immediately placed on ice. Samples were serially diluted

(0.1% peptone) and surface plated on TSA and VRBA. Colonies were counted following incubation at 37°C for 24 h. A similar protocol was followed for non-cold shocked cells.

Cold shocking of cells was accomplished by initially placing flasks containing mid-log grown *E. coli* in an ice bath for ca. 5 min. to quickly drop the temperature to 12°C. The flasks were subsequently transferred to a temperature controlled water bath maintained at 10 ± 1°C for 1.5 h . Non cold shocked cells or controls were prepared in a similar manner, however, once the flask contents reached 10°C the cells were immediately used as inocula. The temperature drop was monitored using a digital thermometer (Tegam) which was placed in a non-inoculated flask.

7. Statistical Analysis

All experiments with the exception of the thermal resistance study were performed in triplicate. Analysis of variance using General Linear Model of the Statistical Analysis System (SAS Institute, Inc., Cary, N.C.) was used to determine significant differences ($p \leq 0.05$) in microbial populations. Mean comparisons were performed using Duncan's Multiple Range Test. Statistics were not performed on % injury calculations because the standard deviations were too high. Therefore, only apparent trends in % injury are reported.

8. Isoelectric Focusing

i. Cell Preparation and Cold Shocking

Mid-exponential cultures of *E. coli* (strain 7283) grown in TSB (pH 7.0) were cold shocked and then harvested by centrifugation (25°C (non-cold shocked), 10°C (cold shocked) 10,000 g, 20 min, Sorvall, Inc., Norwalk, CT).Resultant pellets were washed twice in 10mM Tris buffer (pH 6.8) and transferred to ice-chilled beakers containing 25 ml of 1% CHAPS in 10mM Tris buffer (pH 6.8). Following sonication (Braunsonic 1510, B. Braun, Melsungen, AG) at 250 watts (5 bursts) and centrifugation (4°C, 7800 g, 15 min), the resultant supernatants were transferred to sterile tubes and kept at -20°C until required.

Cold shocking consisted of placing flasks containing *E. coli* in an ice bath in order to rapidly (ca. 5 min) drop their temperature to approximately 12°C. The cultures were then transferred to a temperature controlled incubator maintained at 10±1°C for 1.5 h. The time-temperature decrease was monitored using a digital thermometer (Omega Engineering, Inc., Stanford, CT.) which was placed in a non-inoculated flask. Non-cold shocked cultures (controls) were prepared in a similar manner, however, following growth they were not subjected to a 10°C exposure. Instead cells were directly centrifuged (25°C) and subsequently sonicated.

ii. Protein Determination

Sample protein concentrations were assessed prior to isoelectric focusing using the Pierce Protein Assay Reagent (Pierce Chemical Co., IL.). A series of protein standards (200-1500 µg/ml) were prepared using a stock solution (2 mg/ml) of bovine serum albumen

(Pierce Chemical Co., IL.) diluted with 0.1 N NaOH.

The standards and test protein samples (0.1ml) were reacted with 5 ml of Protein Assay Reagent. Absorbance readings were determined at 595 nm and a standard curve was prepared (Appendix 1).

iii. Isoelectric Focusing

The isoelectric focusing was carried out on an LKB 2117 Multiphor apparatus (Sweden) with an LKB 2197 Constant Power Supply. The temperature was controlled at 10°C with a Haake circulating water bath (West Germany). A Pharmacia Biotech Ampholine PAG (polyacrylamide gel) plate with a pH range of 4.0-6.5 was used with cathode (0.1M β-alanine) and anode (0.1M glutamic acid in 0.5 M H_3PO_4) electrode strips. Protein samples and standards (Pharmacia Biotech standards, 20µl; pH 2.5-6.5) were applied to applicator pieces and placed on the surface of the gel.

Table 1 Isoelectric points of the Pharmacia Biotech standards for isoelectric focusing

PROTEIN	PI (24°C±1.5°C)
pepsinogen	2.80
amyloglucosidase	3.50
glucose oxidase	4.15
soybean trypsin inhibitor	4.55
β-lactoglobulin A	5.20
bovine carbonic anhydrase B	5.85
human carbonic anhydrase B	6.55

The gel was focused for 2.5 h at a constant power of 25 W. The applicator pieces were removed after about 1.25 h Upon completion, the electrode strips were removed and the gel was fixed for 30 min, destained for 5 min, stained for 15 min, destained for 48 h and preserved for 1 h. After the gel was preserved a mylar plastic sheet soaked in preserving solution was placed over the gel and dried at room temperature. The compositions of the solutions used for isoelectric focusing are given in Table 2

A calibration curve (Appendix 2) was established by plotting the isoelectric points of the standards as a function of their migration distances from the cathode. The isoelectric points of the protein samples were determined by measuring the band distances from the cathode.

Table 2 Solutions used in isoelectric focusing

SOLUTION		SOLUTION COMPOSITION
Fixing solution	29g	trichloroacetic acid
	8.5g	sulphosalicylic acid
		adjusted to 250 ml with distilled water
Destaining solution	500 ml	ethanol
	160 ml	acetic acid
		adjusted to 2.0 l with distilled water
Staining solution	0.46 g	Coomassie Brilliant Blue R-250
	400 ml	destaining solution
		mix and filter through Whatman No.4 filter paper
		heat to 60°C before use
Preserving solution	25 ml	glycerol
		make up to 250 ml with destaining solution

9. Proximate Analysis for Meat and Egg Products

i. Moisture Content Determination

Samples were placed into pre-dried weighed aluminum dishes, weighed and freeze dried (Virtis Co., Gardiner, NY) ca. 72h. The samples were then weighed to determine the moisture content. These dried samples were used for all subsequent proximate analyses.

ii. Fat Content Determination

The fat content was determined using a modified Soxhlet method (AOAC, 1975). Three-5g dried samples were placed into cellulose extraction thimbles and covered by glass wool. The thimbles were placed into extracting tubes. Flat bottom flasks (250 ml) containing boiling chips were predried at 100°C (1 h), cooled in a desiccator and weighed. Hexane (150 ml) was added to each flask. The extracting tubes were inserted into the flasks and refluxed (18 h). After refluxing, the remaining solvent was evaporated using a heating mantle and the flasks were placed in a drying oven (100°C, 1 h). The flasks were then cooled in a desiccator and weighed. The % fat was determined as described in AOAC (1975).

iii. Total Ash Determination

The total ash content was determined as described in the AOAC Official Methods (AOAC, 1975).

iv. Protein Determination

The protein content was determined using the micro-Kjeldahl method as described in the AOAC Official Methods (AOAC, 1975). The nitrogen to protein conversion factor used for all samples was 6.25.

RESULTS

1. Isoelectric Focusing

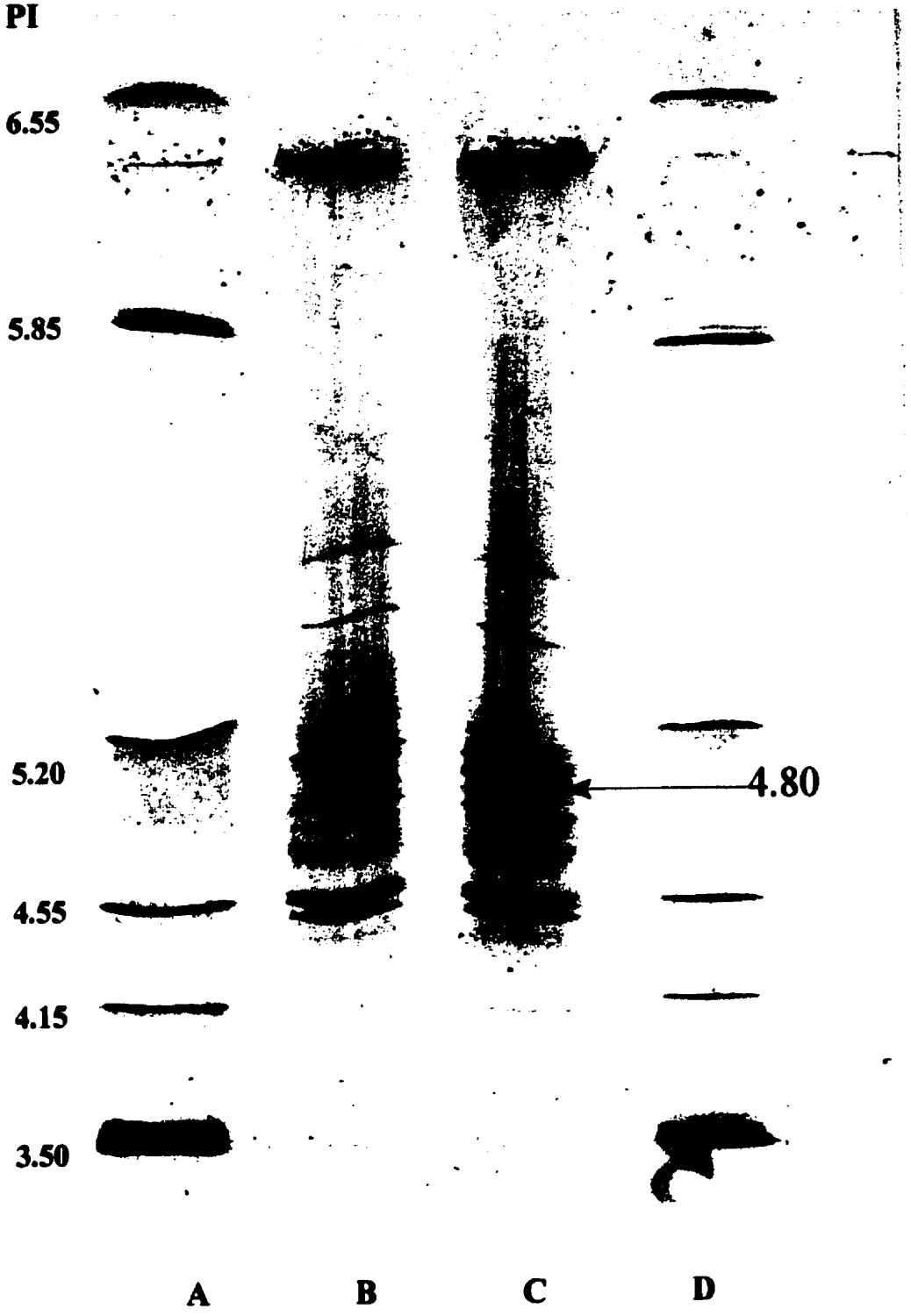
Isoelectric focusing was used to investigate whether the cold shocking conditions used for this study caused a change in the pattern of protein synthesis. The isoelectric focusing patterns of the cold shocked and the non-cold shocked *E. coli* O157:H7 are shown in Figure 1. Goldstein and coworkers (1990) reported that the isoelectric point (pI) of CspA was 5.92. In our experiments we did not localize CspA; however, a novel single band with a pH of 4.8 was found that was produced consistently under cold shocking conditions. Although the identity of this protein is not known, its presence confirmed that the protocol used to induce the cold shock response did cause an alteration in the pattern of protein synthesis.

2. Effect of Cold Shocking on Survival and % Injury of *E. coli* Maintained at -20°C.

i. Ground Beef

The survival and % injury of *E. coli* in ground beef on days 0, 1, 7, and 28 are presented in Tables 3 to 6 respectively. As all *E. coli* strains behaved similarly, only the survival pattern of strain 7110 is illustrated (Fig. 2). In the majority of cases, at each sampling period, the difference in the number of survivors obtained between cold and non- cold shocked strains, recovered on either TSA or VRB, was not significant ($p \leq 0.05$). Over the 28 day storage period the number of survivors declined, but usually not more than 1 $\log_{10}$ CFU/g. Overall, there was not a significant difference observed over the course of storage between cold and non-cold shocked cells regardless of recovery medium.

Fig. 1. Isoelectric focusing of cold and non-cold shocked *E. coli* (strain 7283) in tryptic soy broth. Lanes A & D - standards, lane B - non-cold shocked, lane C - cold shocked


PI
6.55
5.85
5.20
4.55
4.15
3.50
4.80
A
B
C
D

Although the % injury for all strains increased with storage time, there did not appear to be a difference in the injury level between cold and non-cold shocked *E. coli*. In all cases the extent of injury was less than 1 $\log_{10}$ CFU/g.

Table 3. Survival and injury levels of *E. coli* in ground beef on day 0 .

Strain	Source	Survivors $\log_{10}$ CFU/g		% Injury
		TSA[1]	VRBA[1]	
7128	human	5.16 ± 0.03	5.16 ± 0.03	0.91 ± 0.80
7282	food	6.29 ± 0.04	6.33 ± 0.13	1.73 ± 3.00
7110	human	6.35 ± 0.04	6.38 ± 0.04	1.72 ± 2.28
7283	food	6.14 ± 0.03	6.09 ± 0.03	11.15 ± 9.64
7174	bovine	6.16 ± 0.08	6.14 ± 0.10	6.08 ± 5.49
7236	human	6.42 ± 0.11	6.46 ± 0.07	3.43 ± 5.94
my20	food	5.74 ± 0.18	5.65 ± 0.10	17.21 ± 14.67

[1] Results ($\log_{10}$ CFU/ g) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).

Table 4. Survival and injury levels of *E. coli* in frozen (-20°C) ground beef at day 1.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	5.06 ± 0.11[a]	-	4.93 ± 0.12[a]	0.22	26.87 ± 4.44
7128 CS[3]	5.18 ± 0.08[a]		5.15 ± 0.07[b]		7.21 ± 2.75
7282 NS	5.97 ± 0.04[a]	-	5.96 ± 0.11[a]	-	7.85 ± 8.37
7282 CS	6.11 ± 0.10[a]		6.08 ± 0.12[a]		8.10 ± 6.75
7110 NS	6.04 ± 0.09[a]	-	6.02 ± 0.14[a]	-	9.19 ± 9.34
7110 CS	5.94 ± 0.24[a]		5.86 ±0.24[a]		15.75 ± 15.60
7283 NS	6.00 ± 0.08[a]	-	5.94 ± 0.08[a]	-	13.02 ± 2.08
7283 CS	5.98 ± 0.19[a]		5.82 ± 0.34[a]		27.63 ± 23.60
7174 NS	6.03 ± 0.01[a]	-0.15	5.92 ± 0.02[a]	-	21.08 ± 3.01
7174 CS	5.88 ± 0.03[b]		5.86 ± 0.05[a]		7.83 ± 6.82
7236 NS	6.16 ± 0.06[a]	-	6.03 ± 0.04[a]	-	24.89 ± 7.62
7236 CS	6.14 ± 0.10[a]		6.04 ± 0.07[a]		20.01 ± 9.60
my20 NS	5.27 ± 0.32[a]	-	5.18 ± 0.33[a]	-	17.07 ± 3.67
my20 CS	5.44 ± 0.34[a]		5.29 ± 0.43[a]		27.61 ± 18.88

[1] Results ($\log_{10}$ CFU/ g) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same superscript for each medium and strain are not significantly different ($p \leq 0.05$).
[5] Net increase ($\log_{10}$ CFU/g) = Log_{10} CFU/g (CS) - Log_{10} CFU/g (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Table 5. Survival and injury levels of *E. coli* in frozen (-20°C) ground beef on day 7.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	4.76 ± 0.07[a]	-	4.51 ± 0.14[a]	-	44.20 ± 9.21
7128 CS[3]	4.56 ± 0.44[a]		4.39 ± 0.34[a]		31.48 ± 14.92
7282 NS	5.67 ± 0.11[a]	-	5.46 ± 0.24[a]	-	37.03 ± 16.45
7282 CS	5.74 ± 0.17[a]		5.48 ± 0.11[a]		44.20 ± 13.85
7110 NS	6.06 ± 0.12[a]	-	5.97 ± 0.09[a]	-	14.69 ± 12.73
7110 CS	5.90 ± 0.04[a]		5.82 ± 0.07[a]		6.53 ± 5.84
7283 NS	5.57 ± 0.09[a]	-	5.39 ± 0.17[a]	-	33.95 ± 30.90
7283 CS	5.44 ± 0.10[a]		5.27 ± 0.03[a]		30.86 ± 18.71
7174 NS	5.65 ± 0.13[a]	-	5.36 ± 0.13[a]	-	43.68 ± 31.14
7174 CS	5.40 ± 0.23[a]		5.17 ± 0.21[a]		41.11 ± 5.19
7236 NS	5.88 ± 0.14[a]	-	5.74 ± 0.10[a]	-	26.92 ± 8.87
7236 CS	6.01 ± 0.15[a]		5.88 ± 0.10[a]		24.20 ± 22.73
my20 NS	4.96 ± 0.22[a]	-	4.32 ± 0.25[a]	-	76.88 ± 3.73
my20 CS	5.46 ± 0.41[a]		5.25 ± 0.45[a]		37.35 ± 10.54

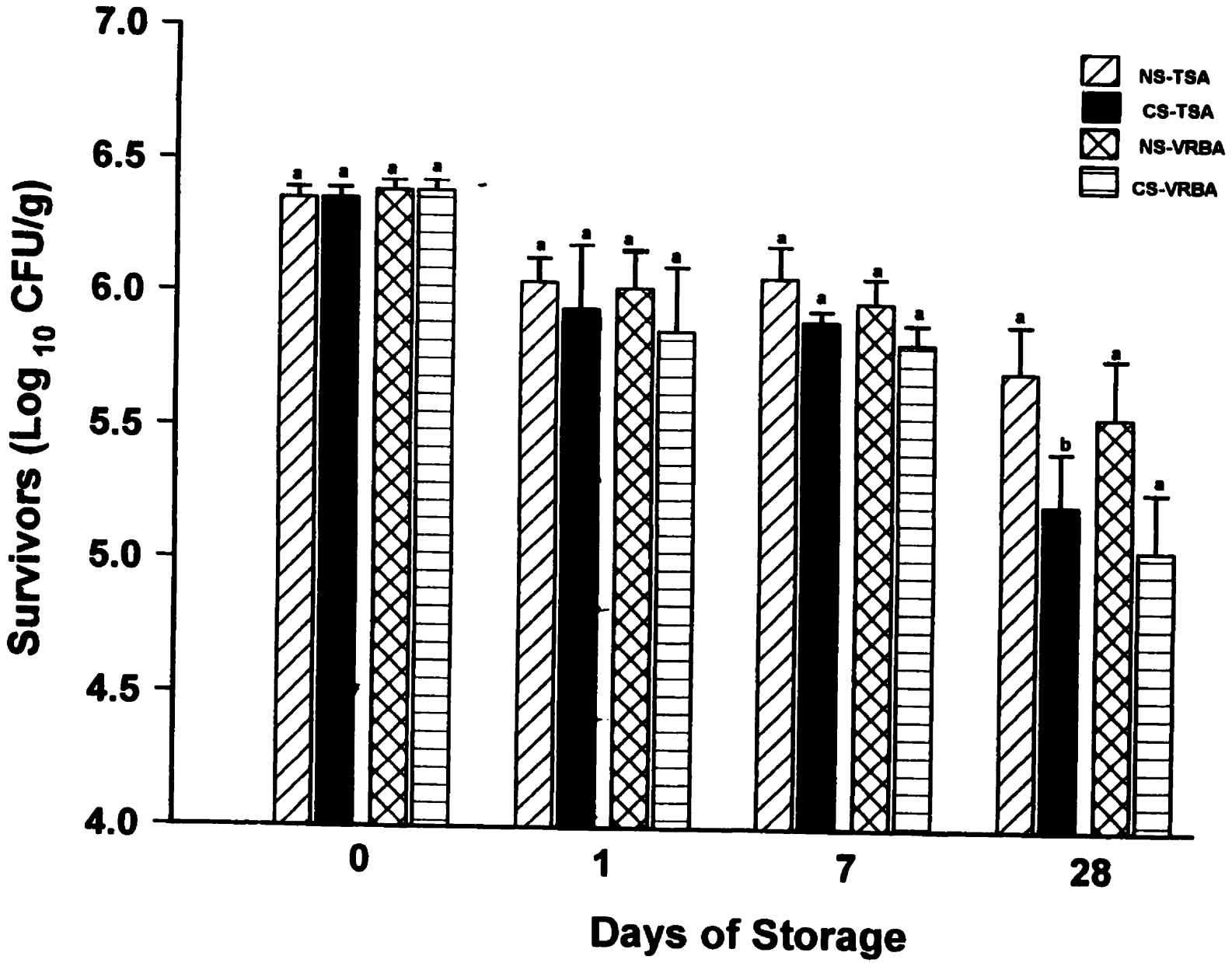
[1] Results ($\log_{10}$ CFU/ g) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same letter for each medium and strain are not significantly different ($p \leq 0.05$).
[5] Net increase ($\log_{10}$ CFU/g) = Log_{10} CFU/g (CS) - Log_{10} CFU/g (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Table 6. Survival and injury levels of *E. coli* in frozen (-20°C) ground beef on day 28.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	4.41 ± 0.12[a]	-	3.85 ± 0.17[a]	0.39	65.96 ± 6.05
7128 CS[3]	4.46 ± 0.07[a]		4.24 ± 0.09[b]		40.27 ± 3.56
7282 NS	5.28 ± 0.19[a]	-	4.97 ± 0.39[a]	-	47.10 ± 23.28
7282 CS	4.89 ± 0.16[a]		4.46 ± 0.18[a]		62.20 ± 5.81
7110 NS	5.72 ± 0.17[a]	-0.50	5.55 ± 0.22[a]	-	32.63 ± 6.65
7110 CS	5.22 ± 0.20[b]		5.05 ± 0.22[a]		32.55 ± 2.59
7283 NS	5.20 ± 0.05[a]	-	4.80 ± 0.19[a]	-	58.97 ± 14.17
7283 CS	5.28 ± 0.23[a]		5.02 ± 0.26[a]		44.78 ± 5.63
7174 NS	5.17 ± 0.08[a]	-	4.74 ± 0.13[a]	-	60.93 ± 14.14
7174 CS	5.07 ± 0.02[a]		4.67 ± 0.06[a]		59.55 ± 7.10
7236 NS	5.09 ± 0.23[a]	-	4.77 ± 0.21[a]	-	51.75 ± 8.51
7236 CS	4.95 ± 0.41[a]		4.69 ± 0.43[a]		45.19 ± 7.06
my20 NS	3.95 ± 0.18[a]	-	3.32 ± 0.21[a]	-	76.55 ± 3.53
my20 CS	5.13 ± 0.38[a]		4.39 ± 0.41[a]		82.11 ± 1.69

[1] Results ($\log_{10}$ CFU/ g) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same letter for each medium and each strain are not significantly different ($p \leq 0.05$).
[5] Net increase ($\log_{10}$ CFU/g) = Log_{10} CFU/g (CS) - Log_{10} CFU/g (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Fig. 2. Effect of cold shocking on the survival of *E. coli* (strain 7110) in ground beef at -20°C. Results with the same superscript for each medium and storage time are not significantly different ($p \leq 0.05$). Bars represent averages of triplicate results each performed in duplicate ($n=6 \pm SD$). Non-cold shocked (NS); cold shocked (CS); tryptic soy agar (TSA); violet red bile agar (VRBA).


7.0
6.5
6.0
5.5
5.0
4.5
4.0
Survivors (Log 10 CFU/g)
NS-TSA
CS-TSA
NS-VRBA
CS-VRBA
a
b
0
1
7
28
Days of Storage

ii. Ground Pork

The impact of cold shocking was examined using ground pork with a cocktail of 3 *E. coli* O157:H7 strains (7282, 7110, 7283) which were randomly chosen from the 6 *E. coli* 0157:H7 strains used in this study. The results are presented in Table 7 and Figure 3. Samples evaluated on the first day with TSA showed significantly higher survivor levels for cold shocked *E. coli.* However, significant differences in survival levels were not observed on VRBA. For all other sampling days, there were no significant ($p \leq 0.05$) differences in survivor levels between the two treatments. As with ground beef, the survivor rates remained high throughout the frozen storage period.

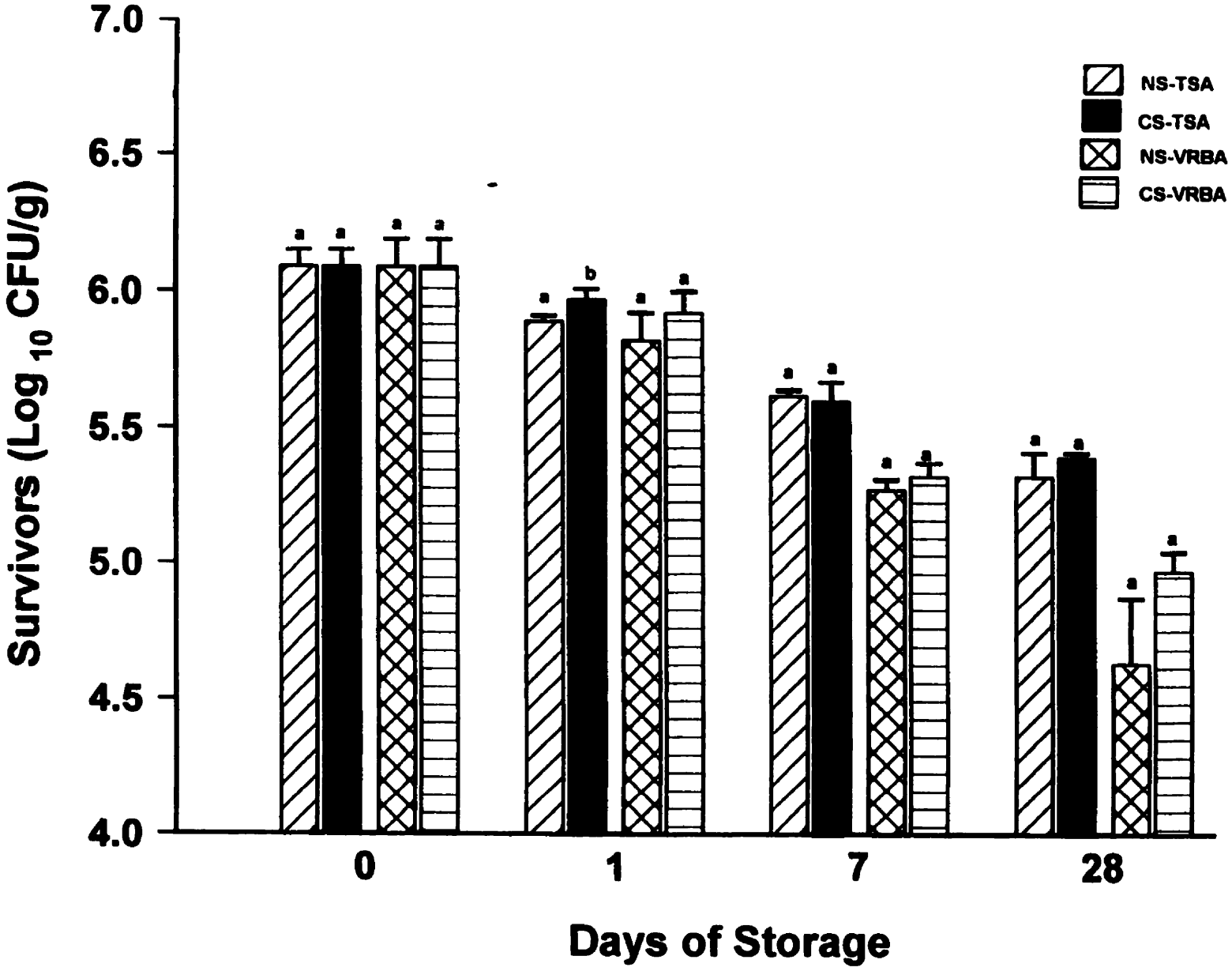
The % injury for both cold and non-cold shocked *E. coli* increased with storage time. In addition, all non-cold shocked cells appeared to exhibit a slightly higher level of injury than the cold shocked cells. The difference in % injury between cold and non-cold shocked cells appeared to increase with time of frozen storage.

Table 7. Effect of cold shocking on survival and injury levels of *E. coli* O157:H7 (strains 7110, 7282, 7238) in ground pork at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
Day 0	6.09 ± 0.06		6.09 ± 0.10		2.74 ± 3.53
Day 1 NS[2]	5.89 ± 0.02[a]	0.08	5.82 ± 0.10[a]	-	15.24 ± 14.16
Day 1 CS[3]	5.97 ± 0.04[b]		5.92 ± 0.08[a]		9.59 ± 10.57
Day 7 NS	5.62 ± 0.02[a]	-	5.27 ± 0.04[a]	-	54.59 ± 1.43
Day 7 CS	5.60 ± 0.07[a]		5.32 ± 0.05[a]		47.00 ± 11.77
Day 28 NS	5.32 ± 0.09[a]	-	4.63 ± 0.24[a]	-	78.64 ± 8.31
Day 28 CS	5.39 ± 0.02[a]		4.97 ± 0.07[a]		61.88 ± 7.08

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/g) = Log_{10} CFU/g (CS) - Log_{10} CFU/g (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Fig. 3. Effect of cold shocking on the survival of *E. coli* (cocktail consisting of strains: 7110, 7282, 7238) in ground pork at -20°C. Results with the same superscript for each medium and storage time are not significantly different ($p \leq 0.05$). Bars represent averages of triplicate results each performed in duplicate ($n=6 \pm SD$). Non-cold shocked (NS); cold shocked (CS); tryptic soy agar (TSA); violet red bile agar (VRBA).


7.0
6.5
6.0
5.5
5.0
4.5
4.0
Survivors (Log 10 CFU/g)
NS-TSA
CS-TSA
NS-VRBA
CS-VRBA
a
a
a
a
a
b
a
a
a
a
a
a
a
a
a
a
0
1
7
28
Days of Storage

iii. Milk

The results of cold shocking on the survival and injury of *E. coli* in frozen (-20°C) milk are presented in Tables 8-12. As all *E. coli* strains behaved similarly, only the survival pattern of strain 7110 is illustrated (Fig. 4). In contrast to ground beef and pork, cold shocking resulted in a significant increase in the survival of *E. coli* for most strains during the entire storage period. By day 1 the difference in survivor levels between cold and non-cold shocked cultures was approximately 1 $\log_{10}$CFU/ml . By day 28 all cold shocked strains exhibited significant increases in survival when recovered on TSA. Strain 7128 appeared to benefit the least from cold shocking. The $\log_{10}$ CFU/ml increase in survival among strains by day 28 as a result of the cold shock treatment ranged from 0.51 to 1.89 and from 0.89 to 2.50 on TSA and VRBA, respectively (Table 12).

The % injury was generally higher in milk compared to results obtained in ground meat. Although the level of injury also increased with the time of storage, no clear pattern was observed between cold and non- cold shocked strains. In all cases injury levels were less than 1 $\log_{10}$CFU/ml.

Table 8. Survival and injury levels of *E. coli* in milk on day 0.

Strain	Source	Survivors $\log_{10}$ CFU/ml		% Injury[1]
		TSA[1]	VRBA[1]	
7128	human	6.60 ± 0.01	5.95 ± 0.07	0
7282	food	5.56 ± 0.06	5.58 ± 0.03	0
7110	human	5.69 ± 0.07	5.66 ± 0.08	8.52 ± 7.97
7283	food	6.24 ± 0.06	5.96 ± 0.09	0
7174	bovine	5.67 ± 0.03	5.43 ± 0.13	40.10 ± 13.83
7236	human	5.82 ± 0.03	5.70 ± 0.13	22.86 ± 20.90
my20	food	5.07 ± 0.11	4.88 ± 0.06	34.68 ± 12.13

[1] Results ($\log_{10}$CFU/ ml) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).

Table 9. Effect of cold shocking on survival and injury levels of *E. coli* in milk on day 1 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7282 NS[2]	3.61 ± 0.03^a	1.38	3.54 ± 0.05^a	1.15	12.77 ± 13.39
7282 CS[3]	4.99 ± 0.11^b		4.69 ± 0.04^b		49.74 ± 10.72
7110 NS	3.86 ± 0.11^a	1.34	3.72 ± 0.08^a	0.98	27.25 ± 12.72
7110 CS	5.20 ± 0.16^b		4.70 ± 0.18^b		68.30 ± 4.06
7174 NS	4.02 ± 0.03^a	1.23	3.83 ± 0.02^a	1.02	34.63 ± 3.08
7174 CS	5.25 ± 0.09^b		4.85 ± 0.04^b		59.70 ± 8.79
7236 NS	4.50 ± 0.09^a	0.97	4.21 ± 0.22^a	1.03	47.21 ± 15.68
7236 CS	5.47 ± 0.02^b		5.24 ± 0.04^b		41.03 ± 3.29

[1] Results ($\log_{10}$ CFU/ ml) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same superscript for each medium and strain are not significantly different ($p \leq 0.05$).
[5] Net increase ($\log_{10}$ CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).

Table 10. Effect of cold shocking on survival and injury levels of *E. coli* in milk on day 7 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	5.09 ± 0.05^{a}	0.48	4.61 ± 0.19^{a}	-	64.11 ± 19.98
7128 CS[3]	5.57 ± 0.16^{b}		5.09 ± 0.26^{a}		66.25 ± 8.93
7282 NS	2.59 ± 0.10^{a}	1.80	2.58 ± 0.05^{a}	1.48	11.76 ± 20.37
7282 CS	4.39 ± 0.027^{b}		4.06 ± 0.14^{b}		50.88 ± 15.46
7110 NS	2.90 ± 0.16^{a}	1.89	2.50 ± 0.07^{a}	1.83	58.91 ± 9.44
7110 CS	4.79 ± 0.13^{b}		4.33 ± 0.05^{b}		63.57 ± 12.76
7283 NS	3.97 ± 0.07^{a}	1.43	3.37 ± 0.10^{a}	1.25	74.91 ± 3.70
7283 CS	5.40 ± 0.06^{b}		4.62 ± 0.17^{b}		82.73 ± 5.72
7174 NS	3.14 ± 0.07^{a}	1.77	2.67 ± 0.06^{a}	2.45	65.64 ± 7.70
7174 CS	4.91 ± 0.07^{b}		5.12 ± 0.05^{b}		73.82 ± 4.56
7236 NS	4.07 ± 0.16^{a}	1.30	3.49 ± 0.31^{a}	1.63	72.21 ± 10.37
7236 CS	5.37 ± 0.07^{b}		5.12 ± 0.05^{b}		43.26 ± 6.34
my20 NS	3.32 ± 0.15^{a}	1.34	2.99 ± 0.10^{a}	1.24	52.33 ± 8.20
my20 CS	4.66 ± 0.02^{b}		4.23 ± 0.02^{b}		45.87 ± 2.30

[1] Results ($\log_{10}$ CFU/ ml) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same superscript for each medium and strain are not significantly different ($p \leq 0.05$).
[5] Net increase ($\log_{10}$ CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Table 11. Effect of cold shocking on survival and injury levels of *E. coli* in milk on day 14 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	4.86 ± 0.07^{a}	0.53	4.41 ± 0.19^{a}	0.48	63.80 ± 10.55
7128 CS[3]	5.39 ± 0.12^{b}		4.89 ± 0.15^{b}		67.98 ± 6.18
7283 NS	4.01 ± 0.15^{a}	1.23	3.58 ± 0.19^{a}	0.74	63.21 ± 3.88
7283 CS	5.24 ± 0.12^{b}		4.32 ± 0.12^{b}		87.99 ± 3.16
my20 NS	4.01 ± 0.03^{a}	0.96	3.69 ± 0.06^{a}	1.02	53.36 ± 6.22
my20 CS	4.97 ± 0.04^{b}		4.71 ± 0.04^{b}		62.54 ± 6.22

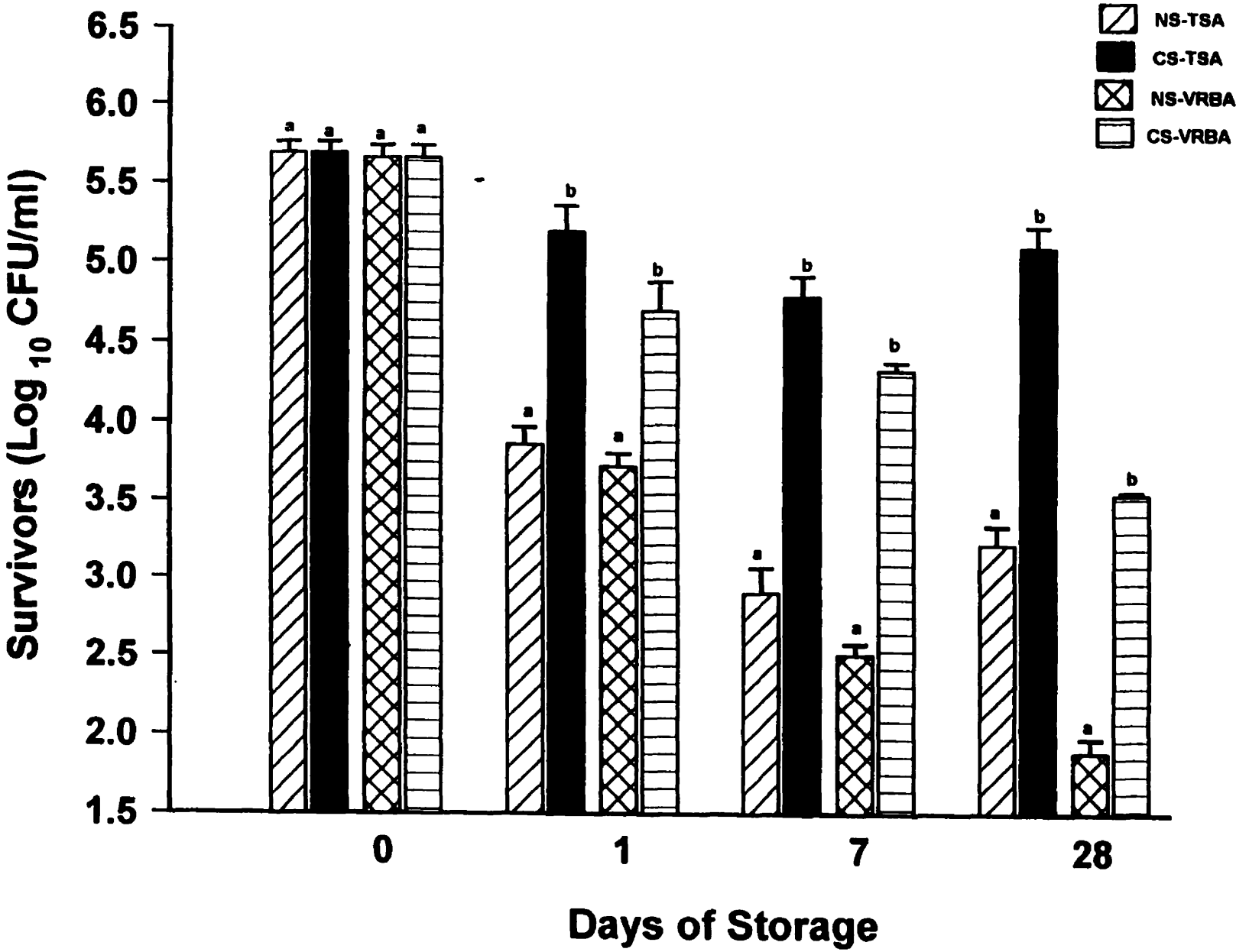
[1] Results ($\log_{10}$ CFU/ ml) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same superscript for each medium and strain are not significantly different ($p \leq 0.05$).
[5] Net increase ($\log_{10}$ CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).

Table 12. Effect of cold shocking on survival and injury levels of *E. coli* in milk on day 28 at - 20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	4.91 ± 0.08[a]	0.51	4.55 ± 0.27[a]	-	53.83 ± 20.07
7128 CS[3]	5.42 ± 0.13[b]		4.95 ± 0.24[a]		65.62 ± 8.63
7282 NS	3.04 ± 0.14[a]	1.58	1.73 ± 0.48[a]	-	94.11 ± 3.59
7282 CS	4.62 ± 0.04[b]		2.86 ± 0.43[a]		97.64 ± 2.29
7110 NS	3.21 ± 0.12[a]	1.89	1.88 ± 0.09[a]	1.66	95.22 ± 1.17
7110 CS	5.10 ± 0.13[b]		3.54 ± 0.02[b]		97.13 ± 0.84
7283 NS	3.77 ± 0.06[a]	1.52	2.94 ± 0.25[a]	0.89	84.37 ± 6.98
7283 CS	5.29 ± 0.03[b]		3.83 ± 0.02[b]		92.00 ± 0.33
7174 NS	3.24 ± 0.07[a]	1.68	1.44 ± 0.20[a]	2.50	98.23 ± 1.12
7174 CS	4.92 ± 0.11[b]		3.94 ± 0.18[b]		89.50 ± 1.72
7236 NS	4.41 ± 0.24[a]	0.98	2.94 ± 0.05[a]	1.80	96.29 ± 1.97
7236 CS	5.39 ± 0.06[b]		4.74 ± 0.17[b]		75.90 ± 10.82
my20 NS	3.56 ± 0.13[a]	0.89	2.06 ± 0.19[a]	1.44	96.81 ± 0.39
my20 CS	4.45 ± 0.07[b]		3.50 ± 0.13[b]		88.47 ± 3.19

[1] Results (log_{10} CFU/ ml) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same superscript for each medium and strain are not significantly different ($p \leq 0.05$).
[5] Net increase (log_{10} CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Fig. 4. Effect of cold shocking on the survival of *E. coli* (strain 7110) in milk at -20°C. Results with the same superscript for each medium and storage time are not significantly different ($p \leq 0.05$). Bars represent averages of triplicate results each performed in duplicate (n=6±SD). Non-cold shocked (NS); cold shocked (CS); tryptic soy agar (TSA); violet red bile agar (VRBA).


NS-TSA
CS-TSA
NS-VRBA
CS-VRBA
Survivors (Log 10 CFU/ml)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
0
1
7
28
Days of Storage

iv) Whole Egg

Results of cold shocking on the survival and injury of *E. coli* in frozen whole egg are presented in Tables 13-16. As all *E. coli* strains behaved similarly, only the survival pattern of strain 7110 is illustrated (Fig. 5). As shown from the data, a significant increase in survival of the *E. coli* strains was observed throughout the storage period as a result of the cold shock treatment. In addition, the number of survivors following cold shocking increased with the duration of storage. By day 28 (Table 16) increases in survival levels of cold shocked bacteria ranged from 0.29 to 0.97 and from 0.89 to 1.84 ($\log_{10}$ CFU/ml) on TSA and VRBA, respectively. Survival of all *E. coli* strains during frozen storage was generally higher in egg than in milk.

The cold shock treatment appeared to reduce the amount of injury incurred by the cells at all sampling periods. By day 28 (Table 16) differences in the % injury between cold and non cold-shocked strains ranged from 33 to 75 %. Similar to previous products the % injury generally increased with an increase in storage time.

Table 13. Survival and injury levels of *E. coli* in whole egg on day 0.

Strain	Source	Survivors $\log_{10}$ CFU/ml		% Injury[1]
		TSA[1]	VRBA[1]	
7128	human	6.07 ± 0.08	6.06 ± 0.11	4.90 ± 5.96
7282	food	6.35 ± 0.05	6.34 ± 0.03	3.36 ± 2.93
7110	human	6.24 ± 0.02	6.22 ± 0.06	5.02 ± 7.75
7283	food	6.26 ± 0.04	6.18 ± 0.01	17.79 ± 5.71
7174	bovine	6.20 ± 0.04	6.18 ± 0.06	5.56 ± 9.62
7236	human	6.16 ± 0.03	6.11 ± 0.02	11.15 ± 10.63
my20	food	5.59 ± 0.05	5.51 ± 0.11	17.79 ± 10.60

[1] Results ($\log_{10}$ CFU/ ml) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).

Table 14. Effect of cold shocking on survival and injury levels of *E. coli* in egg on day 1 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	5.76 ± 0.17[a]	-	5.57 ± 0.12[a]	0.39	34.42 ± 12.08
7128 CS[3]	5.97 ± 0.02[a]		5.96 ± 0.04[b]		2.93 ± 4.23
7282 NS	6.15 ± 0.07[a]	-	6.09 ± 0.09[a]	-	13.25 ± 4.39
7282 CS	6.23 ± 0.04[a]		6.20 ± 0.06[a]		7.04 ± 4.03
7110 NS	5.83 ± 0.01[a]	0.36	5.70 ± 0.03[a]	0.40	23.63 ± 5.29
7110 CS	6.19 ± 0.04[b]		6.10 ± 0.06[b]		13.94 ± 6.53
7283 NS	5.73 ± 0.13[a]	0.39	5.49 ± 0.12[a]	0.65	42.11 ± 3.58
7283 CS	6.12 ± 0.06[b]		6.14 ± 0.05[b]		9.86 ± 4.05
7174 NS	5.93 ± 0.06[a]	0.31	5.89 ± 0.05[a]	0.28	10.14 ± 3.63
7174 CS	6.24 ± 0.01[b]		6.17 ± 0.02[b]		13.60 ± 2.60
7236 NS	6.03 ± 0.09[a]	0.14	5.93 ± 0.12[a]	-	18.89 ± 11.24
7236 CS	6.17 ± 0.02[b]		6.05 ± 0.03[a]		24.72 ± 4.52
my20 NS	5.21 ± 0.15[a]	0.55	5.13 ± 0.17[a]	0.60	16.81 ± 3.94
my20 CS	5.76 ± 0.09[b]		5.73 ± 0.07[b]		17.52 ± 14.27

[1] Results ($\log_{10}$CFU/ ml) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same superscript for each medium and strain are not significantly different ($p \leq 0.05$).
[5] Net increase ($\log_{10}$ CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Table 15. Effect of cold shocking on survival and injury levels of *E. coli* in whole egg on day 7 at-20°C

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	5.29 ± 0.08^a	0.58	4.72 ± 0.15^a	1.10	70.87 ± 14.59
7128 CS[3]	5.87 ± 0.05^b		5.82 ± 0.10^b		11.96 ± 10.70
7282 NS	5.99 ± 0.05^a	0.24	5.51 ± 0.30^a	0.60	63.39 ± 18.89
7282 CS	6.23 ± 0.04^b		6.11 ± 0.04^b		22.89 ± 10.42
7110 NS	5.70 ± 0.07^a	0.36	5.14 ± 0.12^a	0.90	72.38 ± 5.43
7110 CS	6.06 ± 0.04^b		6.04 ± 0.04^b		4.54 ± 3.53
7283 NS	5.75 ± 0.08^a	0.32	5.24 ± 0.06^a	0.76	68.77 ± 3.89
7283 CS	6.07 ± 0.09^b		6.00 ± 0.11^b		13.92 ± 6.79
7174 NS	5.51 ± 0.27^a	0.60	5.08 ± 0.26^a	0.98	63.25 ± 3.44
7174 CS	6.11 ± 0.03^b		6.06 ± 0.00^b		12.12 ± 5.10
7236 NS	5.85 ± 0.05^a	0.29	5.36 ± 0.10^a	0.67	68.00 ± 3.55
7236 CS	6.14 ± 0.03^b		6.03 ± 0.06^b		22.70 ± 6.21
my20 NS	4.71 ± 0.07^a	0.69	4.01 ± 0.13^a	1.11	80.07 ± 3.79
my20 CS	5.40 ± 0.20^b		5.12 ± 0.13^b		46.34 ± 10.64

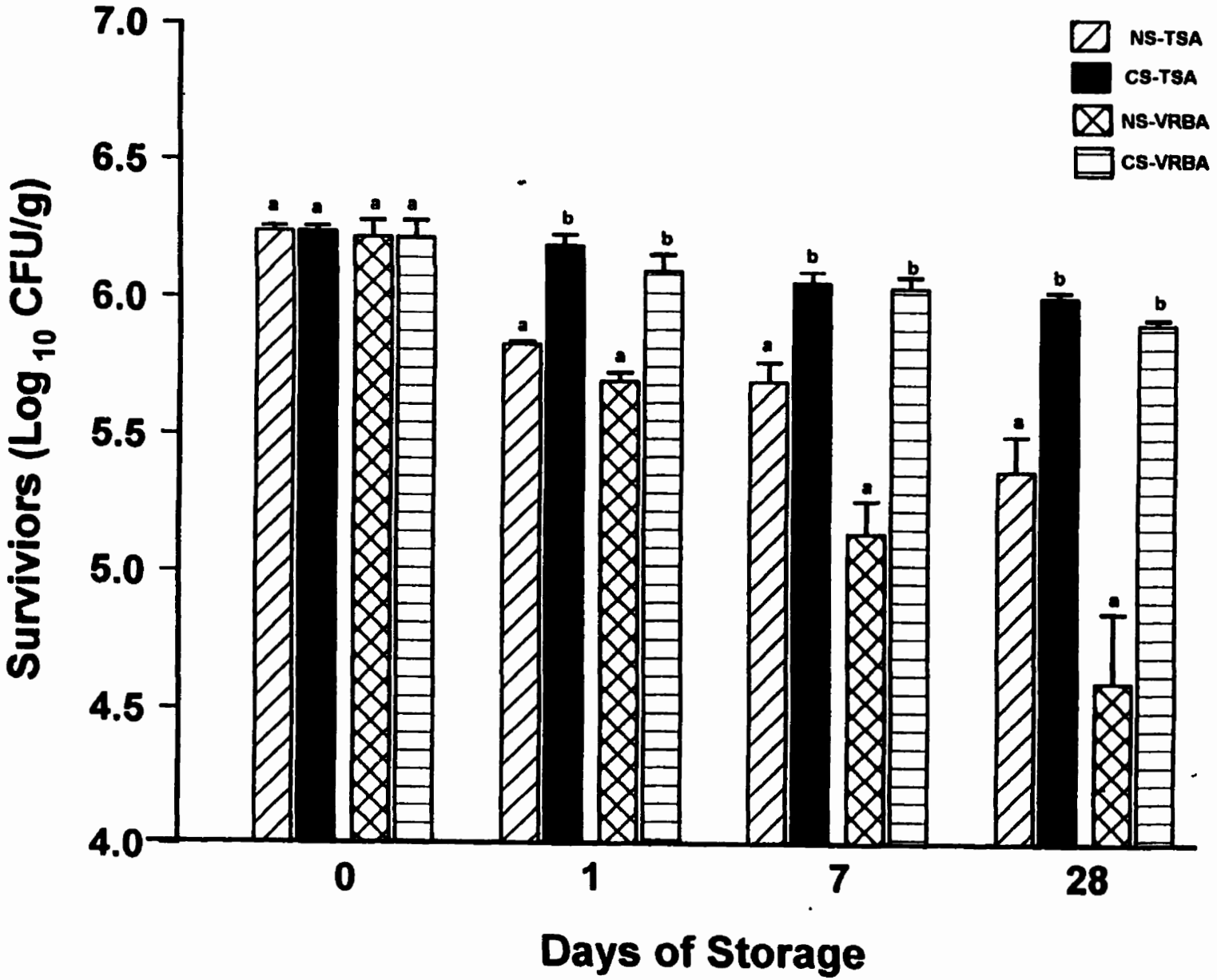
[1] Results ($\log_{10}$ CFU/ ml) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same superscript for each medium and strain are not significantly different ($p \leq 0.05$).
[5] Net increase ($\log_{10}$ CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).

Table 16. Effect of cold shocking on survival and injury levels of *E. coli* in whole egg on day 28 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	4.81 ± 0.09^a	0.97	3.53 ± 0.39^a	1.84	93.86 ± 3.51
7128 CS[3]	5.78 ± 0.04^b		5.37 ± 0.01^b		60.69 ± 4.43
7282 NS	5.64 ± 0.16^a	0.41	5.00 ± 0.14^a	0.89	77.06 ± 3.44
7282 CS	6.05 ± 0.04^b		5.89 ± 0.07^b		29.23 ± 18.70
7110 NS	5.37 ± 0.13^a	0.64	4.60 ± 0.25^a	1.31	82.09 ± 6.38
7110 CS	6.01 ± 0.02^b		5.91 ± 0.02^b		20.67 ± 2.12
7283 NS	5.51 ± 0.24^a	0.51	4.69 ± 0.16^a	1.05	84.87 ± 2.79
7283 CS	6.02 ± 0.02^b		5.74 ± 0.08^b		46.57 ± 7.60
7174 NS	5.48 ± 0.15^a	0.51	4.66 ± 0.41^a	1.06	83.27 ± 8.20
7174 CS	5.99 ± 0.10^b		5.72 ± 0.12^b		44.67 ± 10.57
7236 NS	5.78 ± 0.03^a	0.29	4.98 ± 0.14^a	1.05	83.38 ± 6.64
7236 CS	6.07 ± 0.04^b		6.03 ± 0.06^b		8.94 ± 8.02
my20 NS	4.73 ± 0.15^a	0.68	3.59 ± 0.06^a	-	92.47 ± 2.72
my20 CS	5.41 ± 0.13^b		4.99 ± 0.31^a		58.76 ± 20.32

[1] Results ($\log_{10}$ CFU/ ml) are averages of 3 replicates each performed in duplicate (n = 6 ±SD).
[2] Non-cold shocked.
[3] Cold shocked.
[4] Results followed by the same superscript for each medium and strain are not significantly different ($p \leq 0.05$).
[5] Net increase ($\log_{10}$ CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Fig. 5. Effect of cold shocking on the survival of *E. coli* (strain 7110) in whole egg at -20°C. Results with the same superscript for each medium and storage time are not significantly different ($p \leq 0.05$). Bars represent averages of triplicate results each performed in duplicate (n=6±SD). Non-cold shocked (NS); cold shocked (CS); tryptic soy agar (TSA); violet red bile agar (VRBA).


7.0
6.5
6.0
5.5
5.0
4.5
4.0
Survivors (Log 10 CFU/g)
NS-TSA
CS-TSA
NS-VRBA
CS-VRBA
a
b
0
1
7
28
Days of Storage

v. Sausage

The impact of cold shocking on the survival and injury of *E. coli* in raw sausage is presented in Tables 17-20. As all *E. coli* strains behaved similarly, only the survival pattern of strain 7110 is illustrated (Fig. 6). Overall, survivor levels for *E. coli* were highest following cold shocking. Increases were observed as early as day 1 of frozen (-20^{0}C) storage. By day 28, the net increase in survival levels as a results of the cold shock treatment ranged from 0.71 to 1.53 and from 1.05 to 1.68 (CFU/g) on TSA and VRBA, respectively compared to the non-cold shocked cultures (Table 20).

In some strains, the % injury appeared higher for non-cold shocked *E. coli*, while in other strains there did not appear to be a difference in the injury levels. Overall, the difference in the % injury between cold and non-cold shocked cells decreased with storage time. In all cases the level of injury was less than 1 $\log_{10}$CFU/g. Although the % injury increased during storage, a clear pattern of injury between the cold shocked and the non-cold shocked cells was not apparent, in part due to the high standard deviations obtained. In contrast to other products, particularly ground beef and pork, the % injury appeared relatively high, especially at day 1 of storage (Table 18).

Table 17. Survival and injury levels of *E. coli* in sausage on day 0.

Strain	Source	Survivors Log_{10} CFU/g		% Injury
		TSA	VRBA	
7128	human	5.87 ± 0.14	5.63 ± 0.20	42.05 ± 10.01
7282	food	5.73 ± 0.04	5.52 ± 0.14	36.75 ± 17.19
7110	human	6.00 ± 0.02	5.67 ± 0.07	52.07 ± 8.80
7283	food	6.20 ± 0.03	6.01 ± 0.12	32.57 ± 21.58
7174	bovine	6.17 ± 0.05	6.07 ± 0.12	18.82 ± 16.73
7236	human	6.30 ± 0.07	6.14 ± 0.10	31.58 ± 7.52
my20	food	5.71 ± 0.05	5.43 ± 0.08	46.39 ± 10.01

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD)

Table 18. Effect of cold shocking on survival and injury levels of *E. coli* in sausage on day 1 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	3.72 ± 0.12^a	1.49	2.94 ± 0.30^a	1.84	81.51 ± 9.47
7128 CS[3]	5.21 ± 0.20^b		4.78 ± 0.18^b		62.70 ± 6.21
7282 NS	4.39 ± 0.06^a	0.69	3.89 ± 0.18^a	0.76	68.16 ± 8.15
7282 CS	5.08 ± 0.15^b		4.65 ± 0.32^b		60.79 ± 13.64
7110 NS	4.60 ± 0.32^a	0.70	3.99 ± 0.33^a	0.78	75.13 ± 3.86
7110 CS	5.30 ± 0.07^b		4.77 ± 0.09^b		70.58 ± 2.98
7283 NS	5.79 ± 0.40^a	-	4.90 ± 0.21^a	1.05	81.03 ± 14.33
7283 CS	6.04 ± 0.08^a		5.95 ± 0.04^b		18.11 ± 14.45
7174 NS	4.58 ± 0.29^a	-	3.51 ± 0.51^a	-	89.37 ± 6.49
7174 CS	5.43 ± 0.27^a		5.37 ± 0.36^a		21.58 ± 11.79
7236 NS	5.73 ± 0.15^a	0.44	5.02 ± 0.08^a	0.97	80.05 ± 4.04
7236 CS	6.17 ± 0.09^b		5.99 ± 0.15^b		33.99 ± 9.09
my20 NS	3.67 ± 0.26^a	0.98	2.74 ± 0.47^a	-	87.18 ± 5.84
my20 CS	4.65 ± 0.22^b		4.33 ± 0.28^a		50.91 ± 15.17

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/g) = Log_{10} CFU/g (CS) - Log_{10} CFU/g (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Table 19. Effect of cold shocking on survival and injury levels of *E. coli* in sausage on day 7 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	3.68 ± 0.20^{a}	-	2.47 ± 0.23^{a}	1.61	93.20 ± 3.08
7128 CS[3]	5.07 ± 0.81^{a}		4.08 ± 0.19^{b}		83.54 ± 13.51
7282 NS	3.40 ± 0.05^{a}	0.97	2.37 ± 0.04^{a}	1.30	90.43 ± 1.48
7282 CS	4.37 ± 0.15^{b}		3.67 ± 0.26^{b}		79.52 ± 6.79
7110 NS	3.78 ± 0.40^{a}	-	2.56 ± 0.36^{a}	1.64	93.90 ± 1.29
7110 CS	4.80 ± 0.30^{a}		4.20 ± 0.26^{b}		62.69 ± 29.03
7283 NS	4.95 ± 0.22^{a}	0.71	4.07 ± 0.24^{a}	-	86.93 ± 1.40
7283 CS	5.66 ± 0.02^{b}		5.23 ± 0.31^{a}		56.46 ± 32.32
7174 NS	4.61 ± 0.15^{a}	0.66	2.84 ± 0.19^{a}	2.05	98.23 ± 0.62
7174 CS	5.27 ± 0.09^{b}		4.89 ± 0.13^{b}		57.20 ± 8.89
7236 NS	5.38 ± 0.20^{a}	0.60	4.66 ± 0.37^{a}	1.18	79.81 ± 7.67
7236 CS	5.98 ± 0.04^{b}		5.84 ± 0.01^{b}		26.65 ± 9.38
my20 NS	3.22 ± 0.07^{a}	1.05	2.20 ± 0.14^{a}	1.35	90.38 ± 1.63
my20 CS	4.27 ± 0.21^{b}		3.55 ± 0.19^{b}		76.02 ± 17.43

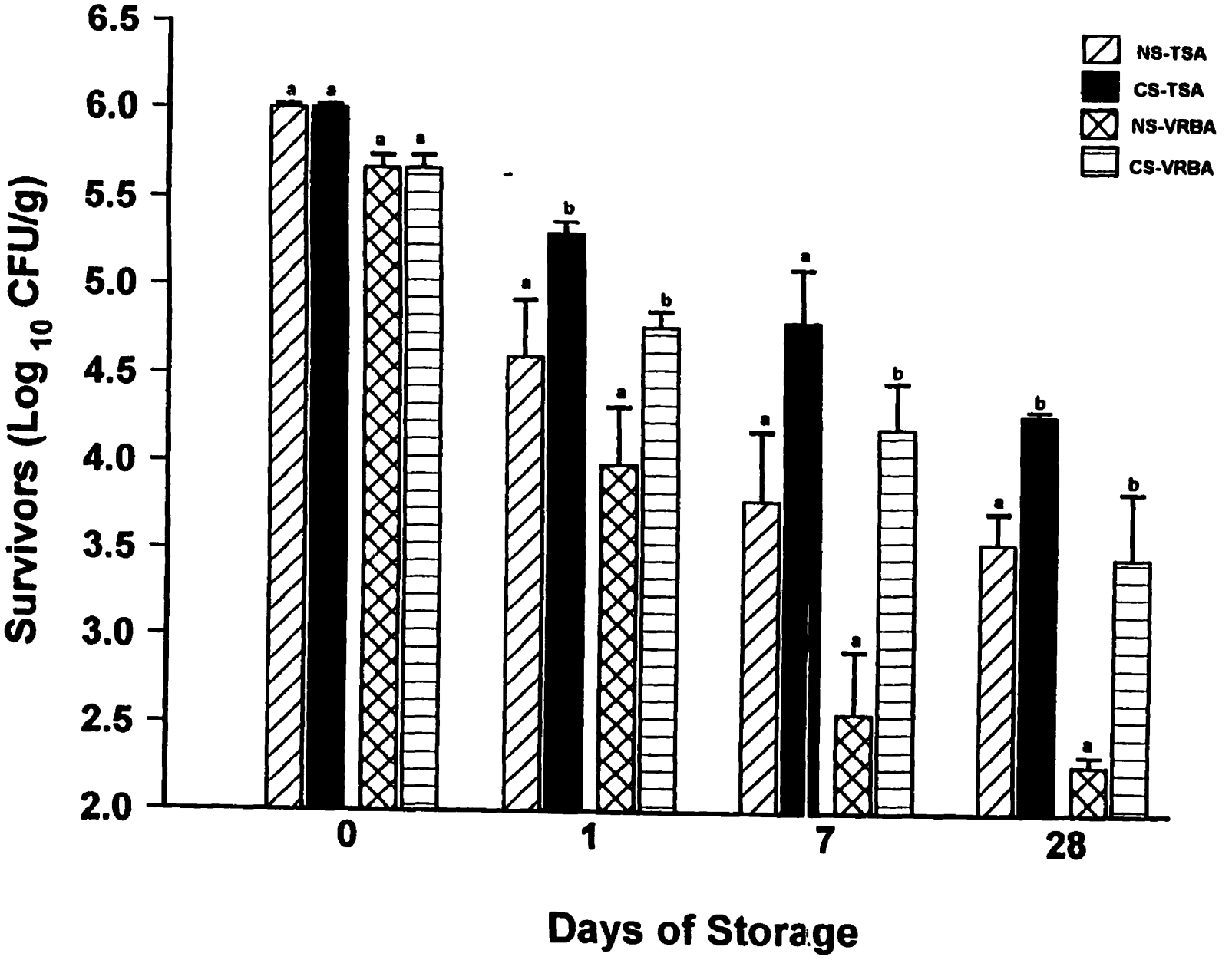
[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/g) = $\log_{10}$ CFU/g (CS) - $\log_{10}$ CFU/g (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Table 20. Effect of cold shocking on survival and injury levels of *E. coli* in sausage on day 28 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
7128 NS[2]	3.28 ± 0.28[a]	1.34	1.73 ± 0.70[a]	1.50	96.12 ± 3.68
7128 CS[3]	4.62 ± 0.23[b]		3.23 ± 0.34[b]		95.85 ± 1.03
7282 NS	3.52 ± 0.03[a]	-	1.48 ± 0.44[a]	-	98.75 ± 0.96
7282 CS	4.18 ± 0.31[a]		2.89 ± 0.44[a]		94.79 ± 1.57
7110 NS	3.54 ± 0.18[a]	0.74	2.27 ± 0.06[a]	-	94.37 ± 2.03
7110 CS	4.28 ± 0.03[b]		3.46 ± 0.38[a]		81.16 ± 14.16
7283 NS	4.67 ± 0.11[a]	0.71	3.71 ± 0.08[a]	1.05	88.74 ± 3.62
7283 CS	5.38 ± 0.15[b]		4.76 ± 0.20[b]		76.14 ± 2.44
7174 NS	3.77 ± 0.21[a]	1.53	2.35 ± 0.18[a]	-	96.10 ± 1.11
7174 CS	5.30 ± 0.03[b]		4.33 ± 0.30[a]		87.73 ± 7.23
7236 NS	5.12 ± 0.24[a]	0.85	4.42 ± 0.72[a]	1.21	72.56 ± 21.94
7236 CS	5.97 ± 0.02[b]		5.63 ± 0.17[b]		52.60 ± 17.24
my20 NS	2.93 ± 0.17[a]	1.22	1.68 ± 0.25[a]	1.68	93.89 ± 2.77
my20 CS	4.15 ± 0.16[b]		3.36 ± 0.06[b]		82.85 ± 7.39

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/g) = $\log_{10}$ CFU/g (CS) - $\log_{10}$ CFU/g (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Fig. 6. Effect of cold shocking on the survival of *E. coli* (strain 7110) in sausage at -20°C. Results with the same superscript for each medium and storage time are not significantly different ($p \leq 0.05$). Bars represent averages of triplicate results each performed in duplicate ($n=6 \pm SD$). Non-cold shocked (NS); cold shocked (CS); tryptic soy agar (TSA); violet red bile agar (VRBA).


6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
Survivors (Log 10 CFU/g)
NS-TSA
CS-TSA
NS-VRBA
CS-VRBA
0
1
7
28
Days of Storage

vi. Tryptic Soy Broth (pH 7.0)

The results of cold shocking on the survival of *E. coli* in tryptic soy broth (pH 7.0) are presented in Tables 21-25. As all *E. coli* strains behaved similarly, only the survival pattern of strain 7110 is illustrated (Fig. 7). The % injury results are not given because the survivor counts were often too low. For the same reason, statistical analyses could not be performed on all samples. For samples where the survivor count was < 10 CFU/ml the number 1 was used for statistical analyses.

Overall, there was a significant increase in the survival levels of cold shocked *E. coli* compared to non-cold shocked cells. This increase was observed as early as day 1 and persisted throughout the storage period. Survival levels, particularly for some of the non-cold shocked cultures were less than 1 $\log_{10}$ CFU/ml by day 7 (Table 23). By 28 days, the survival levels for some cold shocked strains was approximately 2 to 3 $\log_{10}$ CFU/ml higher than the non-cold shocked cells. On day 28, all non-cold shocked cultures had counts of <10 CFU/ml on VRBA.

Many of the strains exhibited injury levels greater than 85% after 1 day of storage. The level increased slightly throughout the storage period and by day 28, non-cold shocked strains, in particular, approached 100% injury.

Table 21. Survival of *E. coli* in TSB (pH 7.0) on day 0.

Strain	Survivors Log_{10} CFU/ml	
	TSA[1]	VRBA[1]
7128	5.81 ± 0.26	5.85 ± 0.23
7282	5.63 ± 0.03	5.63 ± 0.06
7110	5.45 ± 0.12	5.52 ± 0.08
7283	5.83 ± 0.27	5.86 ± 0.22
7174	5.66 ± 0.05	5.72 ± 0.03
7236	5.68 ± 0.04	5.68 ± 0.05
my20	5.53 ± 0.11	5.16 ± 0.07

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).

Table 22. Effect of cold shocking on survival of *E. coli* in tryptic soy broth (pH 7.0) on day 1 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]
7282 NS[2]	2.79 ± 0.27[a]	1.72	1.26 ± 0.24[a]	2.54
7282 CS[3]	4.51 ± 0.09[b]		3.80 ± 0.21[b]	
7110 NS	3.50 ± 0.06[a]	0.89	2.84 ± 0.25[a]	-
7110 CS	4.39 ± 0.08[b]		3.23 ± 0.28[a]	
7174 NS	3.29 ± 0.18[a]	1.53	3.17 ± 0.21[a]	0.86
7174 CS	4.82 ± 0.02[b]		4.03 ± 0.10[b]	
7236 NS	3.54 ± 0.03[a]	1.50	2.28 ± 0.86[a]	1.92
7236 CS	5.04 ± 0.08[b]		4.20 ± 0.22[b]	

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase (log_{10} CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Table 23. Effect of cold shocking on survival and injury of *E. coli* in tryptic soy broth (pH 7.0) on day 7 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]
7128 NS[2]	3.37 ± 0.06[a]	0.85	< 2.00[7]	*
7128 CS[3]	4.22 ± 0.07[b]		0.63 ± 0.59	
7282 NS	<10 CFU/ml[a]	*	<10 CFU/ml[a]	*
7282 CS	2.83 ± 0.07[b]		1.77 ± 0.07[b]	
7110 NS	<1.00[6,a]	*	<10 CFU/ml[a]	*
7110 CS	2.90 ± 0.07[b]		2.23 ± 0.59[b]	
7283 NS	2.77 ± 0.40[a]	0.71	< 2.00[7]	*
7283 CS	3.48 ± 0.16[b]		< 2.00[7]	
7174 NS	<1.00[6,a]	*	<10 CFU/ml[a]	*
7174 CS	3.48 ± 0.12[b]		3.03 ± 0.01[b]	
7236 NS	1.17 ± 0.22[a]	2.54	<10 CFU/ml[a]	*
7236 CS	3.71 ± 0.03[b]		3.28 ± 0.12[b]	
my20 NS	2.86 ± 0.03[a]	1.28	< 2.00[7]	*
my20 CS	4.14 ± 0.15[b]		< 2.00[7]	

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/ml) = $\log_{10}$ CFU/ml (CS) - $\log_{10}$ CFU/ml (NS).
[6] 10^{-1} was the lowest dilution used
[7] 10^{-2} was the lowest dilution used
* Net increase could not be calculated

Table 24. Effect of cold shocking on survival of *E. coli* in tryptic soy broth (pH 7.0) on day 14 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]
7128 NS[2]	2.85 ● 0.07[a]	1.01	< 10 CFU/ml[a]	*
7128 CS[3]	3.86 ± 0.07[b]		2.55 ± 0.22[b]	
7283 NS	2.71 ± 0.31[a]	0.86	<10 CFU/ml	*
7283 CS	3.57 ± 0.05[b]		0.52 ± 0.07	
my20 NS	2.94 ± 0.07[a]	1.48	<10 CFU/ml	*
my20 CS	4.42 ± 0.57[b]		1.54 ± 0.28	

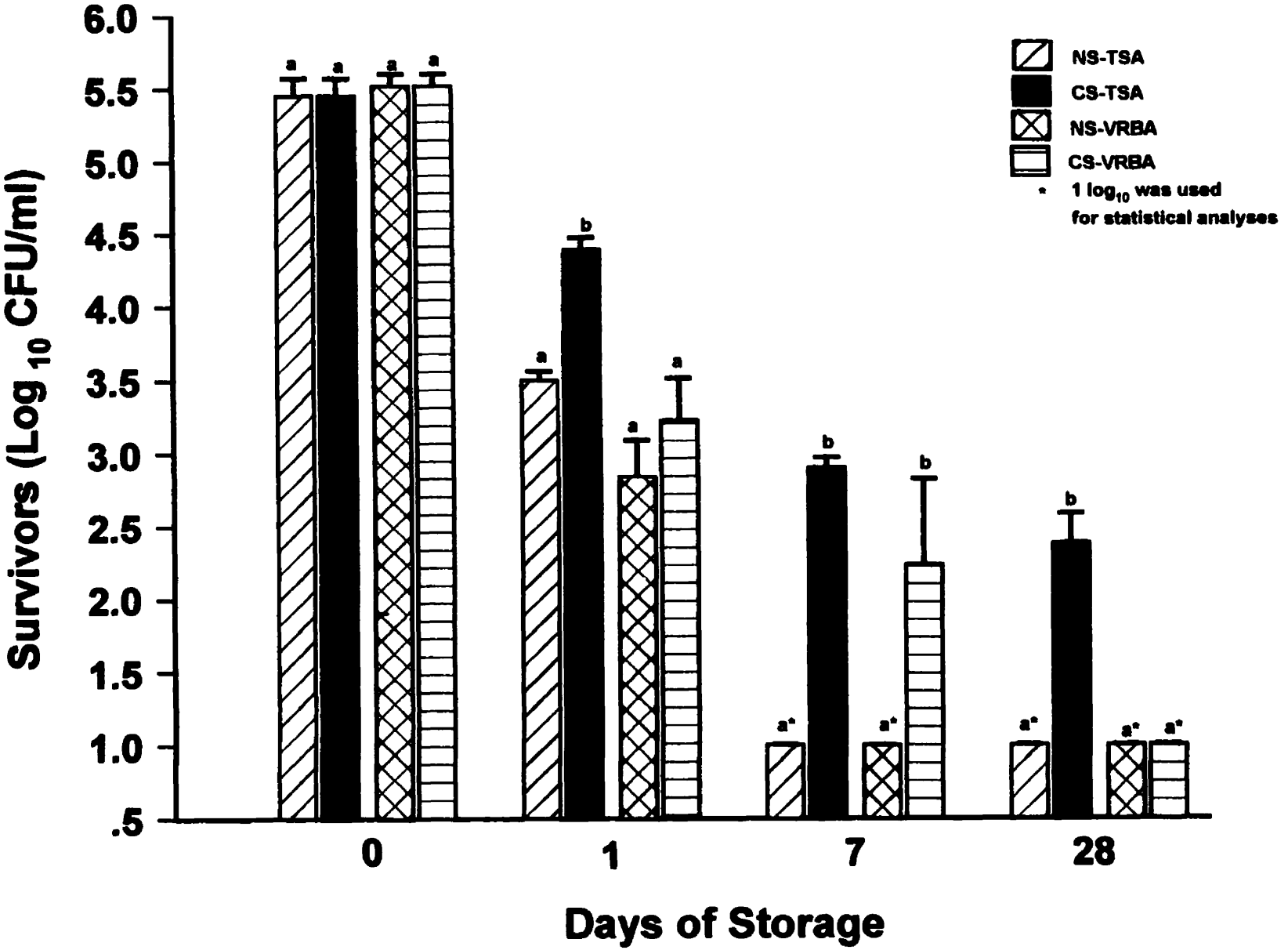
[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
* Net increase could not be calculated

Table 25. Effect of cold shocking on survival of *E. coli* in tryptic soy broth (pH 7.0) on day 28 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]
7128 NS[2]	2.16 ± 0.12[a]	1.06	<10 CFU/ml[a]	*
7128 CS[3]	3.22 ± 0.14[b]		2.07 ± 0.17[b]	
7282 NS	<10 CFU/ml[a]	*	<10 CFU/ml	*
7282 CS	2.25 ± 0.07[b]		<10 CFU/ml	
7110 NS	0.87 ± 0.29[a]	1.51	<10 CFU/ml	*
7110 CS	2.38 ± 0.20[b]		<10 CFU/ml	
7283 NS	1.46 ± 0.06[a]	1.63	<10 CFU/ml	*
7283 CS	3.09 ± 0.06[b]		0.78 ± 0.42	
7174 NS	< 10 CFU/ml[a]	*	<10 CFU/ml	*
7174 CS	2.97 ± 0.10[b]		1.29 ± 0.26	
7236 NS	<10 CFU/ml[a]	*	<10 CFU/ml[a]	*
7236 CS	3.08 ± 0.03[b]		1.70 ± 0.17[b]	
my20 NS	1.35 ± 0.31[a]	2.12	<10 CFU/ml	*
my20 CS	3.47 ± 0.21[b]		<10 CFU/ml	

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
* Net increase could not be calculated

Fig. 7. Effect of cold shocking on the survival of *E. coli* (strain 7110) in tryptic soy broth (pH 7.0) at -20°C. Results with the same superscript for each medium and storage time are not significantly different ($p \leq 0.05$). Bars represent averages of triplicate results each performed in duplicate (n=6±SD). Non-cold shocked (NS); cold shocked (CS); tryptic soy agar (TSA); violet red bile agar (VRBA).


6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
Survivors (Log 10 CFU/ml)
0
1
7
28
Days of Storage
NS-TSA
CS-TSA
NS-VRBA
CS-VRBA
* 1 log10 was used for statistical analyses

vii. Tryptic Soy Broth (pH 5.0)

Results of the impact of cold shocking on the survival of *E. coli* in tryptic soy broth (pH 5.0) are presented in Tables 26-28. As all *E. coli* strains behaved similarly, only the survival pattern of strain 7110 is illustrated (Fig. 8). Samples were only assessed for survivors for 7 days because of low recovery rates with some strains (Table 28). The results are somewhat unique when compared to the previous products because of the large differences in survivors both among strains and between cold and non-cold shocked cells. In particular, at 7 days, survivor levels for strains 7110 and 7174 on TSA and strain 7236 on VRBA were < 10 CFU/ml. In addition, the majority of non-cold shocked strains exhibited a significant increase in survival over those which were cold shocked. Strain 7128 seemed to have an increased acid tolerance compared to the other strains. The survival rate of this strain on both TSA and VRBA remained the highest, also, no significant difference in its survival was exhibited regardless of cold shocking. In contrast, the food strain 7282 appeared the most sensitive. Only strain 7283 showed a significant increase in survival as a result of the cold shock treatment.

Survival rates were consistently low with the exception of strain 7128 when enumerated on VRBA, indicating a high rate of injury. On day 1, injury levels of ca. 75% were observed in both cold and non-cold shocked *E. coli*. By day 7 the % injury approached 100% for all strains.

Table 26. Survival of *E. coli* in TSB (pH 5.0) on day 0.

Strain	Source	Survivors Log_{10} CFU/ml TSA	VRBA
7128	human	5.75 ± 0.08	5.73 ± 0.07
7282	food	5.35 ± 0.08	5.36 ± 0.07
7110	human	5.53 ± 0.03	5.57 ± 0.05
7283	food	5.74 ± 0.08	5.71 ± 0.03
7174	bovine	5.55 ± 0.07	5.59 ± 0.01
7236	human	5.67 ± 0.03	5.71 ± 0.05
my20	food	5.49 ± 0.09	5.49 ± 0.13

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).

Table 27. Effect of cold shocking on survival of *E. coli* in tryptic soy broth (pH 5.0) on day 1 at 20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]
7282 NS	2.80 ± 0.18[a]	-	<10 CFU/ml[a]	*
7282 CS	2.57 ± 0.16[a]		1.71 ± 0.19[b]	
7110 NS	2.84 ± 0.21[a]	-0.66	1.55 ± 0.22[a]	-
7110 CS	2.18 ± 0.22[b]		1.50 ± 0.09[a]	
7174 NS	3.16 ± 0.17[a]	-0.55	2.34 ± 0.02[a]	-0.28
7174 CS	2.61 ± 0.13[b]		2.06 ± 0.15[b]	
7236 NS	4.09 ± 0.06[a]	-0.67	3.47 ± 0.01[a]	-0.54
7236 CS	3.42 ± 0.08[b]		2.93 ± 0.16[b]	

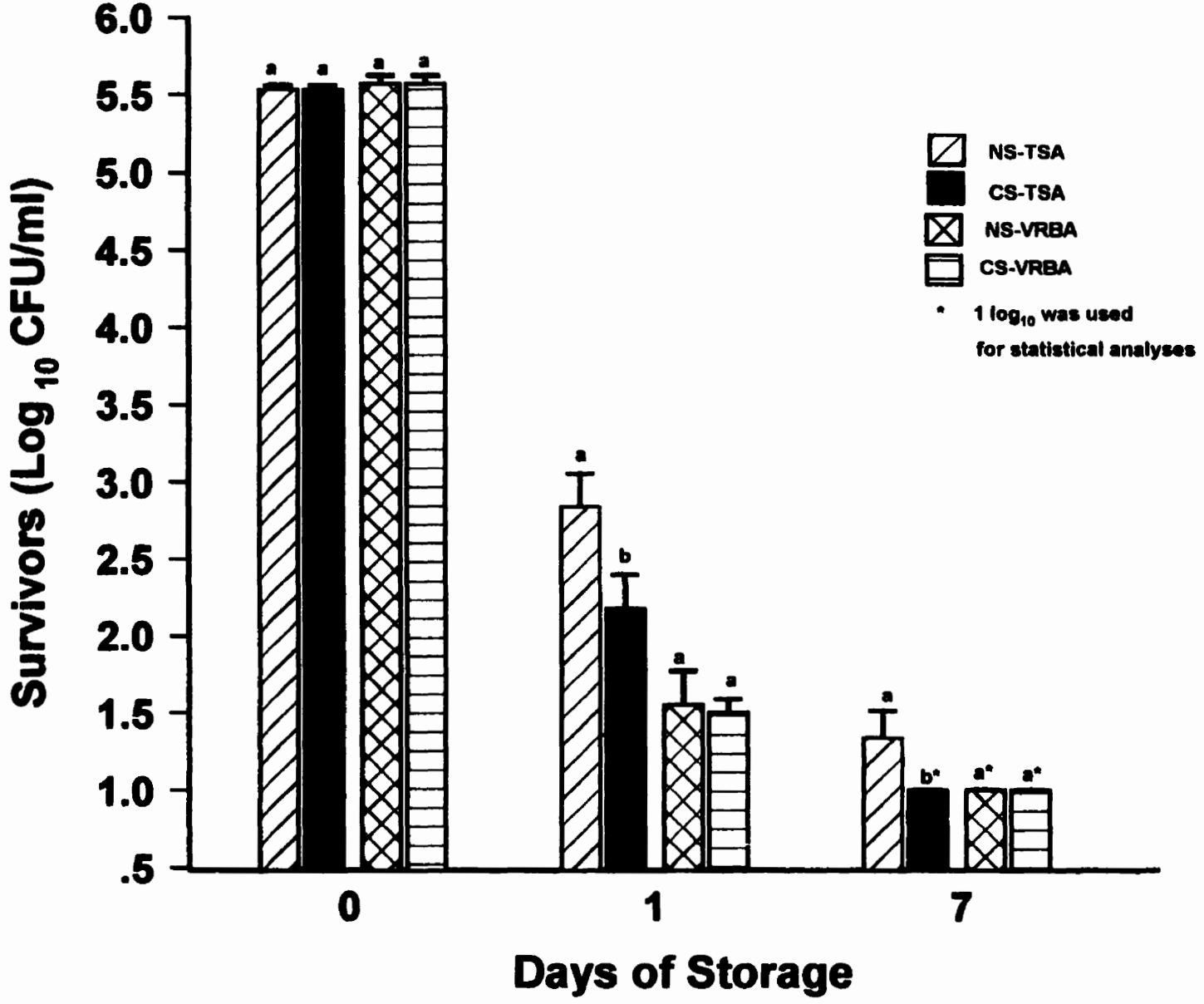
[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase (log_{10} CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
* Net increase could not be calculated
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Table 28. Effect of cold shocking on survival of *E. coli* in tryptic soy broth (pH 5.0) on day 7 at -20°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]
7128 NS	3.89 ± 0.17[a]	-	2.68 ± 0.43[a]	-
7128 CS	3.76 ± 0.19[a]		2.33 ± 0.06[a]	
7282 NS	<10 CFU/ml	*	<10 CFU/ml	*
7282 CS	<10 CFU/ml		<10 CFU/ml	
7110 NS	1.34 ± 0.17[a]	*	<10 CFU/ml	*
7110 CS	<10 CFU/ml[b]		<10 CFU/ml	
7283 NS	2.21 ± 0.35[a]	0.70	<10 CFU/ml	*
7283 CS	2.91 ± 0.14[b]		<10 CFU/ml	
7174 NS	2.03 ± 0.33[a]	*	<10 CFU/ml	*
7174 CS	<10 CFU/ml[b]		<10 CFU/ml	
7236 NS	3.22 ± 0.05[a]	-1.19	1.02 ± 0.48	*
7236 CS	2.03 ± 0.33[b]		<10 CFU/ml	
my20 NS	3.04 ± 0.16[a]	-	<10 CFU/ml	*
my20 CS	3.54 ± 0.34[a]		<10 CFU/ml	

[1] Results are the average of 3 replicates each comprised of duplicate results (n=6±SD).
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase (log_{10} CFU/ml) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
* Net increase could not be calculated
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

Fig. 8. Effect of cold shocking on the survival of *E. coli* (strain 7110) in tryptic soy broth (pH 5.0) at -20°C. Results with the same superscript for each medium and storage time are not significantly different ($p \leq 0.05$). Bars represent averages of triplicate results each performed in duplicate ($n=6 \pm SD$). Non-cold shocked (NS); cold shocked (CS); tryptic soy agar (TSA); violet red bile agar (VRBA).


6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
.5
Survivors (Log 10 CFU/ml)
0
1
7
Days of Storage
NS-TSA
CS-TSA
NS-VRBA
CS-VRBA
* 1 log10 was used for statistical analyses

3. Temperature Shift Required to Enhance Survival.

The results for the minimum temperature shift required to enhance the survival of *E. coli* are presented in Tables 29-32. All temperature shifts with the exception of 20-10^{0}C (Table 32) which were followed by cold shocking, resulted in a significant increase in survival of *E. coli* throughout the storage period. In all cases, the survival levels for *E. coli* following cold shock treatment were at least one log higher. This pattern was observed regardless of the nature of the enumeration medium. The net increase in survival was highest following the 37-10^{0}C shift (Table 29). The net increase in survival of the cold shocked cells appeared to decrease as the temperature range narrowed. Cold shocking after the 20-10^{0}C shift did not increase survival when compared to the non-cold shocked cells.

Statistical analysis was not performed on % injury because of the high variability among replicates, therefore it is difficult to comment on whether the cold shock treatment had an impact on the injury level of the cells. The level of injury among all strains appeared to increase with the time of storage.

Table 29. The impact of cold shocking on survival and injury of *E. coli* O157:H7 with a shift of 37 -10°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
Day 0	5.13 ± 0.09		5.07 ± 0.11		14.19 ± 6.72
Day 1 NS[2]	3.47 ● 0.05[a]	1.43	2.95 ± 0.08[a]	1.55	69.72 ± 2.48
Day 1 CS[3]	4.90 ± 0.03[b]		4.50 ± 0.09[b]		59.62 ± 7.83
Day7 NS	3.35 ± 0.20[a]	1.35	2.58 ± 0.20[a]	1.58	82.97 ± 3.72
Day 7 CS	4.70 ± 0.01[b]		4.16 ± 0.06[b]		70.52 ± 3.38

[1] Results are an average of 3 replicates each comprised of duplicate results (n=6±SD)
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each day are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/g) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).

Table 30. The impact of cold shocking on survival and injury of *E. coli* O157:H7 with a shift of 30 - 10°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
Day 0	5.35 ± 0.04		5.29 ● 0.05		12.64 ± 10.21
Day 1 NS[2]	3.61 ± 0.17[a]	1.23	3.17 ± 0.25[a]	1.22	63.74 ± 8.34
Day 1 CS[3]	4.84 ± 0.09[b]		4.39 ± 0.11[b]		64.28 ± 3.81
Day7 NS	3.59 ± 0.16[a]	1.09	2.97 ± 0.14[a]	1.35	70.76 ± 2.08
Day 7 CS	4.68 ± 0.02[b]		4.32 ± 0.03[b]		56.39 ● 2.41

[1] Results are an average of 3 replicates each comprised of duplicate results (n=6±SD)
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/g) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).

Table 31. The impact of cold shocking on survival and injury of *E. coli* O157:H7 with a shift of 30 - 15°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
Day 0	5.26 ± 0.05		5.23 ± 0.06		6.78 ± 3.52
Day 1 NS[2]	3.57 ± 0.09[a]	1.04	3.26 ± 0.17[a]	0.91	49.25 ± 13.86
Day 1 CS[3]	4.61 ± 0.10[b]		4.17 ± 0.61[b]		20.66 ± 13.89
Day7 NS	3.16 ± 0.05[a]	1.00	2.83 ± 0.02[a]	1.19	53.33 ± 2.77
Day 7 CS	4.16 ± 0.06[b]		4.02 ± 0.02[b]		26.29 ± 7.03

[1] Results are an average of 3 replicates each comprised of duplicate results (n=6±SD)
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each medium within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/g) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).

Table 32. The impact of cold shocking on survival and injury of *E. coli* O157:H7 with a shift of 20 - 10°C.

Strain	TSA[1,4]	Net Increase[5]	VRBA[1,4]	Net Increase[5]	% Injury[1]
Day 0	5.40 ± 0.08		5.32 ± 0.17		17.99 ± 22.34
Day 1 NS[2]	4.59 ± 0.05[a]	-	4.55 ± 0.12[a]	-0.31	14.17 ± 7.28
Day 1 CS[3]	4.47 ± 0.08[a]		4.24 ± 0.08[b]		32.16 ± 5.61
Day7 NS	3.85 ± 0.13[a]	-	3.58 ± 0.19[a]	-	45.96 ± 7.69
Day 7 CS	3.92 ± 0.01[a]		3.46 ± 0.13[a]		64.51 ± 10.21

[1] Results are an average of 3 replicates each comprised of duplicate results (n=6±SD)
[2] Non-cold shocked
[3] Cold shocked
[4] Results followed by the same letter for each media within each strain are not significantly different ($p \leq 0.05$)
[5] Net increase ($\log_{10}$ CFU/g) = Log_{10} CFU/ml (CS) - Log_{10} CFU/ml (NS).
- Net increase was not reported because the difference in survival between the 2 treatments was not significant ($p \leq 0.05$).

4. Proximate analysis

Proximate analyses (Table 33) were performed on the ground beef, whole liquid egg, dinner sausage, and ground pork samples. The moisture, fat, ash and protein concentrations were determined to ascertain whether they exerted a significant contribution to the outcome of some of the results.

Table 33. Proximate analysis of food samples[7].

Samples[1]	Moisture[2]	Fat[3]	Protein[3]	Ash[4]	pH[5]
Ground beef	55.91 ± 0.42[a]	26.10 ± 0.27[a]	15.94 ± 1.50[a]	0.70 ± 0.01[a]	5.74
Sausage	54.40 ± 0.64[b]	26.07 ± 0.46[a]	9.63 ± 1.01[b]	2.40 ± 0.02[b]	6.08
Ground pork	66.02 ± 0.78[c]	14.14 ± 0.64[b]	17.01 ± 0.14[a]	0.94 ± 0.00[c]	5.80
Egg	73.80 ± 0.06[d]	10.41± 0.12[c]	11.90 ± 0.04[c]	0.94 ± 0.01[c]	7.20
Milk[6]	Np	2.00	3.44	Np	6.67

[1] Values expressed on a % fresh weight basis
[2] Each value represents a mean of 5 replicates ±SD
[3] Each value represents a mean a 3 replicates ±SD
[4] Each value represents a mean of 2 replicates ±SD
[5] Each value represents a mean of 2 determinations; differences between individual determinations were ±0.03
[6] The fat and protein content of milk were obtained from the nutritional information on the package. Np (not performed).
[7] Values followed by the same letter for each food component are not significantly different ($p \leq 0.05$)

As shown in Table 33, a significant difference in the moisture content was observed among the food products. Also, the fat content was found to be significantly different for all food products except between ground beef and sausage. With regards to protein, ground beef and the ground pork appeared similar. The ash content was different between all the products except the ground pork and the whole egg.

5. Impact of Cold Shocking on the Thermal Resistance of *E. coli* O157:H7

Preliminary results of the effects of cold shocking on the thermal resistance of *E. coli* O157:H7 are presented in Tables 34 and 35. Statistical analyses were not performed since separate trials instead of duplicate determinations were conducted, This protocol was performed in order to expediate inoculation and sample removal.

Survivors for *E. coli* O157:H7 following heating at 51^{0}C (Table 34) were determined using only TSA. As observed from the data, the % survivors, after 5 min of heating, appeared to be similar between the cold and non-cold shocked cultures. During the first 2 min of heating, however, the lethality rate appeared more pronounced among the non-cold shocked cells. A similar pattern was observed following heating at 48^{0}C (Table 35)

Table 34. % Reduction of *E. coli* O157:H7 at 51°C (initial count, 10^6 CFU/ml).

Heating	Trial 1[3]		Trial 2[3]	
Time(min)	NS[1]	CS[2]	NS	CS
0	0	0	0	0
1	37.0	3.7	35.8	6.9
2	46.5	27.6	50.0	29.2
5	50.4	42.9	55.0	42.9
10	56.5	46.3	56.7	48.7
15	60.06	57.72	58.7	60.2

[1] Non-cold shocked
[2] Cold shocked
[3] Samples enumerated on TSA

Table 35. % Reduction of *E. coli* O157:H7 at 48°C (initial count, 10^6 CFU/ml).

Heating	TSA[3]		VRBA[3]	
Time (min)	NS[1]	CS[2]	NS	CS
0	0	0	0	0
0.5	5.64	0.16	14.52	5.8
1.0	18.08	5.23	35.39	15.59
1.5	28.52	20.75	44.57	42.29
2.0	42.62	31.70	50.75	43.12
3.0	44.44	39.50	50.92	46.93
5.0	47.76	53.43	51.09	53.40
10.0	54.56	53.59	53.76	54.73

[1] Non-cold shocked
[2] Cold shocked
[3] Results are an average of 2 trials

DISCUSSION

Protein banding patterns of cold shocked *E. coli* have been reported by Jones & Inouye (1994). These researchers found that at least 13 polypeptides were induced after a 37-10^{0}C shift followed by a period of cold shocking (1.5 h). In this study, numerous attempts were made to visualize the alteration in protein banding patten as a results of cold shocking. Reducing and non-reducing polyacrylamide gel electrophoresis as well as autoradiography on 1- dimensional gels were used to examine the banding pattern. All attempts to identify the polypeptides were unsuccessful. The attempts may have been unsuccessful because most researchers employ the use of 2-dimensional gel electrophoresis in conjunction with autoradiography to observe these proteins.

All attempts to locate CspA were unsuccessful. Possibly the small size of the protein may have resulted in its migration with the electrophoretic dye front making it difficult to visualize after staining. When using isoelectric focusing, CspA may have been concealed in a precipitate that occurred with sample application. However, a novel single band (pI 4.8) was consistently detected and produced under cold shocking conditions. The identity of this protein has not been reported and did not correspond to CspA (pI 5.92; Goldstein *et al.* ,1990), however its presence confirmed that the protocol used to induce the cold shock response did cause an alteration in protein banding.

Cold shocking *E. coli* in food products with the exception of ground beef and ground pork resulted in significantly higher numbers of survivors when compared with non-cold shocked cultures. In ground beef and ground pork, differences in survivors between the cold shocked and the non-cold shocked (controls) cells were not significant ($p \leq 0.05$). These results

indicated that enhanced survival due to the cold shock treatment appears to be a least partially linked to the substrate composition or more specifically, its ability to protect microorganisms from freeze/thaw damage. In other words, the full impact of the cold shock treatment may be influenced by how well the microorganisms survive during frozen storage in a specified food or broth under normal conditions. For example, in both ground beef and ground pork, the initial population of the controls for all strains decreased an average of only 1.1 and 0.77 $\log_{10}$CFU/g respectively over 28 d of frozen storage (calculated using TSA data). In contrast, the decrease in survival in both milk and sausage over the same period of frozen storage was approximately double (2.07 and 2.16 $\log_{10}$ CFU/g respectively), while in TSB (pH 7.0) the decrease in survival was on average over 4 $\log_{10}$ CFU/ml. It would therefore appear that survival or protection of *E. coli* in ground meats (beef and pork) was more favourable than in milk, sausage or TSB broth. These results were consistent with those reported by Doyle and Schoeni (1984) who found that *E. coli* O157:H7 survived in ground beef during frozen storage at -20^{0}C for up to 9 months with little change in cell numbers. Connor and Hall (1994) conducted similar survival studies in ground chicken breast meat and reported that initial populations decreased by only 0.6 $\log_{10}$ CFU/g during 18 months storage at -20^{0}C, confirming the relatively high survival capability of *E. coli* in muscle meats. Proximate analysis revealed that both ground beef and ground pork contained the highest protein levels of all the products. Since protein is known to exert a cryoprotective effect (Speck & Ray, 1977) the effects of cold shocking *E. coli* in these products may have been overshadowed by the presence of a more favourable survival environment. In contrast, cold shock treatment of *E. coli* in either milk, sausage or neutral broth may offer enhanced survival due to limited participation of cryoprotectants and/or the physical nature of

the food matrix. The conditions employed during freezing and thawing and the participation of food components can also affect the role of the cryoprotectant. It is possible that at least in the case of milk and broth, extracellular crystallization of water coupled with osmotic dehydration may have been major stress factors which far outweighed the presence of cryoprotective agents including proteins and lipids. Alternatively, the breakfast sausage meat contained preservatives such as sodium erythorbate, salt and spices such as sage which have been shown to possess antimicrobial properties (Doyle *et al.*, 1997). In addition they may exert a sensitizing effect on the cells to freezing (Speck & Ray, 1977). This may also explain why sausage with a fat content similar to ground beef, did not provide *E. coli* equal protection. In recovering *E. coli* from frozen ground chicken meat, Connor and Hall (1994) also reported that the presence of salt severely reduced survivor numbers. In addition, milk may not have been able to provide protection to the *E. coli* against freeze damage due to its relatively low fat and protein content (Table 33). Milk also contains natural microbial inhibitors such as lactoferrin and the lactoperoxidase system (Doyle *et al.*, 1997) which may have put an additional stress on the *E. coli* making the bacteria more susceptible to freeze injury and death.

No differences with respect to the rate of cooling was observed between cold and non-cold shocked *E. coli*. In all food products, freezing was very rapid due to the small quantity of sample used. Generally, rapid freezing causes formation of intracellular ice crystals which results in altered permeability or "holes" in the membrane and cell wall (Doyle *et al.*, 1997). The damaged membranes may prevent injured cells from growing on selective media containing inhibitory substances such as bile salts found in VRBA. (Mackey *et al.*, 1980). This would explain why some survivor levels were lower on VRBA as compared with TSA . Functionally,

cold shock proteins may be able to act as antifreeze proteins and prevent or minimize the formation or growth of ice crystals. If they do not directly function as antifreeze proteins the shear number of cold shock protein molecules (250,000/cell) (Tanabe *et al.*, 1992) may have a protective role for the cell by lowering the freezing point and preventing ice crystal formation.

The explanation that cold shocking appeared more effective in foods where *E. coli* had a poor survival rate can not be readily applied to either whole egg or TSB (pH 5). A decrease of only 0.79 $\log_{10}$ CFU/ml was seen in the non-cold shocked bacteria (calculated using TSA data). This data is comparable to the decrease in survivors of ground pork (0.77 $\log_{10}$ CFU/g; calculated using TSA data). Despite the similarity in recovery levels, the cold shock treatment increased the level of survivors in the whole egg. Although protection was afforded to the cells following cold shocking, the level of protection of TSA for this product was the lowest compared to milk, sausage and TSB, pH 7. The survival during frozen storage was generally higher in egg than in milk. This may be due to higher fat and protein content in the egg (10.4% and 11.9% respectively, Table 33) as compared with the milk (2% and 3.44% respectively, Table 33), providing bacteria an increased freeze protection. There may have been an increase in injury and mortality in the egg compared to the ground beef and pork due to antimicrobial compounds such as lysozyme, lactoferrin and avidin that are naturally present in the egg (Doyle *et al.*, 1997).

The average recovery levels for *E. coli* in all products were generally higher on TSA than on VRBA. The number of survivors more than doubled for TSB when enumerated on TSA as compared with VRBA. Since VRBA is a selective medium, in that it contains bile salts and

crystal violet, it is generally assumed that many freeze-injured *E. coli* would fail to grow (Speck & Ray, 1977).

Although the effect of substrate pH on cold shocking was not specifically studied, it is interesting to note that all food products including TSB having a pH value below 6 did not afford *E. coli* protection. In contrast, milk, egg, TSB and sausage with pH values above 6, all rendered *E. coli* protection.

In TSB (pH 5.0) the results are somewhat unique in that the majority of non-cold shocked strains exhibited significantly higher survival compared to those which were cold shocked. In general, the survival of bacteria has been reported to depend on the pH of the substrate in which they are frozen; over a pH range of 2.6-5.0, the lethal effect increases as the pH decreases (Partmann, 1975). Since the freezing protocol was identical for both cold shocked and non-cold shocked cultures, it would appear that the cold shock treatment contributed to the rapid reduction in survivors of some strains during freezing. Prompt freezing of cells as in the case of the non-cold shocked cells, however, appeared to lessen the adverse effects in some strains caused by exposure to acid. *E. coli* O157:H7 strains are known to be aciduric and have been reported to exhibit enhanced survival in acidified TSB broth even at 10^{0}C (Abdul-Raouf, 1993; Conner & Kotrola, 1995). Therefore it is somewhat puzzling to explain why maintenance in an acid environment would sensitize *E. coli* to freezing. It is possible that when cells are acid stressed they become incapable of synthesizing specific cold shock proteins. Repression of cold shock protein synthesis should not, however, contribute to enhanced lethality. Alternatively, even if cells are capable of surviving in an acid environment, changes in metabolism and structure (Leyer & Johnson, 1993) could alter their resistance to subsequent treatment(s).

Results of this study indicate that strain differences may exist with regards to acid tolerance and hence the effect of the cold shock response.

All downshifts in temperature with the exception of the 20-10°C regime provided some level of enhanced survival for *E. coli* cells following frozen storage. Overall, the magnitude of survival appeared to decrease with a decrease in the temperature range. The net increase in survival of cold shocked over non-cold shocked cells following storage was greatest when cells were grown at 37°C and cold shocked at 10°C. Similar findings were reported by Jones & Inouye (1994) who found that minimum of a 13°C decrease in temperature (growth to cold shock temperature) was required to elicit a cold shock response.

As previously mentioned, with the 20-10°C shift, no cold shock based enhancement in survival was observed. This phenomenon may be due to changes that occurred during growth of *E. coli* at 20°C. Specifically, alterations to its fatty acid membrane composition when compared to cells grown at 30 and 37°C could result in additional protection to cells during freezing and lead to downplay in the effect of cold shock proteins. Marr and Ingraham (1962) for example, reported that the proportion of unsaturated fatty acids in the cell membrane decreased as the growth temperature was increased. The increase in the synthesis of unsaturated fatty acids at low temperatures may help to maintain lipids in a fluid state, thereby facilitating membrane function. Paton *et al.* (1978) reported that the injury caused by cooling *Bacillus amyloliquefaciens* could be avoided if the cells were grown at low temperatures. These researchers also observed that cells grown at 20°C had an increase in the ratio of branched- to straight- chain fatty acids and in the level of unsaturation of the branched-chain fatty acids (Paton *et al.*, 1978). Both factors result in a membrane with increased lipid fluidity. Paton *et al.* (1978) also found that the

increase in lipid fluidity could shift the temperature zones for cold shocking, or the temperature where cells lost viability. Cells grown at 30°C and instantly chilled to 3°C totally lost viability; however, those grown at 20°C could be chilled to 3°C with almost no change in viability.

A temperature of 10°C was chosen for the cold shock treatment since below this value synthesis of cold shock proteins is inhibited (Jones & Inouye, 1994). Alternatively, above 15°C the population may increase during cold shocking making it difficult to compare the two treatments. In this study we observed that the population of *E. coli* remained constant at 15°C (results not included) during cold shocking (1.5 h).

Statistical analyses to determine significant differences in % injury between cold and non-cold shocked cells were not performed due to the high variability between replicates. In general, cold shocking did not appear to reduce the incidence of injury, at least the type that could could be detercted using VRBA.. Since VRBA is selective against bacteria that have damage to their cell membrane, other injuries may have gone undetected (Doyle *et al.*, 1997).

The preliminary data resulting from the heat survival studies indicated no clear pattern in regards to resistance. Interestingly, some differences in the rate of destruction within the first 1-5 minutes of heating were observed at both temperatures.

Lelivelt & Kawula (1995) reported that a protein, Hsc66, was a heat shock protein that functioned as a molecular chaperone which was only induced by cold shocking. This indicated that cold shocking may increase the thermal resistance of *E. coli* O157:H7. A subsequent study by Kandror & Goldberg (1997) discovered another cold shock protein that also functioned as a molecular chaperone called the trigger factor (TF) They demonstrated that not only was the TF necessary for the viability of *E. coli* at 4°C, but that its over expression at 50°C decreased cell

viability. Our results do not support this theory because the cold shocked cells did not appear to have reduced viability compared with the non-cold shocked cells.

CONCLUSIONS

In this study, the effectiveness of the cold shock response in providing protection to *E. coli* was shown to depend on the downward temperature range.

Isoelectric focusing revealed that using this temperature range the method used for this study to induce the cold shock response caused an alteration in the protein banding pattern of cold shocked bacteria. A novel band with a pI of 4.8 was identified.

The results of the product inoculation study indicated that cold shocking *E. coli* O157:H7 strains in specific foods resulted in freeze injury protection. Cold shocking provided enhanced survival for cells in milk, whole egg, sausage and tryptic soy broth (pH 7.0). Enhanced survival resulting from cold shocking was not observed with ground pork or ground beef. When *E. coli* was cold shocked in an acidic environment (TSB, pH 5.0), lower survival rates were observed compared to non-cold shocked cells. The presence of acid appeared to sensitize *E. coli* to freeze injury.

The products that did not show an increase in *E. coli* survival following cold shocking and frozen storage had the highest protein content. This may indicate that enhanced survival due to the cold shock response is at least partially linked to substrate composition, or more specifically, its ability to protect microorganisms from freeze/thaw damage.

Cold shocking did not appear to have any clear effect on heat resistance.

The question as to whether cold shocked bacteria pose an increased risk to the safety of our food warrants further investigation. Specifically, cooling and freezing protocols, which are used in the food industry, will have to be examined to determine whether the potential for cold shock-mediated survival of microorganisms exists. Overall there is a greater need to understand

the impact of stress responses on the physiology of pathogenic microorganisms in foods, particulary in regard to their survival.

RECOMMENDATIONS FOR FUTURE STUDIES

1. Use improved analytical procedures such as 2-D electrophoresis to visualize and quantify the induction of the synthesis of cold shock proteins.

2. Examine the rate of cooling as it applies to induction of the cold shock response.

3. Examine the minimum length of time to induce the cold shock response.

4. Examine the effects of cold shocking on growth, survival and injury of bacteria maintained under commercial refrigeration temperatures.

5. Examine the cold shock response in other pathogens such as *Salmonella, Listeria* and *Campylobacter.*

6. Determine whether cold shocking provides cross resistance to acidic environments and the impact of antimicrobials on the cold shock response (i.e. preservatives, salts, lysozyme, etc.)

7. Investigate whether a_w impacts the effect of cold shocking. Since the cold shock response may function to prevent damage due to ice crystal formation, products with different a_w's may elicit different responses.

8. Examine possible benefits of cold shocking to the food (bakery) and beverage industry. For example, cold shocked yeast may be more hardy and therefore assist in the baking industry.

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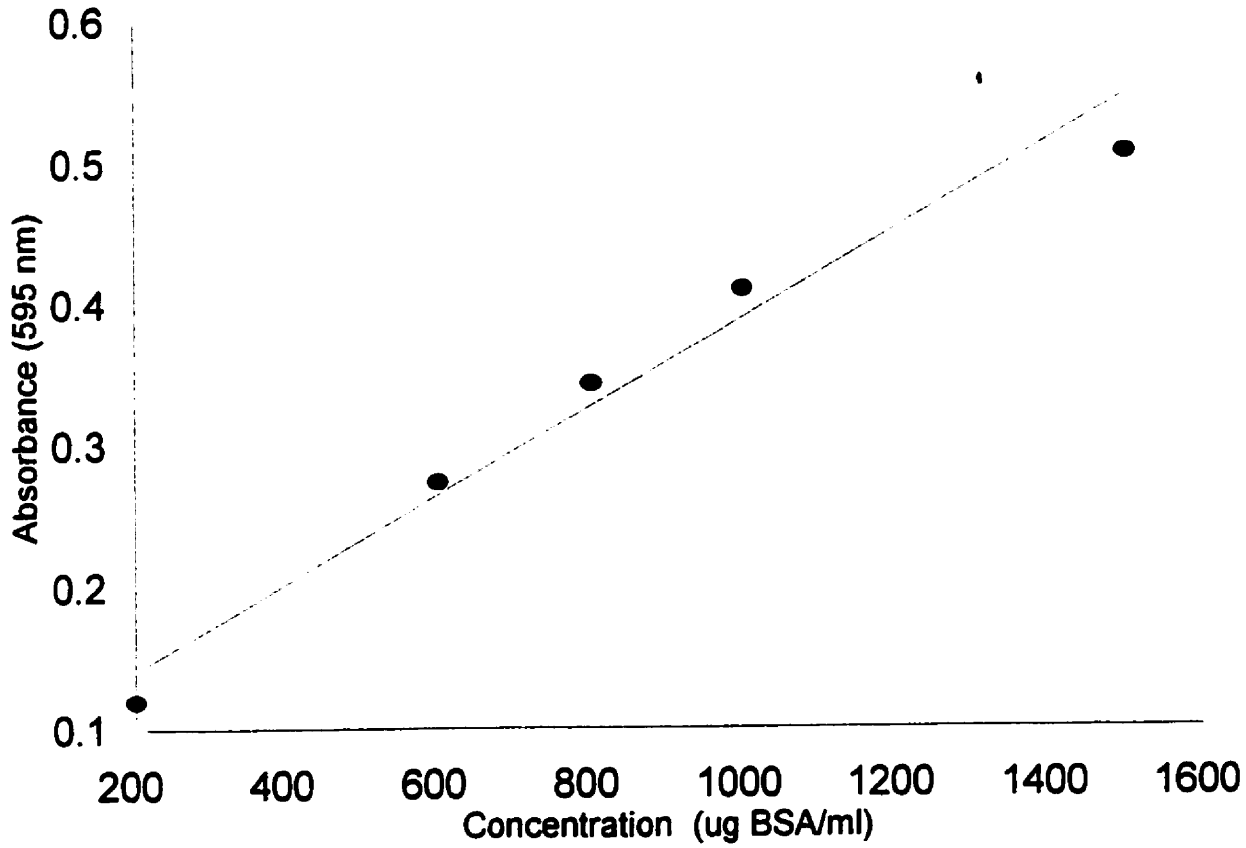
APPENDICES

Appendix 1. Standard curve for determination of the protein concentration of cold and non-cold shocked *E. coli* using Pierce Protein Assay Reagent.



Appendix 2. Calibration curve for isoelectric point determinations of cold shock proteins by isoelectric focusing